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A pathogenic bacterium triggers epithelial signals to form a functional bacterial receptor that mediates actin pseudopod formation

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Enteropathogenic *E.coli* (EPEC) belongs to a group of bacterial pathogens that induce actin accumulation beneath adherent bacteria. We found that EPEC adherence to epithelial cells mediates the formation of finger-like pseudopods (up to 10 μm) beneath bacteria. These actin-rich structures also contain tyrosine phosphorylated host proteins concentrated at the pseudopod tip beneath adherent EPEC. Intimate bacterial adherence (and pseudopod formation) occurred only after prior bacterial induction of tyrosine phosphorylation of an epithelial membrane protein, Hp90, which then associates directly with an EPEC adhesin, intimin. These interactions lead to cytoskeletal nucleation and pseudopod formation. This is the first example of a bacterial pathogen that triggers signals in epithelial cells which activates receptor binding activity to a specific bacterial ligand and subsequent cytoskeletal rearrangement.

Keywords: actin/diarrhea/enteropathogenic *Escherichia coli*/pathogenesis/signal transduction

Introduction

Enteropathogenic *Escherichia coli* (EPEC) is a member of a group of pathogenic organisms that adhere to host cells and cause localized accumulation of host actin beneath adherent organisms (Donnenberg and Kaper, 1992). Other members of this group include enterohemorrhagic *E.coli* (EHEC, the causative agent of hemorrhagic colitis or 'hamburger disease'), *Citrobacter freundii* and *Hafnia alvei*. EPEC is a leading cause of infantile diarrhea in developing nations contributing to high morbidity and mortality (Levine and Edelman, 1984). EPEC form small colonies on the surface of infected epithelial cells, a process called localized or initial adherence. Initial adherence is followed by intimate contact between EPEC and infected cells, and localized degeneration of the epithelial brush border microvilli, resulting in the formation of an attaching and effacing (AE) lesion. The AE lesion (or pedestal) is associated with the assembly of highly organized cytoskeletal structures in the epithelial cells immediately beneath the adherent bacteria (Knutton *et al.*, 1989) and includes the cytoskeletal components α-actinin, myosin light chain, ezrin and talin (Finlay *et al.*, 1992; Manjarrez-Hernandez *et al.*, 1992). The ability of EPEC to induce its own internalization by normally non-phagocytic epithelial cells (invasion) is probably a consequence of this cytoskeletal rearrangement (Andrade *et al.*, 1989; Donnenberg *et al.*, 1990b).

Initial bacterial adherence is dependent on the presence of a 55–70 MDa plasmid that is common to EPEC strains. This process is mediated by a plasmid encoded bundle forming pilus (BFP), and possibly other factors (Donnenberg *et al.*, 1992; Giron *et al.*, 1993). Mutants in EPEC that are defective in initial adherence produce less AE lesions on epithelial cells. Intimin is the product of a bacterial chromosomal locus, *eaeA*, and is a 94 kDa EPEC outer membrane protein that is needed for intimate adherence (Donnenberg and Kaper, 1991). *eaeA* mutants form immature AE lesions and do not organize phosphotyrosine proteins and cytoskeletal components beneath adherent bacteria, although epithelial signals are still transduced to the cell (Rosenshine *et al.*, 1992). Intimin appears to participate in reorganization of the underlying host cytoskeleton after other bacterial factors stimulate epithelial signal transduction. However, how intimin, on the bacterial surface, is linked across the epithelial membrane to the underlying cytoskeleton is not known, nor is the intimin receptor on epithelial surfaces. Signals triggered in epithelial cells infected with EPEC include tyrosine phosphorylation of a 90 kDa host epithelial protein (Hp90) and generation of inositol triphosphate and intracellular calcium fluxes (Rosenshine *et al.*, 1992; Dytoc *et al.*, 1994; Foubister *et al.*, 1994a), although the role of these signals is not known. Moreover, little is known about the function of epithelial Hp90 other than it is tyrosine phosphorylated after EPEC infection. Other EPEC loci, including *sep* (includes cfm mutants) and *eaeB*, are involved in transduction of signals to the host epithelial cell (Rosenshine *et al.*, 1992; Foubister *et al.*, 1994a,b). Mutants that do not activate signal transduction in the host cell cannot form AE lesions and cause no cytoskeletal rearrangement in host cells (Rosenshine *et al.*, 1992; Foubister *et al.*, 1994b).

In this communication we show that intimin-mediated intimate adherence is dependent on prior tyrosine phosphorylation of Hp90, that intimin associates with Hp90 and that tyrosine phosphorylation of Hp90 and its interaction with intimin leads to the assembly of novel actin-containing pseudopods in infected epithelial cells. It appears that EPEC 'creates' an epithelial cell receptor needed for bacterial adherence by activating host signal transduction pathways, a previously undescribed mechanism in microbial pathogenesis. We also show that surface localized EPEC can trigger the formation of specialized underlying cytoskeletal structures (extended pseudopods) which can be used to study actin polymerization, signal transduction and cytoskeletal interactions with epithelial membranes.

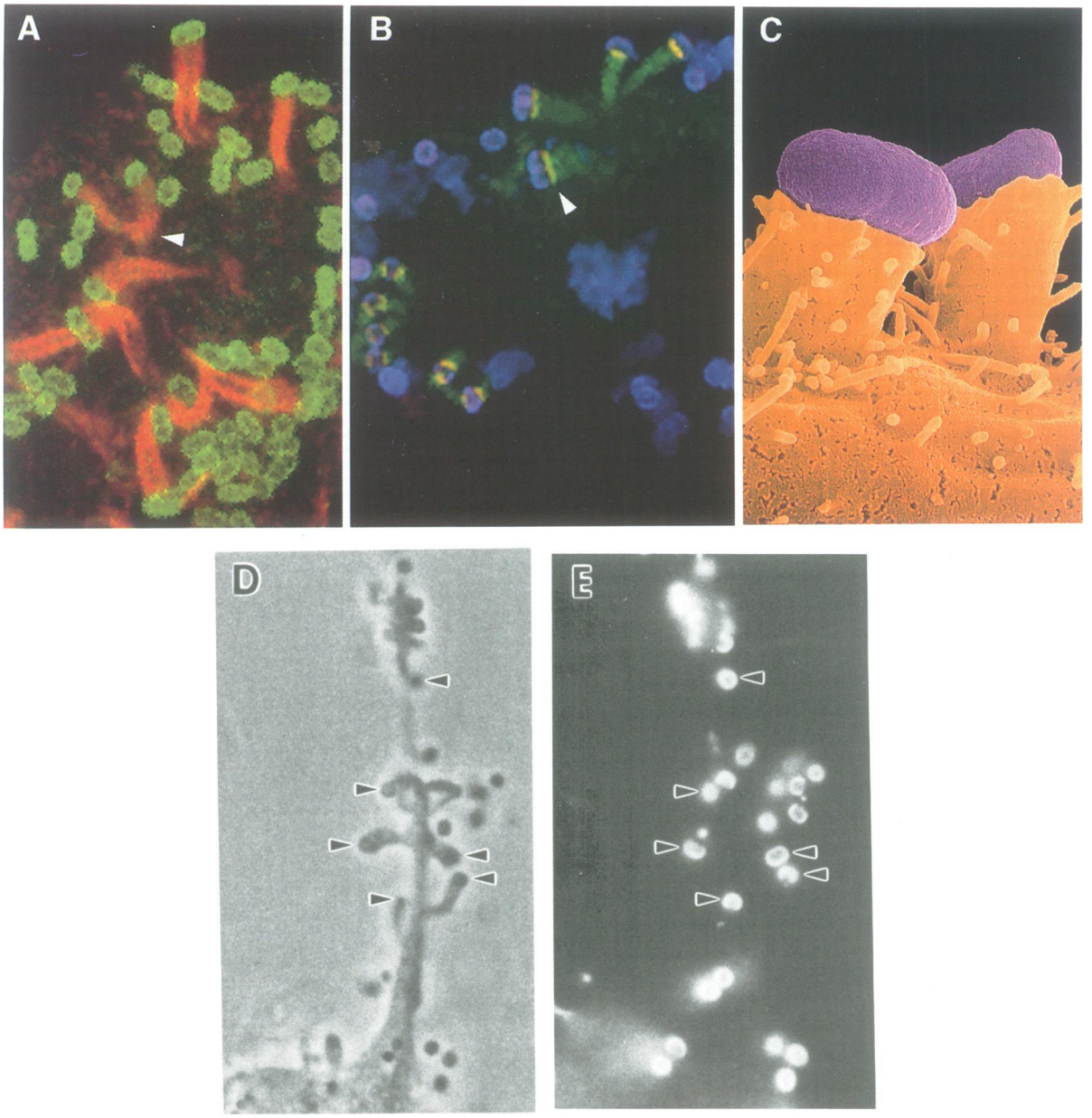

Fig. 1. Surface bound EPEC induces finger-like pseudopods in HeLa cells. Confocal fluorescence microscopy of (**A**) actin tails stained with rhodamine phalloidin (red) in cells infected with EPEC JPN15 (green), or (**B**) triple labeling of actin (green), EPEC JPN15 (blue) and anti-phosphotyrosine antibodies (yellow) illustrating the concentration of host tyrosine phosphorylated proteins immediately beneath the bacteria at the end of the pseudopods (marked by arrow). Arrow in (A) represents a fork in the pseudopod projection. (**C**) is a SEM image of wild type EPEC bound to HeLa cells. Image C was artificially colored to separate EPEC (purple) from the HeLa cell (brown). (**D**) is a phase contrast image of HeLa cells infected with EPEC for 6 h illustrating the pseudopods with EPEC at their tips. (**E**) is the corresponding fluorescence micrograph that had been stained with anti-EPEC antibodies and rhodamine phalloiding without permeabilization, indicating that EPEC are extracellular at the tip of pseudopods. Note that actin filaments are not fluorescently labeled since cells were not permeabilized. Fluorescently labeled extracellular bacteria at the tips of the pseudopods are marked by arrows.

Results

Formation of actin-containing pseudopods by extracellular EPEC

Attachment of EPEC to HeLa epithelial cells results in formation of pedestals beneath the adherent bacterium, often pushing the bacterium slightly above the epithelial surface (Knutton *et al.*, 1989). We found that EPEC-induced pedestals frequently evolve into extended finger-like structures (which we call pseudopods) on epithelial cell surfaces. We examined these pseudopods by scanning electron microscopy (SEM) (Figure 1C). The length of these projections varied from >1 μm to >10 μm (EPEC is ~1 μm in diameter). Pseudopods were also visible using phase contrast microscopy as dark projections protruding out of the cell membrane with bacteria at their tips (Figure 1D and E). Formation of similar pseudopods were also observed in Henle 407 (human intestinal) and MDCK (dog kidney) epithelial cells, although the efficiency and kinetics of pseudopod formation varied between cell lines (data not shown). EPEC strain JPN15, which is cured of its large virulence plasmid (and thus the genes encoding the BFP), adhered to cells to a lesser extent (Table I) and also induced fewer pseudopods per host cell, although the

Table I. Phenotypes of various EPEC mutants

Strain (mutant)	Pseudopod formation	Invasion efficiency (%)	Hp90 tyrosine phosphorylation	PY proteins focused	Adherence to HeLa cells ($\times 10^2$)
E2348/69 (wt)	+	100	+	+	1310 ± 126
CVD206 (Δ*eaeA*)	–	7	+	–	1772 ± 396
UMD869 (Δ*eaeB*)	–	<1	– (+ if preinduced)	–	839 ± 97
cfm 27-3-2(1) (*sep1*::Tn*phoA*)	–	<1	– (+ if preinduced)	–	1129 ± 100
cfm 14-2-1(1)	–	<1	– (+ if preinduced)	–	1087 ± 96
JPN15 (pMAR2 cured)	+	7	+/–	+	365 ± 57

pseudopods formed were indistinguishable from those induced by wild type EPEC.

EPEC-induced pseudopods contained polymerized actin in their stalks, as detected by immunofluorescence microscopy of infected HeLa cells. (Figure 1A and B). In addition to F-actin, the pseudopods also contained α-actinin. We examined whether the EPEC that were inducing these actin containing pseudopods were intracellular (in contact with the host cell cytosol), or extracellular. HeLa cells were infected with EPEC strain JPN15 for 4 h, washed, fixed with paraformaldehyde and the unpermeabilized cells were labeled with rabbit anti-EPEC antisera and rhodamine–phalloidin. This procedure stains extracellular bacteria but the intact host cell membrane prevents staining of intracellular bacteria and actin filaments. All EPEC-induced pseudopods that were visible by phase-contrast microscopy had a fluorescent bacterium on their tip but the underlying actin was not stained by phalloidin–rodamine (Figure 1D and E). This, together with the SEM analysis (Figure 1C), indicates that EPEC located at the tips of these protrusions are extracellular.

The distal tip of pseudopods contains tyrosine phosphorylated proteins

We used confocal immunofluorescence microscopy to examine the distribution of tyrosine phosphorylated proteins in the projections. Tyrosine phosphorylated proteins were restricted to an area immediately beneath the attached bacteria at the tip of the pseudopods (Figure 1B). Several EPEC mutants which are deficient in their ability to induce tyrosine phosphorylation of the host protein Hp90 [*eaeB*, cfm 27-3-2(1) and cfm 14-2-1(1)] or in focusing cytoskeletal components and tyrosine phosphorylated proteins beneath EPEC (*eaeA*) have been described (Donnenberg *et al.*, 1990b; Rosenshine *et al.*, 1992; Foubister *et al.*, 1994b). All of these mutants bind to host cells but do not induce the formation of actin-rich pseudopods, nor do they accumulate tyrosine phosphorylated proteins immediately beneath the bacteria (Table I).

Hp90 tyrosine phosphorylation is independent of actin polymerization

The induction of Hp90 tyrosine phosphorylation and the accumulation of tyrosine phosphorylated proteins at the tip of the actin tail may be dependent on earlier actin polymerization, or, alternatively, tyrosine phosphorylation may precede actin polymerization. We examined the assembly of the actin structure, tyrosine phosphorylation of Hp90 and accumulation of the tyrosine phosphorylated proteins beneath the attached EPEC in HeLa cells treated with the actin depolymerizing agent, cytochalasin D. As expected, cytochalasin D treatment had a strong effect on the HeLa cell shape and prevented specific actin or α-actinin condensation underneath the attached EPEC, as determined by immunofluorescence staining. As a result, pseudopod formation was inhibited. However, condensation of tyrosine phosphorylated proteins underneath attached EPEC was not affected (Figure 2A). More specifically, cytochalasin D treatment did not inhibit the tyrosine phosphorylation of Hp90 (Figure 2B). This indicates that the induction of Hp90 tyrosine phosphorylation and its assembly underneath the attached bacteria is not dependent on prior actin polymerization. Instead, actin polymerization may depend on prior tyrosine phosphorylation of Hp90.

Intimin mediates pseudopod formation

The EPEC *eaeA* gene encodes intimin, an outer membrane protein involved in intimate attachment of EPEC to the host cell membrane (Donnenberg and Kaper, 1991). Intimin is also involved in organizing the tyrosine phosphorylated proteins and actin filaments beneath the attached bacterium (Rosenshine *et al.*, 1992). We found that EPEC *eaeA*$^-$ mutants were also deficient in inducing formation of pseudopods (Table I). The accumulation of actin and tyrosine phosphorylated proteins beneath the attached bacterium might be the outcome of intimate attachment which in turn leads to a localized delivery of signals and localized tyrosine phosphorylation of Hp90. Therefore we examined whether the intimin adherence function can be genetically complemented by other cloned bacterial adhesin genes, including inv_{ent} (pVM101; invasin from *Yersinia enterocolitica*), inv_{ptb} (pBF1001; invasin from *Yersinia pseudotuberculosis*) and *afa* (pIL14; afimbrial adhesin from *E.coli*). EPEC strain CVD206, which contains a deletion in the *eaeA* gene, was transformed with plasmids containing either the *eaeA* gene (pCVD438), or pVM101, pBF1001 and pIL14. Although CVD206 containing all these plasmids exhibited increased adherence, only the *eaeA* positive clone, but not those containing the other cloned adhesins, was able to restore the ability of CVD206 to focus tyrosine phosphorylated proteins and actin filaments beneath the bacteria, and to form pseudopods. This result indicates that intimate bacterial attachment alone is not sufficient to mediate focusing of tyrosine phosphorylated proteins and actin tail formation, suggesting that specific intimin binding to host cell component(s) is important for these processes.

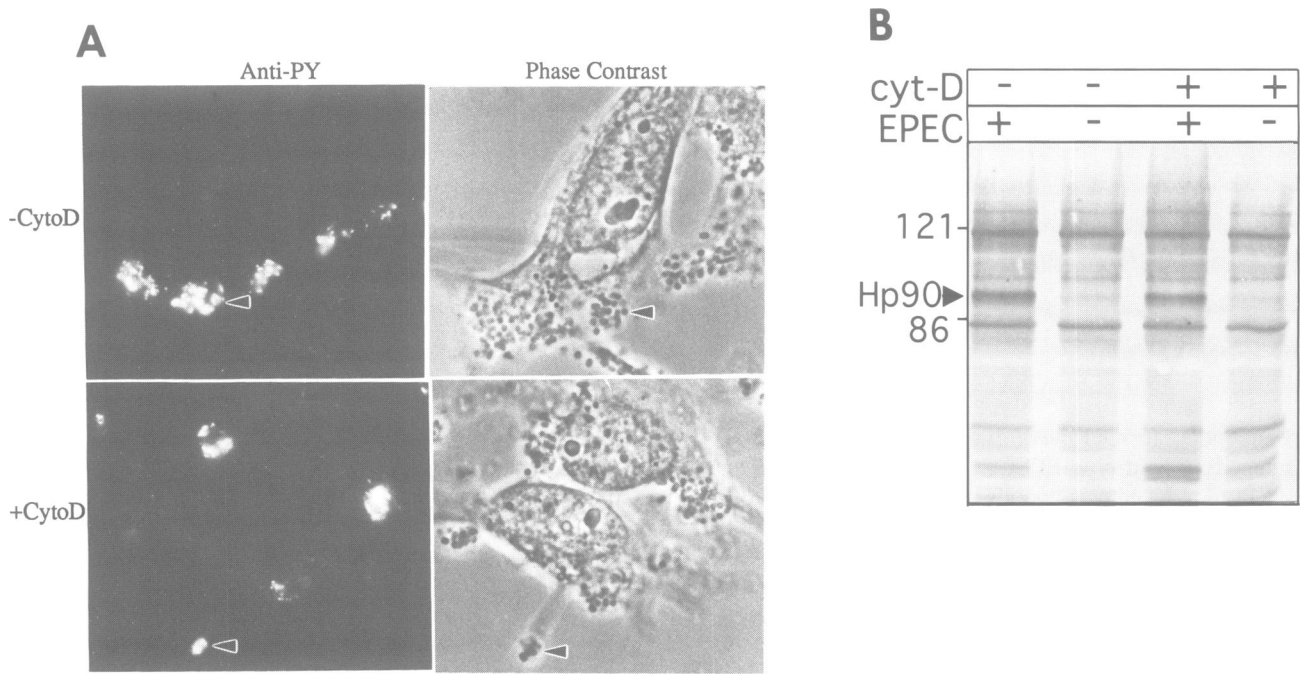

Fig. 2. Cytochalasin D treatment does not inhibit phosphotyrosine localization beneath bacteria or induction of tyrosine phosphorylation of Hp90. (**A**) Phase contrast and corresponding fluorescence microscopy of HeLa cells pretreated or not treated with cytochalasin D that were infected with EPEC, then stained with anti-phosphotyrosine (PY) antibodies. Arrows illustrate groups of bacteria with underlying host tyrosine phosphorylated proteins. (**B**) HeLa cells pretreated with or without cytochalasin D were infected or not for 3 h with EPEC and proteins extracted from HeLa cells with Triton X-100 (the soluble fraction). Twenty micrograms of protein of each preparation were analyzed by SDS–PAGE and immunoblotting with anti-phosphotyrosine antibodies. Tyrosine phosphorylated Hp90 is marked by an arrow, and molecular weight standards are in kDa.

Attachment of intimin-expressing bacteria to host cells is dependent on prior signal transduction

To examine further the role of intimin in adherence, we transformed the non-pathogenic *E.coli* laboratory strain HB101 with pUC19 (control plasmid), pCVD438(*eaeA*), pVM101(inv_{ent}), pBF1001(inv_{ptb}) and pIL14(*afa*), and these strains were used to infect HeLa cells. As expected, large numbers of HB101 containing the inv_{ent}, inv_{ptb} or *afa* genes were associated with epithelial cells. However, HB101 containing the *eaeA* gene did not bind at all to the HeLa cells. Also, none of these cloned adhesins induced any detectable actin condensation. These findings confirmed earlier results obtained by others (Isberg *et al.*, 1987; Jerse *et al.*, 1990; Young *et al.*, 1990).

We then tested the possibility that intimin-mediated adherence required prior EPEC stimulation of host signal transduction pathways by infecting HeLa cells first with the *eaeA* mutant, CVD206, which induces tyrosine phosphorylation of Hp90 but cannot focus it beneath the bacteria (Rosenshine *et al.*, 1992). After 3 h of infection, the infected cells were washed and the CVD206 bacteria were killed by gentamicin and chloramphenicol. Cells were washed again and reinfected with HB101 or HB101 transformed with pCVD438 (containing the cloned *eaeA* gene). After 1 h the reinfected cells were washed extensively, lysed with 1% Triton X-100 and dilutions of the lysate were plated for colony counts on selective media. CVD206 could be distinguished from HB101/*eaeA* since HB101/*eaeA* is Cm^R while CVD206 is Cm^S

In contrast to cells that were not preinfected with CVD206, HB101/*eaeA* bound very efficiently to HeLa cells that were previously infected with the *eaeA* mutant CVD206 (Figure 3B). We also tested HEp-2 and Caco-2 epithelial cells, and found a 51- or 273-fold increase, respectively, in HB101/*eaeA* binding if these cells were pretreated with CVD206. EPEC mutants deficient in inducing tyrosine phosphorylation of Hp90 were also tested for their capacity to induce intimin-mediated binding of HB101. HeLa cells were infected with *eaeB*, cfm 27-3-2(1) or cfm 14-2-1(1) mutants prior to infection with HB101/*eaeA*. None of these mutants could substitute for CVD206 ($eaeA^-$) to prime the HeLa cells for HB101/*eaeA* binding (Figure 3B). Likewise, HB101 did not induce HB101/*eaeA* binding.

We also performed complementary experiments using *E.coli* JM109 containing the cloned intimin and expressing the green fluorescent protein (GFP) to differentiate visually this strain from CVD206. Like HB101, we found that JM109/*eaeA*/pGFP did not bind well to HeLa cells. However, this binding increased 123-fold when monolayers were preinfected with CVD206 prior to adding JM109/*eaeA*/pGFP. Again, preinfection with *eaeB*, cfm 27-3-2(1) or cfm 14-2-1(1) signaling mutants did not increase JM109/*eaeA*/pGFP binding to HeLa cells (1- to 3-fold change in binding levels). To visualize these events, we used fluorescent microscopy following staining with anti-α-actinin antibodies (Figure 3A). JM109 expressing GFP (pGFP) was not visible on monolayers preinfected with CVD206, and α-actinin distribution was unaffected (Figure 3A). However, many JM109 expressing GFP and intimin [JM109(pGFP,pInt)] were visibly adherent to HeLa cells preinfected with CVD206. Additionally, focusing of α-actinin immediately beneath most of the adherent JM109/*eaeA*/pGFP occurred, similar to the rearrangements caused by EPEC (Finlay *et al.*, 1992). We found that 80% of the adherent JM109 expressing cloned intimin and GFP

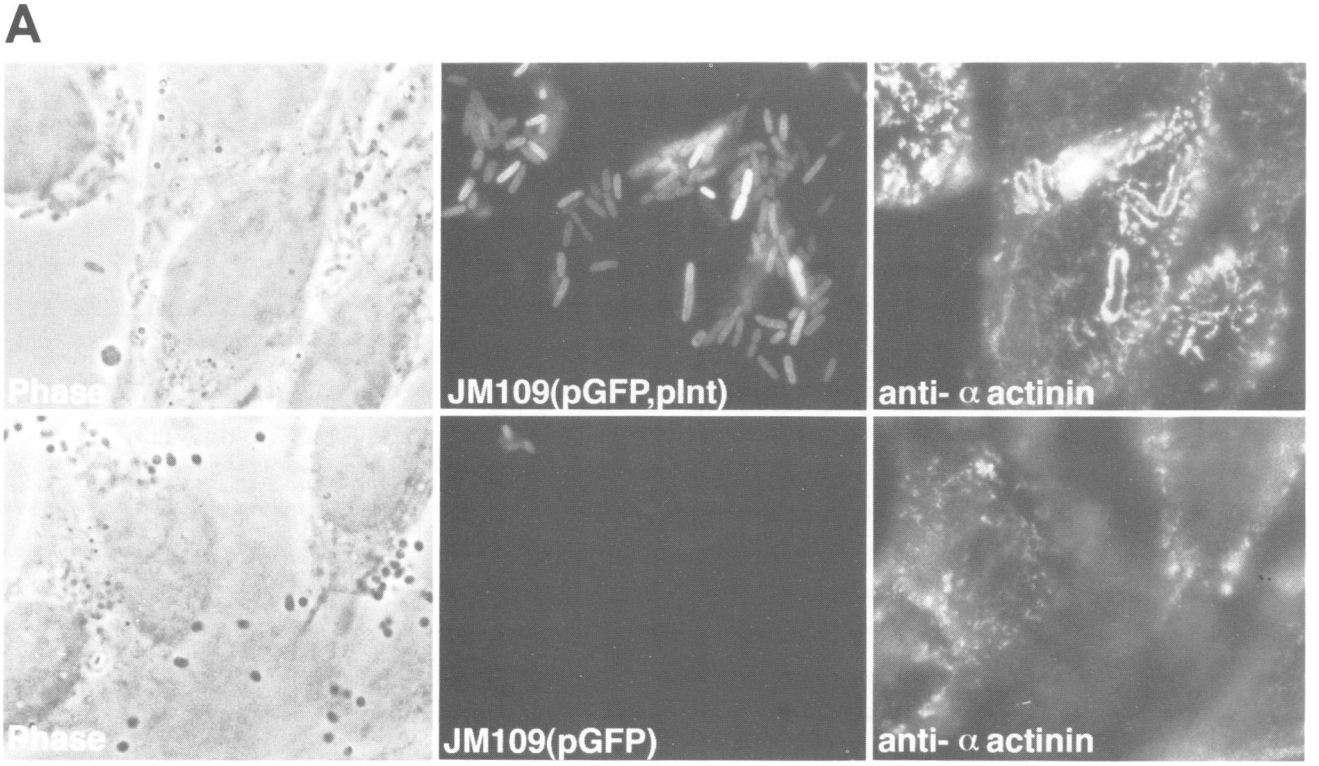

Fig. 3. *Escherichia coli* JM109 expressing cloned intimin mediates α-actinin condensation and binding only to HeLa cells that have been preinduced with the *eaeA* mutant, CVD206. (**A**) Light and immunofluorescence microscopy of JM109 expessing the green fluorescent protein and intimin [JM109(pGFP,pInt)] or JM109 expressing GFP [JM109(pGFP)] binding to HeLa cells preinfected with CVD206 which were killed with antibiotics before adding JM109. JM109 could be distinguished from CVD206 by its GFP fluorescence (middle panels). The corresponding fluorescent images of rhodamine-labeled α-actinin (right panels) indicate locations of α-actinin condensation immediately beneath JM109 expressing intimin. (**B**) Quantitation of HB101/Int binding to HeLa cells preinduced with various EPEC mutants. HeLa cells were infected with various EPEC strains for 3 h, washed and adherent bacteria killed by 1 h of antibiotic treatment and then washed. HB101/Int was then added for 1 h. Monolayers were washed and then lysed with 1% Triton X-100, and lysates plated onto selective agar plates to quantify HB101/Int. The mutants are described in Table I. Values represent the average number of adherent HB101/Int/well of three samples ± SD.

(i.e. fluorescent, to distinguish them from CVD206), caused α-actinin condensation immediately beneath the bacteria. Similar condensation of α-actinin was never observed in cells that had not been preinduced with CVD206, in cells infected with CVD206 alone or in cells preinfected with CVD206 followed by infection with JM109(pGFP).

Collectively, these results suggest that: (i) prior priming of host cell signal transduction pathways, including tyrosine phosphorylation of Hp90, is involved in facilitating intimin binding to the host cell surface; and (ii) presentation of cloned intimin on the surface of non-pathogenic *E.coli* is sufficient for recruitment of cytoskeletal components, but only if the epithelial cells are preinduced with CVD206.

Binding of the C-terminal domain of intimin to host cells is dependent on prior signal transduction

We then further examined the dependency of intimin binding on prior tyrosine phosphorylation of Hp90. We constructed a fusion between the maltose binding protein (MBP) and the carboxyl terminal 280 amino acids of intimin, which contains the putative binding domain of intimin (Frankel *et al.*, 1994). We then examined the binding of the purified fusion product, MBP/Int, to HeLa and HEp-2 cells by two methods. Immunofluorescence of monolayers was examined following addition of MBP/Int to HeLa cells and fluorescent labeling with anti-MBP antibodies (Figure 4). Alternatively, a modified ELISA was employed with anti-MBP antibodies to quantify MBP/Int binding to induced and uninduced monolayers of HeLa cells (Figure 5B). Like intimin-expressing HB101, MBP/Int did not bind to HeLa (Figures 4B and 5B) or HEp-2 cells unless they were preinfected with the *eaeA* mutant, CVD206. The MBP/Int fusion protein bound to the epithelial cell surfaces in cells preinduced with CVD206 (Figure 4A), but was undetectable on uninduced cells (Figure 4B) or on cells induced with the *eaeB* signaling mutant (Figure 5B). MBP alone did not bind when added to preinduced cells (Figure 4E). Cytochalasin D treatment

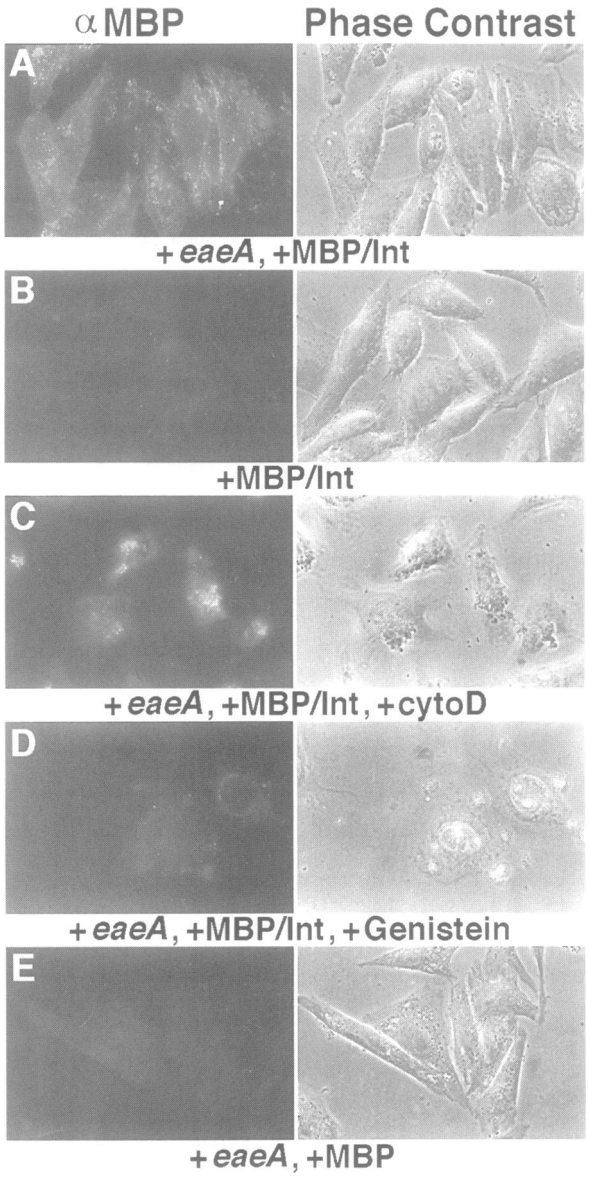

Fig. 4. MBP and MBP/Int binding to HeLa cells. Purified MBP or MBP/Int was added to HeLa cells that had been preinduced with CVD206 ('+*eaeA*') or not followed by immunofluorescence labeling with anti-MBP antibodies. The fluorescent image and the corresponding phase contrast image are shown.

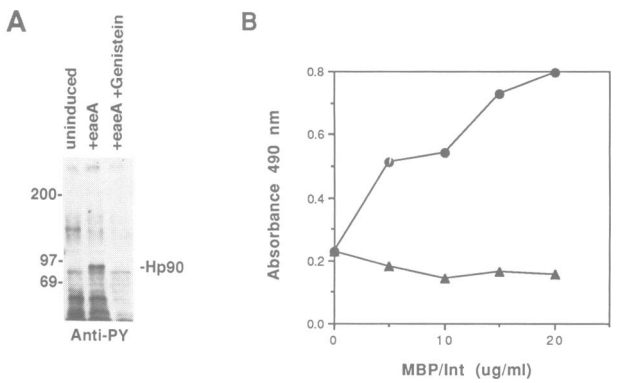

Fig. 5. Western blot of Hp90 with or without genistein (A) and ELISA of MBP/Int binding to HeLa cells (B). (**A**) HeLa cells were preinduced with CVD206 ('+*eaeA*') or not ('uninduced'), with or without gensitein treatment. Following washing, HeLa cell membranes were isolated and then solubilized with Triton-X100 and resolved by SDS–PAGE followed by Western blotting with anti-PY antibodies. Hp90 is marked. (**B**) Alternatively, colorimetric detection of bound MBP/Int to HeLa cell monolayers preinduced with the *eaeA* mutant CVD206 (circles) was quantified with a modified ELISA using anti-MBP antibodies. Preinfection with the *eaeB* mutant did not induce MBP/Int binding to the monolayer (triangles). Values represent the average of two wells, and these results are representative of five experiments.

did not affect MBP/Int binding to cells preinfected with CVD206 (Figure 4C), in agreement with the above results which show that Hp90 tyrosine phosphorylation occurs independently of actin filament polymerization. Again, EPEC mutants deficient in inducing tyrosine phosphorylation of Hp90, *eaeB* (Figure 5B), cfm 27-3-2(1) and cfm 14-2-1(1), did not promote MBP/Int binding to host cell surfaces.

We next tested the effect of blocking tyrosine phosphorylation of Hp90 with the tyrosine protein kinase inhibitor, genistein. This inhibitor significantly decreased EPEC-induced Hp90 tyrosine phosphorylation (Figure 5A), and also decreased MBP/Int binding to HeLa cells that had been preinfected with CVD206 (Figure 4D). Using the ELISA, we also found that genistein decreased MBP/Int binding. When the level of fusion peptide was normalized to the amount of binding to cells infected with the *eaeA* mutant (i.e. 100%), we found that if cells were treated with genistein at a concentration of 250 μM, the amount of fusion peptide (added at 20 μg/ml) bound dropped to 45 ± 24% of the levels seen without genistein (average of five separate experiments done in duplicate). We also found that preinfection with the *eaeB* mutant caused the peptide to bind at 20 ± 15% of the levels seen with CVD206. Similar results were seen with HEp-2 cells. Collectively, these results suggest that tyrosine phosphorylation, presumably of Hp90, plays an essential role in intimin-mediated binding, and that blocking this event inhibits intimin binding.

Tyrosine phosphorylated Hp90 is a surface-exposed host membrane protein

We next examined whether tyrosine phosphorylated Hp90 is a membrane protein. HeLa cells were infected for 3 h with EPEC, followed by cellular fractionation into three fractions: cytosolic, membrane and Triton X-100-insoluble (composed of the cytoskeleton, nuclei and bacteria). The cytosolic and membrane fractions were analyzed by immunoblotting with anti-phosphotyrosine antibodies. As a control, we also examined the distribution of the membrane associated fibronectin receptor (FNR or integrin), with anti-FNR antibody. Tyrosine phosphorylated Hp90 and the α and β subunits of FNR were localized to the membrane fraction and were not in the cytosolic fraction (Figure 6A and B). A similar Hp90 localization was obtained when CVD206 (the *eaeA*$^-$ mutant) was used instead to induce tyrosine phosphorylation of Hp90. Hp90 could not be removed from the membrane with 1.5 M NaCl (data not shown) suggesting that Hp90 is an integral membrane protein. Moreover, gentle treatment of intact HeLa cells with thermolysin resulted in the loss of Hp90 (Figure 6C), consistent with Hp90 containing a surface-exposed domain.

In addition to association with membranes, substantial

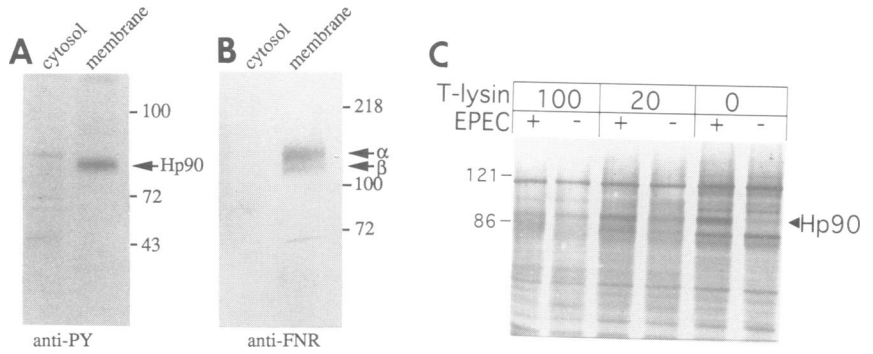

Fig. 6. Membrane location of Hp90. Cytosolic and membrane fractions of EPEC infected HeLa cells were separated by SDS–PAGE followed by Western blot analysis using (**A**) anti-phosphotyrosine or (**B**) anti-fibronectin receptor antibodies. Hp90 and the α and β subunits of the FNR are marked. (**C**) Treatment of intact HeLa cells with 0, 20 or 100 μg/ml of thermolysin (T-lysin) infected (+) or not (–) with EPEC. Note that this treatment cleaved tyrosine phosphorylated Hp90, indicating surface exposure. Hp90 is marked by an arrow, and molecular weight standards are in kDa.

amounts of tyrosine phosphorylated Hp90 were also localized to the insoluble fraction (Rosenshine *et al.*, 1992, and data not shown), which is presumably associated with the detergent-insoluble cytoskeleton and with intimin on EPEC (see below).

Intimin associates with tyrosine phosphorylated Hp90

To determine whether intimin asssociated directly or indirectly with intimin, we used a modified gel overlay (Far Western) approach (Hildebrand *et al.*, 1995). HeLa cell membranes were extracted with detergent, resolved by SDS–PAGE, transferred to nitrocellulose, and proteins bound to the nitrocellulose were renatured by incubating with decreasing concentrations of guanidine hydrochloride. The fusion peptide MBP/Int was overlayed onto the renatured nitrocellulose, washed and developed using anti-MBP antibodies followed by secondary conjugated antibodies. We found that MBP/Int did not bind to membrane extracts of uninfected HeLa cells (Figure 7A). However, if the HeLa cells were preinfected with CVD206 (the *eaeA* mutant), the fusion protein bound to a 90 kDa HeLa membrane protein which had the same molecular weight as tyrosine phosphorylated Hp90 (Figure 7A and B). If HeLa cell membranes were extracted from cells that had been preinfected with a mutant which is defective for secretion of signal transduction proteins (*sep* or CVD452, Jarvis *et al.*, 1995), MBP/Int did not bind to this Hp90 (Figure 7A).

To examine further the interactions of intimin with Hp90, we tested whether Hp90 coprecipitated with either native intimin on EPEC surfaces or recombinant, soluble MBP/Int. HeLa cells were infected either with EPEC or with CVD206 to induce tyrosine phosphorylation of Hp90. After 3 h, bacteria were removed from the epithelial cells with Triton X-100, washed several times, and epithelial proteins which were still associated with the bacteria were eluted with 1.5 M NaCl and probed on a Western blot with anti-phosphotyrosine antibodies. A phosphotyrosine protein that comigrates with Hp90 was eluted from the parental EPEC, but not from the isogenic *eaeA*$^-$ CVD206, which does not express intimin (Figure 8A). This indicates that the tyrosine phosphorylated protein seen in Figure 1B is probably Hp90 and that the specific localization of Hp90 is a result of interaction between Hp90 and intimin.

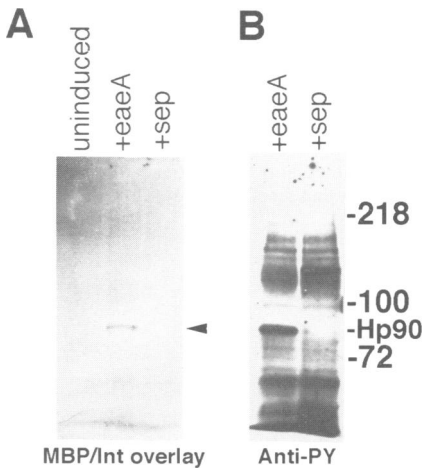

Fig. 7. Intimin binds to Hp90. HeLa cell membranes were collected from cells not infected (uninduced) or from those infected with CVD206 (+*eaeA*) or those infected with a signal-defective EPEC mutant which does not secrete signaling proteins, CVD452 (+*sep*) (Jarvis *et al.*, 1995), separated by PAGE and transferred to nitrocellulose. The nitrocellulose was divided into two, and one half renatured with guanidine hydrochloride followed by overlaying with purified MBP/Int and probing with anti-MBP antibodies (**A**). The other half was probed with anti-PY antibodies in a Western (**B**). Note that MBP/Int binds to a protein of the same molecular weight as Hp90 only in cells that had been preinduced with CVD206. Molecular weights are in kDa, and an arrow marks Hp90.

We further examined the interaction between tyrosine phosphorylated Hp90 and soluble MBP/Int. HeLa cells were preinfected or not infected with CVD206 and treated with affinity purified MBP/Int or MBP. Cells were then lysed, and the cleared lysate was used for immunoprecipitation with anti-MBP antibody and then probed with anti-phosphotyrosine antibodies in Western blots. A phosphotyrosine band that comigrates with Hp90 was coprecipitated only in the MBP/Int sample that was preinduced with CVD206, but not in the sample that was not preinfected with CVD206 (Figure 8B). MBP alone did not immunoprecipitate Hp90, even in cells induced with CVD206. To confirm the specificity of this precipitation, we confirmed that this procedure did not precipitate the fibronectin receptor (Figure 8C). Collectively, these data indicate that Hp90 interacts with MBP/Int only after induction of tyrosine phosphorylation by EPEC-mediated signals. It

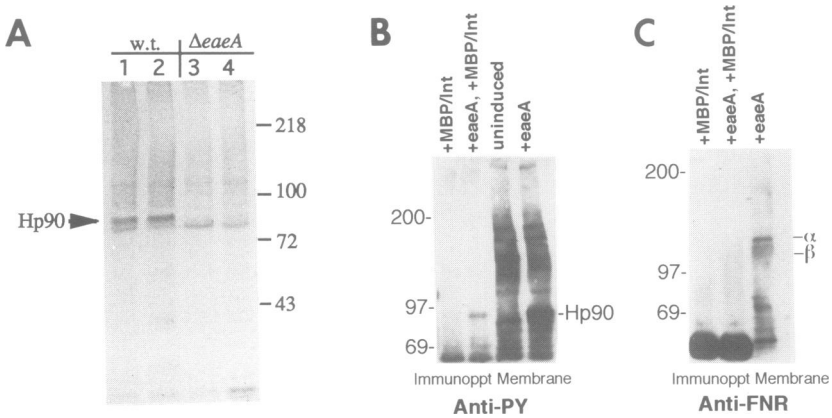

Fig. 8. Hp90 associates tightly with intimin. (**A**) After HeLa cells were infected with EPEC wild type or with the *eaeA* mutant 10-5-1(1), bacteria and associated host cell proteins were separated from the host cell with lysis solution containing 0.4% (lanes 1 and 3) or 0.2% (lanes 2 and 4) Triton X-100 and washed three times with the same buffer. Epithelial proteins that bound to the bacteria were eluted from the bacteria by incubation in lysis solution supplemented with 1.5 M NaCl. Eluted proteins were concentrated, desalted, and analyzed by SDS–PAGE and Western blotting analysis with anti-phosphotyrosine antibodies. Hp90 is marked with the short arrow. (**B**) MBP/Int precipitates Hp90 only from preinduced cells. HeLa cells were preinduced with CVD206 ('+*eaeA*') or not. Purified MBP/Int ('+MBP/Int') was added to cells, incubated, washed, and the monolayers lysed. MBP/Int was immunoprecipitated from the lysate with anti-MBP antisera, and the immunoprecipitate resolved by SDS–PAGE followed by Western blotting with anti-PY antibodies. Note that Hp90 only coprecipitated with MBP/Int when cells were preinduced with CVD206. HeLa cell membranes from cells infected with CVD206 or uninfected (uninduced) were also run on the same gel to mark the mobility to Hp90. (**C**) Equal amounts of the same immunoprecipate and HeLa cell membrane samples used in (B) were analyzed by Western blot analysis using anti-FNR antibodies. The mobility of the α and β FNR receptors are marked. The bands below 50 kDa represent antibodies used in the immunoprecipitation that react in the Western blot.

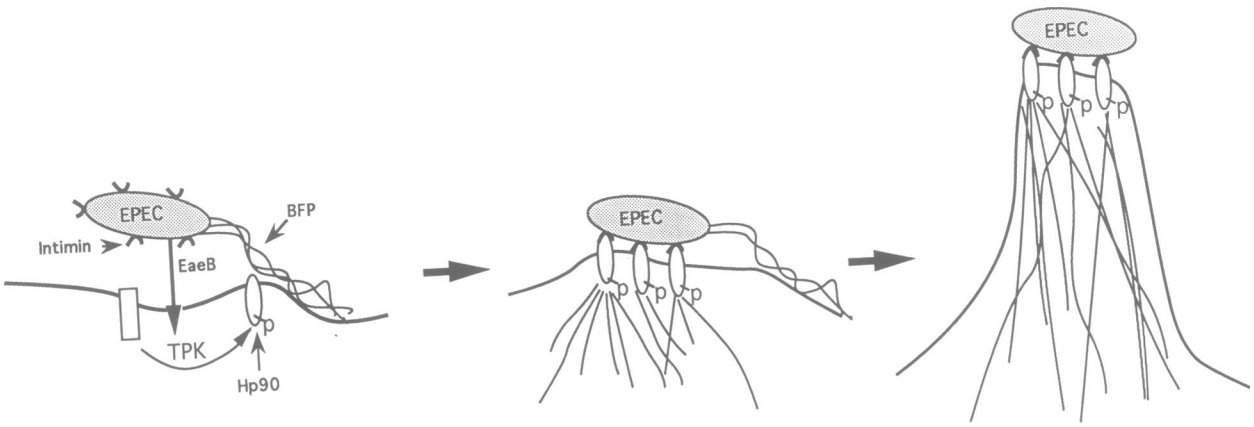

Fig. 9. A model of EPEC interactions with epithelial cells. EPEC initially binds to epithelial cells via bundle forming pili (BFP). EPEC secretes EaeB and other proteins that mediate signal transduction, including tyrosine phosphorylation of Hp90. Once phosphorylated, Hp90 binds to intimin on the bacterial surface and with actin in the cytoplasm, organizing the cytoskeleton beneath the bacteria. Extended pedestal formation or, less frequently, bacterial invasion, then follows. Hp90 remains associated with intimin at the tip of the extended pedestals beneath adherent bacteria.

also indicates that tyrosine phosphorylated Hp90 is most likely the EPEC intimin receptor.

Discussion

Most pathogenic bacteria exert their deleterious effects by attaching to and sometimes invading living host cells. Typically, adherence and/or invasion involves the specific interaction of a bacterial determinant (adhesin or ligand) to a host cell structure (receptor). In all systems studied thus far, the adhesin binds to a receptor that is functional before any contact occurs between the bacterium and the host cell. However, we demonstrate here for the first time that bacterial contact and concomitant signal transduction is needed prior to intimin-mediated EPEC adherence to epithelial cells. Our current working model for EPEC interactions with epithelial cells, which incorporates this concept, is presented in Figure 9. This involves initial EPEC adherence mediated by BFP and other factors. The attached EPEC then induces tyrosine phosphorylation of Hp90 and other host signals such as calcium and inositol phosphate fluxes via EaeB and possibly other secreted bacterial molecules. Tyrosine phosphorylation of Hp90 facilitates association of intimin with surface-exposed Hp90. Tyrosine phosphorylated Hp90 is also probably involved in nucleating cytoskeletal elements perhaps via induction of localized actin polymerization. Intimin–Hp90 interaction might enhance or direct actin polymerization to form the mature actin tail. Formation of pedestals and pseudopods and EPEC internalization (invasion) are probably different manifestations of the same process. All bacterial mutants that are deficient in forming pseudopods are also non-invasive, and both invasion and pseudopod formation are dependent on tyrosine phosphorylation of Hp90 and Hp90–intimin interaction.

Formation of actin comet tails by intracellular *Listeria*

and *Shigella* is believed to propel bacteria from the infected cell to adjacent uninfected cells, thus enabling cell to cell spread without exposure to the extracellular environment (reviewed in Cossart and Kocks, 1994). *Listeria* and *Shigella* mutants that cannot form actin-tails are also strongly attenuated in virulence. Although smaller pedestals have been described for adherent EPEC on several cell types, the EPEC-induced pseudopods reported here have not been previously described. It is probable that these pseudopods are exaggerated pedestals that are more pronounced under the conditions used in these experiments. The biological function of these pseudopods (and the smaller pedestals) is not known, although *eaeA* mutants, which do not form these structures, are much less virulent than wild type EPEC (Donnenberg *et al.*, 1993a). It is possible that pseudopod formation may permit bacteria to move along epithelial surfaces as the underlying actin alters its polymerization state. Experiments to measure EPEC movement on epithelial cell surfaces are in progress. Alternatively, these projections may be static structures upon which the bacteria adhere and manifest their effects. Whichever, these structures will also provide a biological tool to study the dynamic interactions of the actin-containing cytoskeleton with the cell surface. These include studies which examine the role of specific signal transduction events which stimulate localized actin polymerization, and the role of specific proteins such as Hp90 in anchoring the cytoskeleton to the membrane. Because these structures can be specifically and locally induced by an extracellular object (by adding EPEC), they are more readily studied than structures such as focal contacts which are normally present in epithelial cells. These pseudopods will also provide opportunites to examine the distribution of cytoskeletal proteins within these ordered structures, with actin and related proteins forming the stalk and phosphotyrosine proteins, and possibly proteins that link actin filaments to the membrane, present at their distal tip beneath extracellular bacteria.

We have identified three functions of Hp90 that seem to be regulated by its tyrosine phosphorylation: cytoskeletal rearrangement, inositol phosphate fluxes (Foubister *et al.*, 1994a) and association with bacterial intimin. The normal function of Hp90 in uninfected epithelial cells has not been determined, although we have shown here that its tyrosine phosphorylated form is associated with the membrane fraction. High concentrations of tyrosine phosphorylated proteins are also characteristic of focal adhesion contacts (FACs) (Otey and Burridge, 1994). Like the EPEC-induced tails, FACs are also rich in actin and other cytoskeletal elements. One of the FAC tyrosine phosphorylated proteins is an autophosphorylating TPK, $pp125^{FAC}$ or FAC-kinase (Lo and Chen, 1994). FAC-kinase is believed to play some role in the assembly of FACs and perhaps in actin reorganization. However, in EPEC infected cells we could not detect any elevation of FAC-kinase tyrosine phosphorylation (unpublished observations). Hp90 does not seem to be involved in FAC formation, as it is not normally tyrosine phosphorylated in several cell lines that contain FACs, such as the HeLa cells used in these experiments. Although the role of Hp90 in uninfected cells has not been established, it is probably involved in linking the actin-containing cytoskeleton to the membrane. Since we do not normally detect phosphorylated Hp90 in uninfected epithelial cells, it may be involved in short lived linkages that are mediated by transient Hp90 tyrosine phosphorylation. Alternatively, tyrosine phosphorylated Hp90 may play a role in normal cellular processes that are not activated during cell culture growth, and thus we are unable to detect phosphorylated Hp90 in uninfected cells. We are currently using the immunoprecipitation procedures presented here to purify and identify Hp90 which will allow us to begin to address the function of Hp90 in uninfected cells.

Several EPEC loci are involved in inducing Hp90 tyrosine phosphorylation. This includes the *sep1* and *sep2* genes, which encode components for a type III bacterial protein secretion system, the cfm*14-2-1(1)* loci which also seem to be involved in this secretion system, and the *eaeB* gene which encodes one of the proteins that is secreted by this pathway (Jarvis *et al.*, 1995; Kenny and Finlay, 1995). Treatment of host cells with soluble secreted EPEC proteins is not sufficient to induce tyrosine phosphorylation of Hp90. This induction seems to be dependent on direct contact of EPEC with the host cell membrane and results from coordinate actions of several bacterial factors. In *Yersinia* species, a homologous type III secretion system mediates contact-dependent injection of an actin cytotoxin, YopE, and perhaps a tyrosine phophatase, YopH, into the host cytosol (Rosqvist *et al.*, 1994; Sory and Cornelis, 1994). By analogy with *Yersinia*, the factor that induces tyrosine phosphorylation of Hp90 (possibly EaeB) might be injected by adherent EPEC into the host cytosol.

Intimin is a member of the invasin/intimin-like protein family. In addition to EPEC intimin, this family includes the product of the *eaeA* genes of several pathogens, including EHEC, *Hafnia alvei* and *Citrobacter freundii*, and the *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* invasins (Jerse *et al.*, 1990; Frankel *et al.*, 1994). The *inv* genes encoding both *Yersinia* invasins could not phenotypically restore *eaeA* mutants in genetic complementation assays. Thus, invasin and intimin do not seem to bind to the same host ligand. Invasin binds to several β1 integrin receptors (Isberg and Leong, 1990). Intimin appears to associate directly with tyrosine phosphorylated Hp90, based on the gel overlay experiments and coprecipitation with MBP/Int. Unlike invasin, intimin binding is dependent on prior tyrosine phosphorylation of Hp90, its putative ligand. Tyrosine phosphorylation of Hp90 may affect the conformation or localization of the intimin recognition site. Priming the host cell with EPEC-induced signals increases the binding efficiency of both intimin-expressing bacteria and MBP/Int. However the bacterially induced attachment was much more enhanced than that of the MBP/Int. This might be due to higher avidity of the bacteria; it is also possible that membrane anchored intimin has a higher binding affinity than the intimin C-terminal domain fused to MBP. It will also be interesting to examine whether the product of the *eaeA* genes of EHEC, *H.alvei* and *C.freundii* can complement intimin function in EPEC *eaeA* mutants, or if they diverge in the ligand that they recognize.

Recently Frankel *et al.* (1994) reported contradictory results regarding the capability of MBP/Int to bind to host cells. In agreement with our results and the results of Jerse *et al.* (1990), they found that HEp-2 cells do not bind to MBP/Int immobilized on plastic substrates. In

contrast to this, they found that beads coated with MBP/Int and soluble MBP/Int bound to HEp-2 cells (without preinduction with EPEC). We found, using the same MBP/Int fusion, that MBP/Int bound to HEp-2 cells (and several other epithelial cell lines) only after preinduction with EPEC or the *eaeA* mutant. The reasons for these differences remain undefined.

In conclusion, we have found that intimin-mediated adherence to Hp90 requires prior bacterially induced signal transduction in the host epithelial cell. This finding has significant implications in the study of bacterial adherence, since it was previously thought that bacterial ligands bound to receptors that were already present on the host cell surface. We have also shown that intimin–Hp90 association is required for pseudopod formation. These unique structures will facilitate the study of the organization and interactions of actin structures that are linked to the cell membrane. The complex interplay between bacterial and host components emphasizes the intimate interactions that occur between bacterial pathogens and their host cells. Knowledge of the mechanisms used to cause these changes in the host cells will suggest new approaches in designing new antibacterial agents that can block these processes.

Materials and methods

Bacterial strains and plasmids

EPEC strains E2348/69, JPN15, CVD206, CVD452, cfm 27-3-2(1), cfm 14-2-1(1) and UMD869 have been described elsewhere (Donnenberg *et al.*, 1990a, 1993b; Jerse *et al.*, 1990; Donnenberg and Kaper 1991; Rosenshine *et al.*, 1992; Foubister *et al.*, 1994a,b; Jarvis *et al.*, 1995). The bacterial phenotype and genotype of each of these strains are summarized in Table I. The bacteria were grown in LB agar or LB broth at 37°C without shaking. For infection, overnight bacterial cultures were diluted 1:100 in MEM and added to cultured epithelial cells. The plasmids pCVD438 (Jerse *et al.*, 1990), pVM101 (Young *et al.*, 1990) and pIL14 (Labigne-Roussel *et al.*, 1985) have been described. pBF1001 is *inv* gene from *Y.pseudotuberculosis* (Isberg and Leong, 1990) cloned into the vector pACYC184.

Scanning electron microscopy

HeLa cells, grown on glass cover slips, were infected with wild type EPEC for 7 h. The infected cell were washed twice with cold PBS and fixed with cold 5% gluteraldyde in PBS at 4°C for 1 h. The samples were than postfixed with 1% OsO_4 for 1 h, and dehydrated in a critical-point drying apparatus. Samples were coated with gold and examined with a Hitachi S-4100 scanning electron microscope.

Confocal and immunofluorescence microscopy

Epithelial cells were seeded and grown overnight on glass coverslips. After infection or other treatment, cells were washed with PBS and fixed 30 min in 2% paraformaldehyde in PBS. The fixed cells were washed three times with PBS, permeabilized with 0.1% Triton X-100 in PBS for 5 min and washed as before. Cells were incubated with 20 µl of primary antibodies, including monoclonal anti-phosphotyrosine (4G10, UBI Inc.), anti-α-actinin (BM-75.2, Sigma), polyclonal rabbit anti-EPEC antibodies or phalloidin conjugated to FITC or rhodamine (Molecular Probes Inc.) for 60 min. Cells were then washed and incubated with 20 µl of secondary antibody, including anti-mouse IgG and IgM (FITC, Texas Red or Cy5 conjugated), or anti-rabbit IgG (FITC or Texas Red conjugated), from ICN Inc. Samples were visualized and photographed as described (Finlay *et al.*, 1991). For confocal laser scanning we used a Bio-Rad MRC-600 microscope, and optical sections were usually taken at 0.5 µm intervals.

Green fluorescent protein labeling. Escherichia coli JM109 was transformed with pGFP (Clontech, Palo Alto, Ca) which encodes the green fluorescent protein and pCVD438 which encodes intimin. Bacteria were grown overnight at 26°C prior to addition to epithelial cells preinfected with CVD206 for 3 h or uninfected. Monolayers were washed and fixed for microscopy as described above.

Cytochalasin D treatment

HeLa cells were grown on cover slips for microscopy, or in 100 mm tissue culture Petri dishes (3×10^6 cells/plate) for protein extraction. HeLa cells were preincubated with MEM containing 2 µM cytochalasin D or MEM only, for 10 min and then infected with 10 µl of a standing overnight EPEC culture. After 3 h infection, the samples were either fixed and processed for immunofluorescence microscopy, or used for protein extraction and immunoblotting analyses with anti-phosphotyrosine monoclonal antibody (mAb) 4G10.

Preinduction of EPEC-mediated signaling

HeLa cells were first infected with CVD206. After 3 h, infected cells were washed three times with PBS and attached bacteria were killed by incubating in MEM containing 100 µg/ml gentamicin, 30 µg/ml chloramphenicol, and diluted (1:500) heat-inactivated rabbit anti-EPEC antisera was used to stain extracellular CVD206. After 1 h, antibiotics were washed away with PBS and the cells were reinfected with the HB101 transformants in MEM supplemented with either chloramphenicol (30 µg/ml; for infection with HB101 transformed with CVD438 and pBF1001) or ampicillin (50 µg/ml; for infection with HB101 transformed with pVM101 or pIL14). After 1 h the reinfected cells were either (i) washed extensively with PBS, lysed with 1% Triton X-100 and dilutions of the lysate were plated for colony counting; or (ii) fixed and used for staining with rhodamine conjugated anti-rabbit IgG antibody, and either anti-phosphotyrosine mAb or FITC–phalloidin.

Quantitation of HB101/Int binding after preinduction

Epithelial cells were seeded (1×10^5/ml) in 24 well tissue culture plates and grown overnight. Cells were either preinfected with CVD206 (as above) or not for 3 h, followed by washing and antibiotic killing for 1 h prior to infection with various HB101 transformants or EPEC mutants. After 1 h incubation, cells were washed with PBS, and the bacteria were released by 1% Triton X-100 treatment (Tang *et al.*, 1993) and quantified by growing on selective agar plates.

Protein extraction and fractionation

HeLa cells were seeded at a density of 2×10^6/plate (100 mm tissue culture Petri dishes). After 12 h, cells were infected with 100 µl of late logarithmic bacterial culture (OD_{600nm} ~1.5) and incubated for the indicated time at 37°C, 5% CO_2. The infected cells were washed three times with 10 ml cold PBS and then scraped into 1.5 ml of PBS, spun down (60 s 4000 *g*) and lysed in 0.2 ml of lysis solution (1% Triton X-100; 50 mM Tris–HCl, pH 7.6; 0.4 mM $NaVO_4$; 0.1 mg/ml phenylmethylsulfonyl fluoride and 10 µg/ml leupeptin). The lysate was spun (60 s, 15 000 *g*), and the supernatant (soluble fraction), was mixed with 50 µl of 5× loading buffer. The insoluble pellet was dissolved in 100 µl of 2.5× gel loading sample buffer. The lysates were boiled for 7 min and cleared (5 min, 15 000 *g*) prior to resolution by SDS–PAGE. For fractionation into membrane, cytosol and insoluble fractions, the scraped infected cells were suspended in sonication buffer (50 mM Tris–HCl pH 7.6, 0.4 mM $NaVO_4$, 0.1 mg/ml phenylmethylsulfonyl fluoride and 10 µg/ml leupeptin) instead of lysis buffer. Cells were then sonicated for two 1 s bursts. This sonication procedure ruptures the HeLa cell membranes but not the nuclei or EPEC. The sonicate was then spun (15 min, 103 000 *g*), the supernatant containing the cytosolic proteins was removed and the pellet (containing cell membrane, bacteria and cell nuclei) was washed once with sonication buffer. The pellet was than suspended in lysis buffer, the lysate was spun 5 min, 103 000 *g*, and the supernatant containing membrane proteins was harvested. The remaining pellet containing cytoskeletal proteins, bacteria and nuclei was dissolved in 100 µl of 2.5× loading buffer. The lysates were boiled for 7 min and cleared (5 min, 15 000 *g*) prior to resolution by gel electrophoresis. Using this protocol we usually obtained 0.3–0.5 mg of membrane proteins and ~1 mg of cytosolic proteins.

Western immunoblotting

Samples were subjected to SDS–PAGE and transferred to nitrocellulose membrane (AB-S 83 Schleicher & Schuell Inc.) using a NovaBlot electrophoretic transfer unit (LKB) according to the manufacturer's recommendations (Rosenshine *et al.*, 1992). Monoclonal anti-phosphotyrosine (4G10, UBI Inc.) and polyclonal rabbit anti-maltose binding protein (New England BioLabs) were diluted in TBS (150 mM NaCl, 20 mM Tris–HCl pH 7.5) containing 1% BSA (Sigma). Binding of secondary anti-mouse IgG or anti-rabbit IgG(Amersham) horseradish

peroxide conjugated antibody was detected using the ECL system (Amersham).

Thermolysin treatment

HeLa cells were either infected or not infected with CVD206. The cells were placed on ice, washed three times with cold PBS and suspended in cold PBS or cold PBS supplemented with 100 or 20 μg/ml thermolysin (Sigma). Cells were incubated with the protease for 15 min on ice. Cells were then washed three times with PBS supplemented with 10 μg/ml leupeptin, 100 μg/ml PMSF and 200 μM VO_4. The washed cells were lysed in 150 μl lysis solution. Protein concentration in the lysates were determined and 20 μg protein per lane of the lysate were used for SDS–PAGE and Western analysis using an anti-phosphotyrosine antibody.

Association of Hp90 with intimin on EPEC surfaces

HeLa cells grown in 100 mm Petri dishes were infected with either EPEC or with the *eaeA* mutant CVD206. After 3 h, the cells were washed three times in PBS, harvested by scraping into 1 ml PBS and spun (1 min, 4000 *g*) in a microfuge. The pellets were extracted with 0.5 ml lysis solution containing 0.2 or 0.4% Triton X-100. The Triton-insoluble pellet was washed three times with lysis solution. The pellet was resuspended in lysis solution containing 1.5 M NaCl and incubated for 10 min. Samples were then centrifuged (30 min, 14 000 r.p.m.) and the supernatants were collected, and concentrated and desalted using a Centricon 30 (Amicon Inc.). Samples were analyzed by SDS–PAGE and immunobloting with anti-phosphotyrosine mAb 4G10.

Construction and purification of MBP/Int fusion

The MBP/Int genetic fusion was constructed, and MBP and MBP/Int fusion proteins were purified, as described elsewhere (Frankel *et al.*, 1994).

MBP/Int binding to epithelial cells

MBP or MBP/Int binding to HeLa cells was determined in two ways. Immunofluorescence detection was done exactly as described (Frankel *et al.*, 1994) by adding 5 μg purified MBP or MBP/Int to epithelial cells, incubating for 45 min, 37°C, washing, fixing with 2% paraformaldehyde and staining with rabbit anti-MBP antisera followed by secondary anti-rabbit antibodies conjugated to FITC. For preinduced samples, CVD206 was added for 3 h, washed and killed with antibiotics as described above. Cytochalasin D treatment was done as described above, and for genistein treatment, genistein (250 μM final concentration) was added 15 min prior to addition of CVD206, and this concentration maintained for the remainder of the experiment.

For the ELISA, 1 ml of 1×10^6 HeLa cells/ml was added to each well of a 24 well microtiter plate and grown overnight. Monolyers were either preinfected with or without broth-grown CVD206 or *eaeB* (10 μl) for 3 h followed by addition of gentamicin (1 h) and washing as above. When used, genistein (250 μM final concentration) was added with the gentamicin. After washing, purified MBP/Int fusion protein was added at various concentrations in binding buffer (Frankel *et al.*, 1994) and incubated with the monolayers for 60 min. Following washing with PBS, cells were fixed with 2.5% paraformaldehyde as described above. Rabbit anti-MBP was added (diluted 1:1000) for 30 min, 37°C, followed by washing and addition of goat anti-rabbit horseradish peroxidase (30 min, 37°C) and then substrate addition and colorimetric reading (absorbance at 490 nm).

Gel overlay/Far Western

These were performed as described (Hildebrand *et al.*, 1995). Briefly, HeLa cells were infected with CVD206, CVD452 or not infected for 3 h, followed by washing with PBS and gentamicin treatment for 1 h. Membranes were purifed and resolved by 6% polyacrylamide gel electrophoresis, followed by transfer to nitrocellulose. One half of the nitrocellulose was analyzed by Western analysis using anti-PY antibodies to verify the mobility of Hp90. The other half was renatured using decreasing concentrations of guanidine hydrochloride as described (Hildebrand *et al.*, 1995). The nitrocellulose was incubated overnight, 4°C, with 10 ml of maltose binding solution (Frankel *et al.*, 1994) containing 30 μg of MBP/Int. Following extensive washing, the blot was incubated with rabbit anti-HRP and developed using the ECL system (see above).

MBP/Int-Hp90 coprecipitation

Three plates of 2.5×10^6 HeLa cells/plate were either preinfected with or without CVD206 (0.5 ml) for 3 h followed by addition of antibiotics and washing as above. MBP or MBP/Int (75 μg/plate) was added for 1 h, 37°C. Plates were placed on ice and washed extensively with cold PBS, harvested by scraping into 1.5 ml lysis solution and centrifuged. The supernatant was collected and 5 μl rabbit non-specific antisera was added for 30 min, 4°C, with gentle agitation. Uncoated Sepharose-A beads were added (30 min, 4°C), and then removed by centrifugation. Goat anti-rabbit IgG coated Sepharose-A beads and 5 μl rabbit anti-MBP (diluted 1:10) were added and incubated overnight at 4°C. Samples were then centrifuged, the beads washed five times in lysis buffer, and then boiled for 5 min in 2× SDS sample buffer. The beads were removed by centrifugation and the supernatant resolved on SDS–PAGE, followed by Western blot analysis.

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