Brief Definitive Report

A Diarrheal Pathogen, Enteropathogenic *Escherichia coli* (EPEC), Triggers a Flux of Inositol Phosphates in Infected Epithelial Cells

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Summary

Enteropathogenic *Escherichia coli* (EPEC) is a bacterial pathogen that causes diarrhea in infants by adhering to intestinal epithelial cells. EPEC induces host cell protein phosphorylation and increases intracellular calcium levels that may function to initiate cytoskeletal rearrangement. We found that EPEC triggers the release of inositol phosphates (IPs) after adherence of bacteria to cultured epithelial cells. We also demonstrated that the EPEC-induced flux of IPs precedes actin rearrangement and bacterial invasion. EPEC mutants and tyrosine protein kinase inhibitors were used to establish that formation of IPs is dependent on tyrosine phosphorylation of a 90-kD HeLa protein. Collectively these results suggest that EPEC-induced tyrosine phosphorylation of a host cell substrate(s) leads to release of IPs, which may then trigger cytoskeletal rearrangement.

Enteropathogenic *Escherichia coli* (EPEC)[1] is a leading cause of infantile diarrhea, particularly in developing countries (1). It is thought that intimate attachment of EPEC to host cell leads to malabsorption and diarrhea. EPEC attaches to cells in distinct microcolonies by a process termed localized adherence which is mediated by a plasmid-encoded bundle-forming pilus and probably by other factors (2–4). EPEC's attaching and effacing (A/E) activity mediates intimate contacts between the bacterium and host cells (5). Intimin, the product of the *eaeA* locus, is a 94-kD outer membrane protein that is required for this tight adherence (6–8). The A/E contact lesion is characterized by loss of microvilli and accumulation of cytoskeletal components beneath the attached bacterium (9). EPEC is also capable of entering into (invading) normally nonphagocytic epithelial cells (10).

EPEC-induced cytoskeletal rearrangement and invasion is dependent on tyrosine phosphorylation of a 90-kD host protein (Hp90) (11). The *cfm* gene(s) is needed for invasion and tyrosine phosphorylation, but not for adherence (7, 11). The *eaeA* gene is not needed for tyrosine phosphorylation of Hp90, but is necessary for intimate attachment and invasion (6, 11). Adherence of EPEC to cultured cells also causes localized elevation of cytoplasmic calcium ($[Ca]_i$) in the host cell (12). Inhibition of this elevation by dantrolene suggests that the calcium is released from inositol 1,4,5-trisphosphate (IP_3)–sensitive stores (12). This $[Ca]_i$ elevation stimulates calcium-dependent serine/threonine protein kinases to phosporylate several host cell proteins, including the myosin light chain (13–15). In this communication we examined whether EPEC triggered the formation of IPs in infected epithelial cells.

Materials and Methods

Bacterial Strains. EPEC strains E2348/69, JPN15, CVD206, 10-5-1(1), 14-2-1(1), and 27-3-2(1) are described elsewhere (7–9) and were grown in Luria-Bertani (LB) agar or LB broth at 37°C without shaking.

Tissue Culture. Epithelioid HeLa (CCL 2) cells, Caco-2 (HTB 37) cells, and Henle-407 (CCL 6) cells were obtained from American Type Culture Collection (Rockville, MD). All cells were maintained in MEM (GIBCO BRL, Gaithersburg, MD), 10% FCS (GIBCO BRL), nonessential amino acids, penicillin (100 μg/ml), and streptomycin (100 μg/ml).

Inhibitors. Inhibitors were dissolved in dimethyl sulfoxide. Stock solutions of cytochalasin D (1 mg/ml; Sigma Chemical Co., St. Louis, MO), staurosporine (1 mM; Boehringer Mannheim, Mannheim, Germany), and genistein (100 mM; Upstate Biotechnology Inc., Lake Placid, NY) were diluted in tissue culture fluid before use.

Measurement of IP Release. IP release was determined as described by Ruschkowski et al. (16). Cells were seeded onto 60-mm petri dishes and labeled with [^{3}H]myoinositol (18.4 Ci/mmol; Amersham Life Science, Arlington Heights, IL). Cells were washed and either infected with 40 μl of a fresh overnight bacterial culture ($\sim$1.5 $\times$ 10^6 bacteria), treated with inhibitors (250 μM genistein, 1 μM staurosporine, or 2.5 μg/ml cytochalasin D), infected with bacteria in the presence of inhibitors, or left untreated. After specified times, cells were washed with cold PBS and harvested. IPs were collected off a Dowex anion exchange column (AG 1-X8 resin, 100–200 mesh formate form; Bio-Rad Laboratories, Richmond, CA) either collectively with 1 M ammonium formate in 20 ml of 0.1 M formic acid, or separately as follows: inositol monophosphate (IP), 20 ml of 0.1 M formic acid/0.2 M ammonium formate; inositol bisphosphate (IP_2), 20 ml of 0.1 M formic acid/0.4 M ammonium formate; inositol trisphosphate (IP_3), 30 ml of 0.1 M

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formic acid/0.8 M ammonium formate; and inositol tetrakisphosphate (IP_4), 20 ml of 0.1 M formic acid/1.0 M ammonium formate. The counts per minute represent the release of IPs.

Invasion and Cell Association Assays. Invasion and cell association levels were determined as described by Finlay and Falkow (17). To determine the number of cell-associated (adherent and intracellular) bacteria, the eukaryotic cells were lysed without gentamicin treatment. These assays were carried out in quadruplicate.

Results

EPEC Induces a Flux of IPs in Infected Human Epithelial Cells. To determine if EPEC triggers a flux of IPs, HeLa cells were labeled with [^{3}H]myoinositol and infected with bacteria for increasing times. The level of total [^{3}H]IPs began increasing ~2 h after EPEC addition, reaching a maximum of 2.6-fold over uninfected at 3 h (Fig. 1). Addition of 200 ng/ml of epidermal growth factor (EGF) (as a positive control) for 10 min before IP extraction caused a 1.9-fold increase in [^{3}H]IP release. Similar to the flux of IPs, the rate of association (adherence and invasion) of EPEC to HeLa cells peaked at 3–3.5 h (Fig. 2 *A*), and bacterial entry into HeLa cells reached a maximum rate at 3.5 h (Fig. 2 *B*).

Individual IPs were also measured and the levels of IP, IP_3, and IP_4 all peaked ~3 h after EPEC infection (Table 1). The inability to detect an increase in IP_2, and the small increases in IP_3 and IP_4 are probably a result of the rapid conversion of most forms of these different species of IP (18), although the conversion of IP to inositol is prevented by Li^+ in the assay buffer. The levels of IP, IP_2, IP_3, and IP_4 are known to increase after stimulation with EGF (19).

It has previously been reported that preinducing EPEC can shorten the time required to establish LA (3, 20). We tested whether preinducing the bacteria in tissue culture medium, or spinning them onto the cell monolayers and allowing them to adhere on ice before warming and measurement of IPs, would decrease the time required by EPEC to induce a flux of IPs. These conditions decreased the time needed for EPEC-induced release of IPs by ~2 h. However, even under these conditions, EPEC-induced formation of IPs still required a minimum of 60 min before increasing, a much longer time than an EGF-induced flux (10 min).

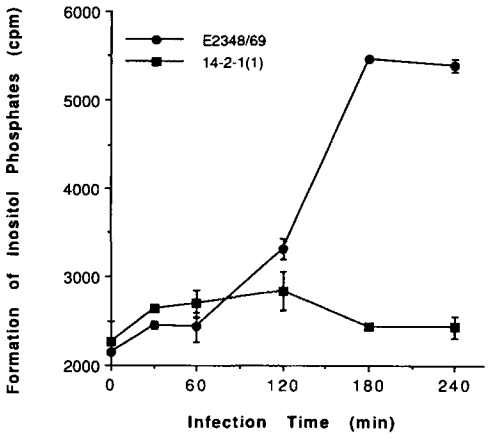

Figure 1. Formation of IPs in HeLa cells infected with EPEC or a *cfm*::Tn*phoA* mutant [14-2-1(1)]. Formation time points were an average of two samples ± error and are representative of one of six different experiments.

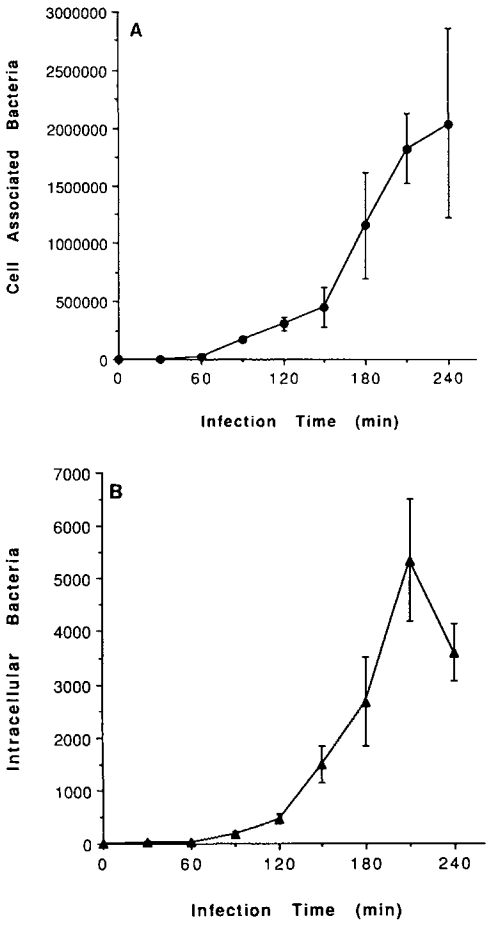

Figure 2. EPEC association with (*A*) and entry into (*B*) HeLa cells. Values are the average of four samples ± SD and represent the results of two independent experiments.

The ability of EPEC to induce a flux of IPs in two other human epithelial cell lines was examined. Formation of IPs was found to increase 1.3-fold in Caco-2 and 1.9-fold in Henle-407 cells (Fig. 3). The kinetics of these fluxes of IPs were similar to that observed for EPEC-infected HeLa cells, with maximal levels of IPs induced 3 h after infection. HeLa cells were used for the remainder of the experiments.

Formation of IPs Is Not a Consequence of Actin Rearrangement or Bacterial Invasion. To determine if EPEC-induced release of IPs triggers, or is a result of, bacterial internalization, we analyzed the effect of blocking EPEC invasion on levels of IPs after infection. Cytochalasin D inhibits actin polymerization and blocks entry of EPEC into human cells, without affecting adherence (21, 22). We found that 2.5 μg/ml cytochalasin D decreased invasion by 99.5%. 2.5 μg/ml cytochalasin D was added to [^{3}H]myoinositol-labeled HeLa cells either independently or simultaneously with EPEC and

Table 1. *Release of Separate IPs* in HeLa Cells After Different Treatments*

Treatment	Time	IP	IP_2	IP_3	IP_4
Untreated		3,610.7 ± 1,596.5	2,320.0 ± 276.1	740.4 ± 84.0	154.1 ± 10.0
EPEC	3 h	11,841.1 ± 446.8	2,329.9 ± 273.7	865.4 ± 179.9	170.9 ± 1.3
eaeA::Tn*phoA*	3 h	9,607.86 ± 206.56	2,229.4 ± 233.3	796.7 ± 144.7	156.9 ± 16.8

* Representative of three separate experiments.

incubated for 3 h. Formation of [^{3}H]IPs was induced by EPEC in the presence of cytochalasin D, demonstrating that cytoskeletal rearrangement and bacterial invasion are not prerequisites for formation of IPs (Fig. 4).

EPEC Mutants That Fail to Induce Host Cell Protein Tyrosine Phosphorylation Do Not Trigger a Flux of IPs. We next examined whether noninvasive mutants of EPEC, which differ in their ability to stimulate tyrosine phosphorylation of a 90-kD host protein, Hp90, induce a flux of IPs. Several classes of these mutants were examined: (*a*) two strains of *eaeA* mutants: a Tn*phoA* insertion [10-5-1(1)] and an internal deletion (CVD206); (*b*) two different Tn*phoA* class 4 insertion mutations (*cfm*) [14-2-1(1) and 27-3-2(1)]; and (*c*) JPN15, a nonadherent EPEC strain that is cured of its large plasmid.

The *eaeA*::Tn*phoA* and Δ*eaeA* mutants, which still induce tyrosine phosphorylation of Hp90, also induced the flux of IPs at 3 h (Table 2). Although the amount of released IPs was lower than wild type EPEC, the increase in individual IPs was proportionately similar (Table 1). JPN15, the plasmidless strain, which is reduced in adherence and induction of Hp90 phosphorylation (11, 23), did not induce release of IPs (Table 2). Both *cfm*::Tn*phoA* mutants, which adhere normally but do not induce tyrosine phosphorylation, did not trigger a flux of IPs (Fig. 1 and Table 2).

Host Cell Tyrosine Phosphorylation Precedes EPEC-Induced Formation of IPs. EPEC-induced tyrosine phosphorylation of Hp90 and bacterial invasion are both attenuated after treatment with the protein kinase inhibitors genistein and staurosporine. Staurosporine, however, inhibits Hp90 tyrosine phosphorylation less efficiently than genistein. We examined whether these drugs also inhibited the EPEC-induced flux of IPs. 1 μM staurosporine or 250 μM genistein was added either independently or simultaneously with bacteria to [^{3}H]myoinositol-labeled HeLa cells. After a 3-h infection, formation of [^{3}H]IPs was measured. Genistein completely inhibited the formation of EPEC-induced IPs, whereas treatment with staurosporine had only a small effect (Fig. 5).

Formation of IPs was also measured at increasing times in the presence of 250 μM genistein with or without EPEC. Unexpectedly, genistein alone induced a flux of IPs that peaked 2 h after drug addition and then gradually declined (Fig. 6). The level of IPs in cells infected with EPEC in the presence of genistein increased in a manner similar to that induced by the drug alone, but after 2 h the level of IPs declined sharply

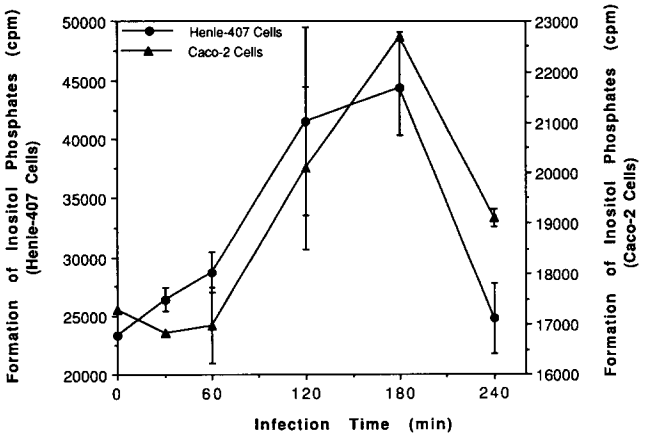

Figure 3. EPEC-induced formation of IPs in Henle-407 and Caco-2 human epithelial cells. Values are the average of four samples (Henle-407) or two samples (Caco-2) ± error and represent the results of two different experiments.

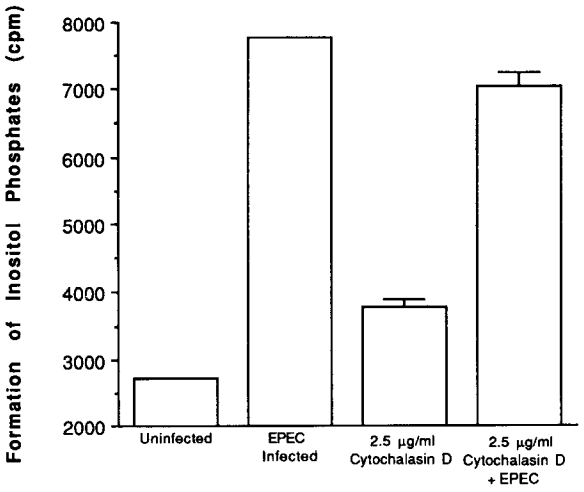

Figure 4. Effect of cytochalasin D on EPEC-induced flux of IPs. [^{3}H]myoinositol-labeled HeLa cells were infected for 3 h with or without 2.5 μg/ml cytochalasin D. Values are the average of two samples ± error and represent the results of two independent experiments.

Table 2. *Release of IPs in HeLa Cells Infected for 3 h with Different Noninvasive EPEC Mutants*

Genotype	Phenotype	Localized adherence	A/E lesions	Hp90 tyrosine phosphorylation	Release of IPs at at 3 h after infection
					cpm
Uninfected					3,156.2 ± 532.7
E2348/69	Wild type	+	+	+	8,440.0 ± 649.8
CVD206	Δ*eaeA*	+	−	+	6,703.7 ± 764.1
10-5-1(1)	*eaeA*::Tn*phoA*	+	−	+	6,386.7 ± 395.7
14-2-1(1)	*cfm*::Tn*phoA*	+	±	−	4,144.0 ± 689.2
27-3-2(1)	*cfm*::Tn*phoA*	+	±	−	4,467.3 ± 271.7
JPN15	plasmid cured	−	±	±	3,117.0 ± 145.5

* Average of a minimum of three assays.

(Fig. 6). In contrast, EPEC infection without genistein caused the level of IPs to increase after 2 h, reaching a even higher level at 3 h (Fig. 6). These results suggest that genistein does not inhibit the EPEC-induced peak of IPs at 3 h by a direct mechanism such as phospholipase C (PLC) inhibition, as this drug alone induces the level of IPs to increase at 2 h. Instead, it suggests that genistein inhibits a protein tyrosine phosphorylation event(s) that is required for downstream activation by EPEC.

Discussion

We found that EPEC infection triggers an increase in the level of IPs within cultured epithelial cells. In contrast to the rapid formation of IPs that occurs within seconds or minutes in response to many growth factors and hormones, EPEC induced the release of IPs only after several hours of infection. The different kinetics are not solely a result of the time needed for EPEC to establish adherence, as release of IPs takes a minimum of 60 min to commence after bacterial attachment. It is interesting to note that other host signals triggered by EPEC, including Hp90 tyrosine phosphorylation and increased $[Ca]_i$, are also induced a similar times (11, 12). These signals do not appear to be transient, but instead are relatively stable and continuous. Therefore EPEC may use a novel mechanism(s) to manipulate defined host signals to establish infection.

The *cfm*::Tn*phoA* mutants do not induce tyrosine phosphorylation of Hp90 or formation of IPs, suggesting that Hp90 tyrosine phosphorylation may be involved in the release of IPs. In addition, the effectiveness of two different kinase inhibitors to block Hp90 tyrosine phosphorylation also corresponds with their ability to inhibit a flux of IPs (11). Genistein efficiently inhibits both Hp90 tyrosine phosphory-

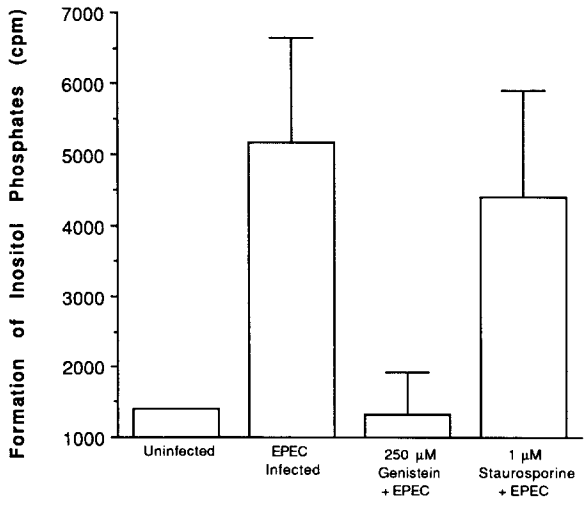

Figure 5. Effect of staurosporine and genistein on formation of IPs by EPEC in HeLa cells. 250 μM genistein or 1 μM stauroporine were added or with bacteria to [^{3}H]myoinositol labeled HeLa cells and incubated for 3 h. Samples are the average of three values ± SD and represent the results of four independent experiments.

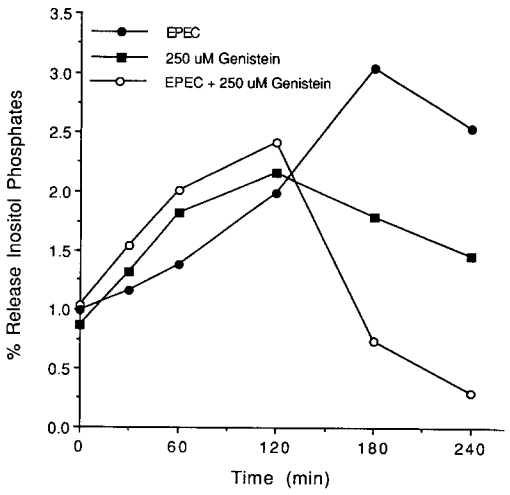

Figure 6. Rate of release of IPs in genistein-treated, uninfected, or EPEC-infected HeLa cells. All samples were the average of two values.

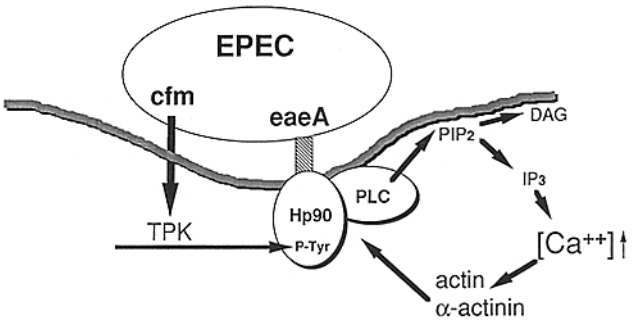

Figure 7. A model of the interaction of EPEC with HeLa cells. EPEC attaches to the cell surface and uses the *cfm* gene(s) product(s) to trigger Hp90 tyrosine phosphorylation by inducing a host tyrosine protein kinase (*TPK*) activity. This, possibly with other signals, activates a host PLC to cleave PIP_2 to DAG and IP_3. IP_3 mobilizes $[Ca^{2+}]_i$, which initiates cytoskeletal rearrangement beneath the attached bacterium. The *eaeA* gene product, intimin, is involved in nucleating the subbacterial structure that contains both signaling and cytoskeletal proteins.

lation and release of IPs, whereas staurosporine incompletely inhibits Hp90 tyrosine phosphorylation and has little effect on formation of IPs. Since staurosporine only partially inhibits Hp90 phosphorylation but does not effectively block release of IPs, the phosphorylation event may not be a rate limiting step. Alternatively, genistein may specifically inhibit the tyrosine phosphorylation of a substrate that is not affected by staurosporine that participates in the release of IPs by EPEC. Both of these conclusions support the involvement of host tyrosine phosphorylation in the release of IPs by EPEC.

The IPs released after EPEC infection contain many species of phosphorylated inositol including IP, IP_3, and IP_4. The different IPs are proposed to have roles as second messengers, although the role of IP_3 is the best characterized since binding of IP_3 to its receptor calcium from intracellular stores (24). Therefore, our results suggest that the EPEC-induced increase in IPs occurs upstream of the reported intracellular calcium flux (13). It is likely that the intracellular changes induced by increased calcium levels initiate cytoskeletal rearrangement, disrupt the function of the brush border microvilli, affect epithelial permeability (25), and ultimately may contribute to diarrhea.

Based on our findings, we propose a model in which intimate contact between EPEC and the host cell is needed to induce host tyrosine kinase activity (Fig. 7). The activated tyrosine kinase phorphorylates Hp90, and possibly PLC. Activated PLC would generate IP_3, thereby leading to the release of calcium from intracellular stores. The increased calcium levels could then induce cytoskeletal rearrangements and activate calcium-dependent kinase(s) resulting in morphological changes in the host cell.

Salmonella typhimurium and *Helicobacter pylori* also induce the formation of IPs in infected cells (16, 26). *S. typhimurium* rapidly stimulates release of IPs in epithelial cells, with maximal formation occurring 30 min after infection, simultaneous to its invasion rate (16). However tyrosine kinase inhibitors do not decrease entry of *S. typhimurium* into host cells (27). Therefore, although both EPEC and *S. typhimurium* trigger an IP flux, different host cell mechanisms appear to be activated both downstream and upstream of this common signal messenger.

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