Enteropathogenic *Escherichia coli*-Induced Epidermal Growth Factor Receptor Activation Contributes to Physiological Alterations in Intestinal Epithelial Cells[▽]

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The diarrheagenic pathogen enteropathogenic *Escherichia coli* (EPEC) is responsible for significant infant mortality and morbidity, particularly in developing countries. EPEC pathogenesis relies on a type III secretion system-mediated transfer of virulence effectors into host cells. EPEC modulates host cell survival and inflammation, although the proximal signaling pathways have not been well defined. We therefore examined the effect of EPEC on the epidermal growth factor receptor (EGFR), a known upstream activator of both the prosurvival phosphoinositide 3-kinase/Akt and proinflammatory mitogen-activated protein (MAP) kinase pathways. EPEC induced the autophosphorylation of EGFR in intestinal epithelial cells within 15 min postinfection, with maximal phosphorylation being observed at 4 h. Filter-sterilized supernatants of EPEC cultures also stimulated EGFR phosphorylation, suggesting that a secreted component(s) contributes to this activity. EPEC-induced EGFR phosphorylation was blocked by the pharmacological inhibitor tyrphostin AG1478, as well as by EGFR-neutralizing antibodies. Inhibition of EGFR phosphorylation by AG1478 had no effect on bacterial adherence, actin recruitment to sites of attachment, or EPEC-induced epithelial barrier function alteration. EPEC-mediated Akt phosphorylation, however, was inhibited by both AG1478 and EGFR-neutralizing antibodies. Correspondingly, inhibition of EGFR activation increased the apoptosis/necrosis of infected epithelial cells. Inhibition of EGFR phosphorylation also curtailed EPEC-induced ERK1/2 (MAP kinase) phosphorylation and, correspondingly, the production of the proinflammatory cytokine interleukin-8 by infected epithelial cells. Our studies suggest that EGFR is a key proximal signaling molecule during EPEC pathogenesis.

Enteropathogenic *Escherichia coli* (EPEC) is a diarrheagenic pathogen responsible for significant morbidity and mortality, especially among infants in developing countries (9, 34). EPEC is an extracellular gram-negative pathogen that forms microcolonies on intestinal epithelial cells. Infected epithelial cells display histopathological alterations known as attaching and effacing (A/E) lesions characterized by a loss of the enterocyte brush border (17).

A ~35-kb pathogenicity island, known as the locus of enterocyte effacement (LEE), was demonstrated to be necessary and sufficient for EPEC to induce A/E lesions, form pedestal-like structures, and alter epithelial barrier function (16, 30, 41). The LEE encodes components of a type III secretion system, as well as several of the secreted effector proteins. The type III secretion system, elaborated by many pathogenic bacteria, includes a syringe-like complex that conveys various effector proteins directly into the host cytosol (10, 46). Mutations that inactivate the secretion system result in a substantial attenuation of EPEC-induced host effects (23, 31). One of the secreted proteins, the translocated intimin receptor (Tir), inserts into the host cell membrane, engages the bacterial surface adhesin, intimin, and subsequently promotes host cell actin polymerization and the formation of a pedestal-like structure (7).

While the precise mechanism by which EPEC causes diar-

rhea is presently not known, numerous studies have identified specific effects of the pathogen on host epithelial cells (21). At the histological level, EPEC alters the actin cytoskeleton, intermediate filaments, and microtubule network of epithelial cells (1, 7, 29, 43, 45). EPEC triggers the localized polymerization of actin within host cells, eventually leading to the formation of a pedestal-like structure below the attached bacteria (7). At the level of host cell function, EPEC stimulates pro- and anti-inflammatory pathways, disrupts epithelial barrier function and alters epithelial ion and water transport, and stimulates pro- and antiapoptotic pathways (3, 11, 13, 14, 18, 20–22, 38). The bacterial factors responsible for mediating these changes and the signaling pathways involved are only beginning to be characterized.

The EPEC secreted protein F (EspF) contributes to the apoptosis/necrosis of intestinal epithelial cells (12, 33, 35). EPEC, however, is a relatively weak inducer of cell death, possibly because it is also known to stimulate prosurvival pathways (11). The phosphoinositide 3-kinase (PI3K)/Akt pathway is known to contribute to cell survival by inactivating proapoptotic proteins such as BAD (4). Indeed, the PI3K inhibitor wortmannin caused a significant increase in the death of EPEC-infected epithelial cells (11). While PI3K activation has been reported in EPEC-infected macrophages, this has not been directly examined in epithelial cells (8).

Based on various observations, we hypothesized that the epidermal growth factor receptor (EGFR) is involved in EPEC pathogenesis. EGFR, a well-known activator of PI3K (4), is a key signaling molecule engaged by various bacterial pathogens (5, 25, 49). The oral administration of epidermal growth factor,

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by an unknown mechanism, reduced colonization of the rabbit intestinal epithelium by the A/E pathogen rabbit diarrheagenic *Escherichia coli* 1 (6). Additionally, the internalization of EPEC into renal epithelial cells was inhibited by the EGFR kinase inhibitor AG1478 (36). These observations suggest that A/E pathogens likely engage the EGFR signaling axis. The aim of this study, therefore, was to explore EGFR phosphorylation in EPEC-infected intestinal epithelial cells. The contribution of EGFR transactivation to EPEC-induced signaling cascades and the consequences to host effects, including barrier function alteration, apoptosis/necrosis, and proinflammatory signaling, were explored. Our data strongly support a role for EGFR as a key signaling molecule in EPEC pathogenesis.

MATERIALS AND METHODS

Chemicals and antibodies. Chemicals for standard laboratory procedures, including antiactin and horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin G antibody, were purchased from Sigma Chemical Company (St. Louis, MO). Control EGF-treated A431 cell extracts and antibody to total EGFR were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against phosphorylated EGFR pY1068 were obtained from Cell Signaling (Beverly, MA). EGFR-neutralizing antibody was purchased from Upstate (Charlottesville, VA). The Bradford protein assay reagent was obtained from Bio-Rad (Hercules, CA). The EGFR tyrosine kinase inhibitor tyrphostin AG1478 was purchased from EMD Biosciences, Inc. (San Diego, CA).

Cell lines. Caco-2 BBE (C2_{BBE}) intestinal epithelial cells were used for the experiments reported in this study. EGFR transactivation was independently confirmed in HeLa, Caco-2 (parental), HT-29, and T84 cells (data not shown). HeLa cells elaborate a more pronounced inflammatory response to EPEC infection and were therefore employed to examine interleukin-8 (IL-8) production. C2_{BBE} cells were cultured in high-glucose (25 mM) Dulbecco's modified Eagle's medium, 10% fetal bovine serum, 20 mM HEPES, 100 IU/ml penicillin, and 100 µg/ml streptomycin at 37°C in the presence of 5% CO₂. Cells employed in these studies were between passages 25 and 45 and were used for experiments at days 7 to 10 postplating. For epithelial barrier function studies, C2_{BBE} cells were grown on permeable supports (Costar Transwells).

Growth of bacteria and infection of C2_{BBE} cells. Wild-type (WT) EPEC used for all experiments was the *Escherichia coli* O127 strain E2348/69. The nonpathogenic human fecal isolate (HS-4) has been described elsewhere (32). We utilized an established model for infecting epithelial cells (41, 45). Briefly, bacterial cultures grown in Luria-Bertani broth were subcultured in antibiotic- and serum-free Dulbecco's modified Eagle's medium and grown to mid-log growth phase. Approximately 2×10^7 to 4×10^7 bacteria were added to the apical surface of C2_{BBE} monolayers, corresponding to an initial multiplicity of infection (MOI) of 20 to 40. For assessing the effects of bacteria-conditioned medium on EGFR activation, similarly grown bacteria were separated from the supernatant using a 0.22-µm filter and the sterile solution was added to C2_{BBE} cells. The plates were then incubated in antibiotic- and serum-free medium at 37°C in a 5% CO₂ water-jacketed incubator for 1 h. At this time, unattached bacteria were washed away and replaced with fresh medium and incubated for additional periods. Times indicated in the figures are from the point of initial addition of bacteria. To evaluate bacterial attachment, monolayers were infected with EPEC (MOI, ~30) for 1 h, washed, scraped into Tris-buffered saline containing 1% Triton X-100, serially diluted, and plated to determine the number of CFU. Where used, AG1478 (1 µM) or wortmannin (100 nM) and EGFR-neutralizing antibody (10 µg/ml) were added to the epithelial cells 1 hour prior to infection and maintained through the course of the infection.

Western blot analysis. At specific time points postinfection, the cells were washed three times with sterile phosphate-buffered saline and scraped, and total protein was extracted in cell lysis buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na₃VO₄, 1 µg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride). Samples were sonicated on ice three times for 20 seconds to facilitate cell lysis. Protein concentrations of extracts were determined by the Bradford method. For EGFR immunoblot analysis, extracts (300 µg) were separated on 6% sodium dodecyl sulfate-polyacrylamide gel electrophoresis using the Protean II XI apparatus (Bio-Rad, Richmond, CA). For detection of total and phosphorylated ERK1/2, 25 µg of each extract was separated on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The separated pro-

teins were transferred to 0.2-µm nitrocellulose membranes (Transblot cell apparatus; Bio-Rad), blocked with 5% nonfat milk in Tris-buffered saline containing Tween 20 (TBST), and then incubated with total EGFR antibodies, phospho-specific EGFR antibodies, or actin antibodies at appropriate dilutions in membrane blocking solution (Zymed, San Francisco, CA) overnight at 4°C. Blots were incubated in HRP-conjugated secondary antibody for 1 h at room temperature. Membranes were washed three times for 5 min in TBST between each incubation step. The membranes were then developed with the ECL Western blotting substrate for HRP (Amersham Biosciences, Piscataway, NJ).

Cell death assays and immunofluorescence microscopy. Host cell death was evaluated using the Live/Dead viability/cytotoxicity kit (Invitrogen, Carlsbad, CA) as per the manufacturer's instructions. Only ethidium homodimer uptake, reflecting dead cells, is shown below in Fig. 6C. Fluorescent (dead) cells were counted from a minimum of five separate fields from two independent experiments and reported as a percentage of the total number of cells in the corresponding field. To monitor actin distribution, confluent C2_{BBE} monolayers grown on glass coverslips were infected with EPEC and fixed with 3.75% paraformaldehyde. Cells were rinsed twice with phosphate-buffered saline and permeabilized with 100% acetone for 4 to 5 min at -20°C. The monolayers were air dried and then incubated with fluorescein phalloidin (Invitrogen, Carlsbad, CA) for 30 min in the dark at room temperature. After washing, monolayers were mounted using a Prolong antifade kit (Invitrogen, Carlsbad, CA) and assessed using a Nikon Opti-Phot microscope. Images were captured using the Spot-RT digital imaging system.

Measurement of TER. C2_{BBE} monolayers on 0.33-cm² collagen-coated Transwell inserts were infected with bacteria grown to mid-log phase, and transepithelial electrical resistance (TER) was measured at hourly intervals for up to 6 h postinfection. TER was measured by passing a 25-µA current across the cell monolayers as described by Madara et al. (28). The resulting voltage deflection was measured, and resistance was calculated using Ohm's law ($V = IR$). TER values, reported as means ± standard errors, were obtained from three measurements per condition; the data are representative of two independent experiments.

IL-8 assays. HeLa monolayers grown in 24-well plates (Corning, Corning, NY) were infected with WT EPEC (MOI, ~30). Unattached bacteria were washed away after 1 hour, and the infection was continued for an additional 5 h. Culture supernatants were collected and assayed for IL-8 using the Quantikine IL-8 immunoassay kit (R&D Systems, Minneapolis, MN).

RESULTS

EPEC promotes tyrosine phosphorylation of EGFR. To determine the effect of EPEC on EGFR activation, C2_{BBE} cells were infected with WT EPEC or the nonpathogenic commensal bacterium HS-4 for 1 to 4 h, and total protein was extracted and immunoblotted with anti-Y1068 antibody. EPEC infection resulted in a progressive increase in EGFR autophosphorylation with maximal phospho-Y1068 being observed following 4 h of infection (Fig. 1A and B). EPEC-induced EGFR activation was earlier, and more robust, than that observed with HS-4 treatment. Indeed, activation could be observed as early as 15 min postinfection in EPEC-treated cells (see Fig. 4, below). There was no significant difference in the number of EPEC and HS-4 cells attached to C2_{BBE} cells at 1 h postinfection (EPEC, $6.77 \times 10^6 \pm 5.77 \times 10^5$ CFU, versus HS-4, $8.17 \times 10^6 \pm 3.54 \times 10^5$ CFU at 1 h postinfection; $P = 0.072$). Mock-infected cells (medium alone) did not display EGFR phosphorylation at any of the corresponding time points (data not shown). Equal loading of samples was verified by immunoblotting for total EGFR and actin.

A secreted component contributes to EPEC-induced EGFR activation. To examine the role of bacterial attachment in EPEC-induced EGFR phosphorylation, C2_{BBE} monolayers were treated with filter-sterilized supernatants of WT EPEC. As shown in Fig. 2A and B, conditioned EPEC supernatants stimulated EGFR activation, with maximal phosphorylation being observed at 4 h postinfection. This suggests that a se-

FIG. 1. EPEC transactivates EGFR in intestinal epithelial cells. (A) Cell extracts (300 μ g) from C2_{BBE} cells infected with WT EPEC or the commensal strain HS-4 for 60, 120, and 240 min were immunoblotted against EGFR pY1068, total EGFR, and actin. (B) Densitometric analysis was performed on three such blots to determine pEGFR abundance relative to total EGFR and normalized to levels in control uninfected cells ($P < 0.05$ by analysis of variance for control versus EPEC and HS-4 for all time points).

creted component in EPEC supernatants contributes to EGFR activation. Not surprisingly, activation was comparatively less than that induced in the continued presence of EPEC (Fig. 1). We also observed similar activation of EGFR by filter-sterilized supernatants of HS-4 (data not shown), raising the possibility that a toll-like receptor (TLR) agonist may contribute to this activity.

EPEC-induced EGFR activation is delayed in polarized epithelial cells. EGFR is predominantly localized to the basolateral sides of polarized intestinal epithelial cells (2, 44). EPEC, on the other hand, is an extracellular pathogen that attaches to the apical side of epithelial cells (34). Tight junctions between polarized epithelial cells have both a barrier function that restricts free luminal-serosal diffusion of solute molecules and a fence function that maintains the distinction between apical and basolateral membrane compartments. To determine whether EPEC can transactivate basolateral EGFR in polarized epithelial cells, we utilized Caco-2 cells grown on permeable filters. The cells were infected apically, and protein ex-

tracts isolated following specific times of infection were immunoblotted for pY1068. Unlike in cells grown on plastic (Fig. 1 and 2), EGFR Y1068 phosphorylation in polarized epithelial cells grown on permeable filters was evident only after 4 h of infection (Fig. 3A and B). This suggests that EPEC-induced barrier function alteration may be a prerequisite for a component(s) in the supernatant to access and activate the basolateral EGFR.

EPEC-induced EGFR activation is inhibited by AG1478 or EGFR-neutralizing antibodies. To determine the specificity of EGFR activation by EPEC, we examined the effect of tyrphostin AG1478, a highly selective inhibitor of EGFR kinase activity (50% inhibitory concentration, 3 nM, compared to $>50 \mu$ M for other receptor tyrosine kinases) (26). Alternatively, cells were pretreated with neutralizing antibodies that specifically bind to the ectodomain of EGFR, as described earlier (48). EGFR autophosphorylation was inhibited with both treatments in EPEC-infected intestinal epithelial cells (Fig. 4). Unlike the neutralizing antibodies, treatment with preimmune

FIG. 2. A secreted EPEC component induces EGFR autophosphorylation. (A) C2_{BBE} cells were treated with filter-sterilized EPEC supernatants for 60, 120, and 240 min. The monolayers were washed and the cell extracts (300 μ g) immunoblotted against EGFR pY1068, total EGFR, and actin. (B) Data from three similar experiments were evaluated by densitometry ($P < 0.05$ by analysis of variance for 240 min).

FIG. 3. EPEC-induced EGFR activation is delayed in polarized epithelial cells. (A) Polarized C2_{BBE} cells grown on permeable supports were infected with WT EPEC, and the extracts isolated at specific time points were immunoblotted against EGFR pY1068. EGF-treated A431 cell extract was included as a positive control to confirm the identity of the band. (B) Data from three similar experiments were evaluated by densitometry ($P < 0.005$ by analysis of variance for 240 min).

serum had no effect on EPEC-induced EGFR phosphorylation (data not shown). These reagents were therefore employed to investigate the role of EPEC-induced EGFR activation in the alteration of host cell physiology.

FIG. 4. EPEC-induced EGFR transactivation is inhibited by AG1478 and EGFR-neutralizing antibodies. Confluent C2_{BBE} monolayers were treated with medium alone (A and B, lanes 1 to 5), AG1478 (1 μ M; A), or EGFR-neutralizing antibody (10 μ g/ml; B) for 1 h, and the cells were infected for up to 4 h in the continued presence of the inhibiting compounds. Extracts isolated at specific time points were immunoblotted for pEGFR, EGFR, and actin.

EGFR phosphorylation has no role in bacterial attachment, actin recruitment to attached bacteria, or EPEC-induced barrier function alteration. Inhibition of EGFR phosphorylation did not significantly reduce EPEC attachment to intestinal epithelial cells (CFU, $1.04 \times 10^6 \pm 4.52 \times 10^5$ in the dimethyl sulfoxide [DMSO] control versus $5.33 \times 10^5 \pm 2.63 \times 10^5$ in AG1478-treated cells; $n = 3$; $P > 0.1$). A key event in EPEC pathogenesis is tyrosine phosphorylation of Tir by the kinases Fyn, Arg, or Abl and subsequent pedestal formation (37, 42). To explore a potential direct or indirect role for the EGFR tyrosine kinase activity in this process, C2_{BBE} cells grown on coverslips were infected with EPEC for 1 h in the presence or absence of AG1478 and evaluated for actin distribution using fluorescein phalloidin. Actin accumulation was evident around attached bacteria, and AG1478 did not interfere with this phenotype (Fig. 5), suggesting that EGFR transactivation is not essential for pedestal formation. To examine the role of EGFR autophosphorylation on EPEC-induced epithelial barrier function alteration, polarized C2_{BBE} monolayers grown on permeable filters were infected with WT EPEC or treated with medium alone in the presence or absence of AG1478, and the TER was monitored. While EPEC infection of C2_{BBE} monolayers resulted in a drop in TER (uninfected control, 26.64 ± 3.01 ; EPEC, -74.34 ± 0.29 at 6 h postinfection), AG1478 treatment had no effect on this phenotype (EPEC plus AG1478, -77.61 ± 0.65 at 6 h postinfection). This suggests that EPEC-induced EGFR activation does not play a role in barrier function alteration. These experiments also confirm that AG1478 does not have a generalized and nonspecific effect on EPEC infection.

Inhibition of EGFR transactivation attenuates EPEC-induced Akt phosphorylation and reduces survival of infected epithelial cells. Celli et al. demonstrated transient PI3K activation in EPEC-infected macrophages (8). PI3K activation has not been directly examined in epithelial cells; however, the PI3K inhibitor wortmannin significantly increased death of EPEC-infected intestinal epithelial cells (11). To explore PI3K activation in infected intestinal epithelial cells and determine the role of EGFR in this pathway, extracts from

FIG. 5. EPEC-induced EGFR phosphorylation does not affect actin accumulation around attached bacteria. Confluent C2_{BBE} monolayers grown on glass coverslips were infected with EPEC in the presence or absence of AG1478. Cells were fixed with paraformaldehyde and then incubated with fluorescein phalloidin to stain for actin. Images were captured using a Nikon Opti-Phot microscope and the Spot-RT digital imaging system.

EPEC-treated cells were immunoblotted for total and phospho-Akt. EPEC infection resulted in Akt phosphorylation, with maximal activation being observed at 4 h postinfection (Fig. 6A and B). Prior treatment of C2_{BBE} cells with AG1478 significantly attenuated EPEC-induced Akt phosphorylation. EGFR-neutralizing antibody, but not preimmune serum, similarly attenuated Akt phosphorylation in infected cells (data not shown). This supports the notion that EPEC-induced EGFR phosphorylation contributes to activation of the PI3K/Akt pathway. The increased death of EPEC-infected epithelial cells in the presence of the PI3K inhibitor wortmannin suggests that this pathway contributes to host cell survival (11).

Since EGFR contributes to PI3K activation, the effect of EGFR inhibition on the survival of EPEC-infected C2_{BBE} cells was examined. C2_{BBE} monolayers were infected with EPEC for 2 hours in the presence of DMSO (vehicle), AG1478, or EGFR-neutralizing antibody. Since EPEC is a relatively weak inducer of apoptosis/necrosis, a larger inoculum (MOI, 100), comparable to that used by Crane et al. (11), was employed in these studies. Dead cells were identified by their permeability to ethidium homodimer. DMSO (vehicle), AG1478, or the neutralizing antibody alone did not induce significant host cell death (Fig. 6C and D). EPEC infection induced about 4% host cell death, and this was considerably increased in the presence of AG1478 or EGFR-neutralizing antibody. The greater number of dead cells in the presence of AG1478 compared to treatment with the neutralizing antibody may reflect incomplete inhibition of EGFR phosphorylation with the latter (Fig. 4B, 120' in the presence of the antibody). Consistent with a previous report, the PI3K inhibitor wortmannin also promoted the death of EPEC-infected cells (11); the number of dead cells was comparable to that seen with AG1478 treatment (percent dead cells, 21.92 ± 1.79 for EPEC plus wortmannin versus 20.09 ± 1.38 for EPEC plus AG1478). These studies suggest that EPEC-induced EGFR activation contributes to host cell survival, possibly via activation of the PI3K/Akt pathway.

EPEC-induced EGFR phosphorylation contributes to ERK activation and IL-8 production. EPEC promotes ERK1/2 phosphorylation, and inhibition of this pathway resulted in a partial reduction of IL-8 production in infected cells (13, 39). EGFR is a well-known activator of ERK1/2 phosphorylation in many systems (27). To assess the role of EGFR transactivation in EPEC-induced ERK phosphorylation, C2_{BBE} cells were infected for specific time periods in the presence or absence of AG1478, and the corresponding extracts were immunoblotted for phospho-ERK1/2. EPEC infection resulted in ERK1/2 phosphorylation, and this was inhibited by AG1478 (Fig. 7A and B), suggesting that EPEC-induced EGFR transactivation contributes to this pathway.

Since ERK1/2 phosphorylation contributes to IL-8 production, the effect of AG1478 on IL-8 secretion was examined. Since C2_{BBE} cells display a relatively weak IL-8 response (51.98 ± 6.10 pg/ml in EPEC with DMSO versus 24.20 ± 2.18 pg/ml in EPEC with AG1478; $P = 0.013$), these studies were also performed in HeLa cells. Monolayers were infected with EPEC in the presence or absence of AG1478; following 6 h of infection, the medium was assessed for secreted IL-8 by ELISA. Consistent with earlier reports, EPEC promoted the secretion of IL-8 by infected epithelial cells (Fig. 7C). EPEC-induced IL-8 production was significantly inhibited by AG1478, suggesting that EGFR transactivation contributes to this proinflammatory pathway, possibly via phosphorylation of ERK1/2.

DISCUSSION

The experiments in this study suggest, for the first time, a role for EGFR in EPEC pathogenesis. EPEC promotes the phosphorylation of EGFR tyrosine residue Y1068 in intestinal epithelial cells. Our studies suggest that a component(s) in EPEC supernatants contributes to EGFR phosphorylation. Given the activation of EGFR by the commensal strain HS-4 (although delayed and attenuated), it is possible that pY1068 phosphorylation is mediated via a TLR ligand. To our knowl-

FIG. 6. Inhibition of EGFR phosphorylation curtails Akt phosphorylation and promotes host cell death. $C2_{BBE}$ cells were infected with WT EPEC for the specified time periods in the presence of DMSO or AG1478 (1 μ M). (A) The monolayers were then washed, and the cell extracts (300 μ g) were immunoblotted against Akt pS473, total Akt, and actin. (B) Densitometric analysis was performed on three such blots to determine pAkt abundance relative to total Akt and normalized to levels in control uninfected cells. ($P < 0.05$ by analysis of variance [ANOVA] for EPEC versus control at 240 min.) (C) To evaluate the viability of infected cells, $C2_{BBE}$ cells grown on coverslips were infected with WT EPEC in the presence of DMSO (vehicle), AG1478 (1 μ M), or EGFR-neutralizing antibody (10 μ g/ml) for 2 h, and ethidium homodimer uptake was monitored. Similarly treated uninfected cells were included as controls. (D) Quantitation of dead cells (fluorescent) as a percentage of the total cells in a given field. Each value, indicated as the mean $\pm$ standard error, was derived from a minimum of five fields from two independent experiments. *, $P < 0.001$ by ANOVA.

edge such cross-activation of EGFR by a TLR ligand has not hitherto been reported. Preliminary studies suggest that EPEC flagellin, the TLR5 ligand, is not involved in EGFR transactivation (data not shown). Delayed phosphorylation of EGFR in

polarized epithelial cells suggests that the activating component may depend on barrier function disruption to access the receptor directly.

We employed the EGFR-specific inhibitor tyrphostin AG1478

FIG. 7. The EGFR kinase inhibitor AG1478 inhibits ERK1/2 phosphorylation and curtails IL-8 secretion. (A) To examine the role of EGFR activation in ERK phosphorylation, C₂B_{BE} cells were infected with WT EPEC in the presence or absence of 1 μ M AG1478. The monolayers were then washed, and the cell extracts (25 μ g) were immunoblotted against pERK1/2, total ERK, and actin. (B) Densitometric analysis was performed on three such blots to determine pERK1 and pERK2 abundance relative to total ERK1 and -2, respectively, and normalized to levels in control uninfected cells. ERK1 and -2 were undetectable in tyrphostin-treated cells. (C) To explore the role of EPEC-induced EGFR autophosphorylation in IL-8 secretion, HeLa cells were infected with WT EPEC for 6 h in the presence or absence (DMSO) of 1 μ M AG1478. The supernatants were then isolated and assayed for IL-8 by ELISA.

and EGFR-neutralizing antibodies to evaluate the physiological role of EPEC-induced EGFR transactivation. Both approaches resulted in the inhibition of infection-dependent EGFR phosphorylation. Inhibition of phosphorylation did not have a generalized nonspecific effect on infection, since bacterial attachment, actin recruitment, and epithelial barrier disruption were not influenced by AG1478.

Our experiments identify EGFR as an upstream component in signaling pathways previously determined to play a role in EPEC pathogenesis (8, 13, 39). EPEC-induced EGFR autophosphorylation contributes to the activation of the prosurvival PI3K/Akt pathway. Thus, AG1478 and EGFR-neutralizing antibodies inhibited Akt phosphorylation and promoted the death of infected epithelial cells. It is possible that apoptosis/necrosis of host cells is detrimental at the early stages of infection by extracellular pathogens. Consistent with this notion, both *Helicobacter pylori* and *Pseudomonas aeruginosa* also enhance host cell survival by activating the EGFR/PI3K/Akt pathway (47, 49; F. Yan and D. B. Polk, personal communication). In macrophages, a relatively rapid, type III secretion-dependent, tyrosine phosphatase-mediated, down-regulation of PI3K activity was reported (8). While such a rapid attenuation was not seen in epithelial cells, we did observe down-

regulation of PI3K activity following infection for more than 4 h (data not shown).

As noted for the gastrointestinal pathogens *H. pylori* and *Clostridium difficile*, EPEC-induced EGFR activation promotes ERK1/2 phosphorylation and consequent IL-8 release (39, 25, 47). Shc, an EGFR substrate and an upstream regulatory protein in the ERK1/2 pathway, is also known to be activated by EPEC (13). While our studies suggest that EGFR activation promotes IL-8 release, other pathways also likely contribute to EPEC-induced inflammation (40, 50). EGFR activation may also play a role in ERK1/2-dependent activation of the transcription factor Egr-1 in EPEC-infected cells, a signaling cascade also reported in *H. pylori*-infected gastric epithelial cells (15, 24).

In summary, our studies demonstrate EGFR autophosphorylation in EPEC-infected epithelial cells. Inhibition of EGFR activation results in greater susceptibility of infected cells to apoptosis/necrosis and also results in attenuation of proinflammatory signaling. Taken together, our studies support a role for EGFR autophosphorylation in EPEC pathogenesis. Studies are under way to identify the EPEC molecules involved in EGFR phosphorylation and to determine the mechanism of activation.

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