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Intimin-Dependent Binding of Enteropathogenic *Escherichia coli* to Host Cells Triggers Novel Signaling Events, Including Tyrosine Phosphorylation of Phospholipase C-γ1

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Enteropathogenic *Escherichia coli* (EPEC) interactions with HeLa epithelial cells induced the tyrosine phosphorylation of a host protein of approximately 150 kDa, Hp150. Phosphorylation of this protein band was dependent on the interaction of the EPEC protein intimin with epithelial cell surfaces and was correlated with pedestal formation. Hp150 phosphorylation was specifically inhibited by the addition of cytochalasin D, an inhibitor of actin polymerization, although this appeared to be an indirect effect preventing interaction of intimin with its receptor, tyrosine-phosphorylated Hp90, and thus triggering Hp150 phosphorylation. This suggests the involvement of an actin-based movement of membrane-bound tyrosine-phosphorylated Hp90 to allow its interaction with intimin. Analysis of the tyrosine-phosphorylated Hp150 protein demonstrated that it is heterogeneous in composition, with phospholipase C-γ1 (PLC-γ1) being a minor component. Activation of PLC-γ1 by tyrosine phosphorylation leads to inositol triphosphate and Ca^{2+} fluxes, events detected following EPEC infection. EPEC also induced tyrosine dephosphorylation of host proteins, including a 240-kDa host protein (Hp240), following EPEC infection. Protein dephosphorylation appears to be a signaling event which occurs independently of intimin. Inhibition of host tyrosine dephosphorylation events by the addition of the tyrosine phosphatase inhibitor sodium vanadate did not prevent actin accumulation beneath the adherent bacteria. We conclude that EPEC induces two sets of signaling events following infection. One set is dependent on EPEC proteins secreted by the type III secretion pathway (EspA and EspB) which induces Hp90 tyrosine phosphorylation and dephosphorylation of host phosphotyrosine proteins. The second set, which is also dependent on the first signaling events, requires intimin interaction with its receptor, tyrosine-phosphorylated Hp90, to trigger Hp150 and PLC-γ1 tyrosine phosphorylation as well as pedestal formation. Inhibition of pedestal formation by tyrosine kinase inhibitors indicates an important role for tyrosine phosphorylation events during EPEC subversion of host processes.

Enteropathogenic *Escherichia coli* (EPEC) is a gram-negative bacterium responsible for both acute and persistent infantile diarrhea (24) and remains a major cause of morbidity and mortality in underdeveloped countries. Both in vivo and in vitro studies indicate that EPEC initially attaches nonintimately to host epithelial cells, which leads to localized effacement of host cell microvilli (15, 18, 22). This event is then followed by intimate contact with the host cell and the accumulation of host cytoskeletal proteins beneath the bacteria, resulting in the formation of a pedestal-like structure (9, 19, 25, 27). Several host and bacterial factors that are associated with these events have been identified. These include the induction of host cell inositol triphosphate (IP_3) and Ca^{2+} fluxes as well as host protein phosphorylation (1, 8, 10, 21), including tyrosine phosphorylation of a 90-kDa epithelial membrane protein (Hp90) (25). These signaling events are dependent on the secretion of at least two proteins, EspA and EspB, by a dedicated type III export pathway (11, 14, 16, 17). The EPEC transposon mutant Cfm-14 is defective for activating signaling in host cells (4) and is deficient in type III-mediated EspA and EspB protein secretion (16). In addition to these EPEC-secreted proteins, a bacterial outer membrane protein, intimin (EaeA), is required for the organization of the host cytoskeletal proteins beneath adherent bacteria (15, 25). Recent experiments have shown that tyrosine-phosphorylated Hp90 is the epithelial receptor for the intimin protein (27). Other EPEC loci which play a more indirect role in host signaling, have been identified, including *bfp*, a plasmid-encoded locus which mediates bundle-forming pilus production required for microcolony formation and nonintimate adherence (6). Also located on the pMAR plasmid is the *per* regulon, which is involved in the positive regulation of intimin and Bfp expression as well as the regulation of EPEC protein secretion (13, 16).

In this study, we report previously unidentified tyrosine phosphorylation and dephosphorylation events that occur in HeLa cells following EPEC infection. We identified one of the host proteins which becomes tyrosine phosphorylated but only after intimin interacts with the host cell. We also investigated the nature and role of these tyrosine phosphorylation events in EPEC-triggered signaling and pedestal formation.

MATERIALS AND METHODS

Bacterial strains. In this study, we used the wild-type EPEC strain E2348/69 and mutant strains UMD864 (*eaeB espB*), CVD206 (*eaeA*), Cfm-14 (14-2-1 [1]), and UMD872 (*espA*) described elsewhere (4, 5, 7, 17). Bacteria were grown on Luria broth agar plates or Luria broth with kanamycin (50 μg/ml), nalidixic acid (50 μg/ml), or tetracycline (10 μg/ml) added as appropriate.

Cell lines. HeLa (ATCC CCL2), Caco-2 (ATCC HTB37), and HEp-2 (ATCC CCL23) cells were grown at 37°C in 5% CO_2 in Dulbecco's modified Eagle's medium) supplemented with 10% (vol/vol) fetal calf serum (GIBCO).

Cellular fractionation and protein extraction. HeLa monolayers were infected with EPEC strains (multiplicity of infection, 100) for 3 h. In experiments involving presignaling of HeLa cells, monolayers were incubated with the intimin-negative mutant (CVD206) for 2.5 h prior to washing and adding strain Cfm-14

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for a further 3 h. The monolayers were washed two or three times in cold phosphate-buffered saline (PBS), and the HeLa cells were permeabilized by adding 0.2% saponin (Calbiochem) (23) buffer (in 50 mM Tris [pH 7.5] containing 0.4 mM $NaVO_4$, 1 mM NaF, and 0.1 mM phenylmethylsulfonyl fluoride). After 5 min of incubation on ice, the samples were centrifuged (16,000 relative centrifugal force, 5 min, 4°C), and the soluble cytoplasmic protein fraction was removed. The insoluble pellet was washed in PBS, and the membrane proteins were separated from the insoluble components by the addition of 1% Triton X-100 lysis buffer (same as saponin buffer described above but with the addition of Triton X-100) as described before (25). Samples were resuspended in Laemmli sample buffer (20). With experiments using the epidermal growth factor (EGF) to stimulate the tyrosine phosphorylation of phospholipase C-γ1 (PLC-γ1), EGF was added at 100 ng/ml (final concentration) for 10 min prior to protein extraction as described above.

Inhibitors of signal transduction. Staurosporine (1 μM [final concentration] in dimethyl sulfoxide; Sigma), genistein (250 μM [final concentration] in H_2O; Bethesda Research Laboratories), and cytochalasin D (1 μg/ml [final concentration in dimethyl sulfoxide; Sigma) were used in these studies. Monolayers were infected simultaneously with EPEC and the various inhibitors. Since there is a 60- to 90-min lag prior to EPEC adherence and signal induction (10, 16, 25, 30), the drug has sufficient time to inhibit host processes. When HeLa monolayers were preinduced with the intimin-negative mutant as described above, the drugs were added at the same time as the Cfm-14 bacteria. Trypan blue (GIBCO; 0.1% [wt/vol] in PBS) exclusion assays were used to confirm that the combination of drugs and EPEC did not induce HeLa cell death over the course of the experiment, except in the case of genistein, where 20 to 40% cell death was observed.

Immunoprecipitation studies. A total of 2.5 μg of anti-bovine PLC-γ1 antibodies conjugated to protein A-agarose (mixed immunoglobulins G; Upstate Biotechnology, Inc.) or 15 μg of agarose-conjugated antiphosphotyrosine antibodies (immunoglobulin G2bκ; Upstate Biotechnology, Inc.) was added to the HeLa cytoplasmic and/or membrane fractions derived from 3×10^6 HeLa cells prepared as described above. After overnight incubation at 4°C with gentle rotation, the beads were pelleted (6,000 relative centrifugal force) for 1 min and then washed four times in 1% Triton X-100 lysis buffer (see above) prior to resuspension in Laemmli sample gel buffer (20).

Western blot (immunoblot) analysis. Protein samples were resolved by sodium dodecyl sulfate–6% polyacrylamide gel electrophoresis (SDS–6% PAGE) (20), and the proteins were transferred to nitrocellulose as described elsewhere (28). Blots were blocked in 5% bovine serum albumin–0.02% Tween 20 in PBS prior to incubation with antiphosphotyrosine (4G10; Upstate Biotechnology, Inc.; 1:2,000 dilution), PLC-γ1 (1:5,000), or PLC-γ2 (Santa Cruz Biotech; 1:200) specific antibodies. Bands bound by these antibodies were detected by alkaline phosphatase-conjugated secondary antibodies as described previously (25).

Immunofluorescence microscopy. HeLa cells seeded on glass coverslips were infected with various EPEC strains in the presence or absence of drugs as described above. After infection, the monolayers were washed and fixed in 2.5% paraformaldehyde. Cells were permeabilized with 0.1% Triton X-100 in PBS and stained for filamentous actin (with phallodin-Texas red; Molecular Probes) or with antiphosphotyrosine antibodies (4G10; Upstate Biotechnology, Inc.) with an appropriate secondary fluorescein-conjugated antibody as described previously (25).

Data imaging. Photographic negatives or raw data of the figures were scanned into Adobe Photoshop with an AGFA Studio Scanner or a Kodak professional RFS 2035 Plus film scanner, where they were labeled before printing with a Mitsubishi S3600-30U color printer.

RESULTS

Tyrosine phosphorylation events induced by EPEC in HeLa epithelial cells. Rosenshine et al. (25) previously described the tyrosine phosphorylation of three HeLa cell proteins, a prominent band of 90 kDa (Hp90) and two others (72 and 39 kDa), following infection with EPEC. Further examination of cytoplasmic, membrane, and insoluble (host nuclei, cytoskeleton, and adherent EPEC) protein extracts derived from EPEC-infected or control HeLa cells by Western blot analysis revealed several additional EPEC-induced tyrosine phosphorylation events (Fig. 1). In addition to Hp90, EPEC induced the tyrosine phosphorylation of a band of ca. 150 kDa, Hp150, found in both the cytoplasmic and membrane fractions. In contrast to Hp150, Hp90 fractionated in the membrane and insoluble fractions, while the EPEC tyrosine-phosphorylated Ep85 protein fractionated solely in the insoluble fraction as described previously (25). Figure 1 also shows two distinct series of tyrosine-phosphorylated bands in uninfected cells, one in the cytoplasmic fraction (series I) and the other in the membrane fraction (series II), which become dephosphorylated following infection with EPEC. EPEC also induced tyrosine dephosphorylation of a ca. 240-kDa protein found in both the cytoplasmic and membrane fractions, Hp240, following infection. Similar results were observed when HeLa cells were fractionated after using a Dounce homogenizer to release the cytoplasmic proteins rather than detergents (data not shown). EPEC infection of human colon adenocarcinoma, Caco-2, and human laryngeal carcinoma, HEp-2, cell lines triggered similar dephosphorylation events. However, only minor levels of tyrosine-phosphorylated Hp90 could be detected with these cell lines (data not shown).

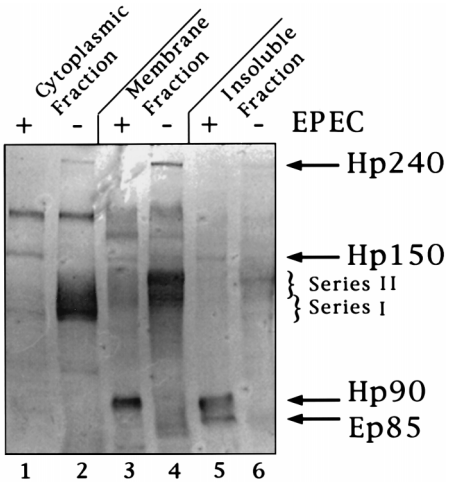

FIG. 1. HeLa cell tyrosine phosphorylation events induced in EPEC-infected HeLa cells. HeLa cells were fractionated into cytoplasmic, membrane, and insoluble fractions as described in Materials and Methods. Samples were resolved by SDS–6% PAGE and transferred to nitrocellulose before probing with antiphosphotyrosine specific antibodies. The host tyrosine phosphorylation events altered after EPEC infection are indicated on the right, and Ep85 represents an EPEC tyrosine-phosphorylated protein (25). +, EPEC-infected cells; −, uninfected cells.

Host tyrosine phosphorylation profiles following infection with EPEC mutants. A number of EPEC mutants that have been isolated and described can be grouped into the following two basic categories: (i) those which are defective for secreting one or more proteins and do not induce signaling events, including IP_3 fluxes and Hp90 tyrosine phosphorylation (these include UMD864 and UMD872, deleted for *espB* and *espA*, respectively, and Cfm-14, which is defective in the secretion of EspA, EspB, and additional proteins); and (ii) those that induce Hp90 phosphorylation and IP_3 fluxes but do not induce pedestal formation (CVD206) due to a mutation in the *eaeA* gene which encodes the EPEC outer membrane protein intimin. The mutants described above were used to infect HeLa cells, and the cells were fractionated as before. Analysis of the resulting protein extracts by Western blot analysis with antiphosphotyrosine specific antibodies demonstrated that the signaling defective mutants (UMD864 [*espB*], UMD872 [*espA*], and Cfm-14 [type III secretion-defective mutant]) did not induce the tyrosine phosphorylation or dephosphorylation events that normally follow wild-type infection (Fig. 2). As expected, CVD206, the intimin-negative mutant, induced Hp90 phosphorylation, although Hp90 remained almost exclusively in the membrane fraction (Fig. 2). However, CVD206 did not induce Hp150 phosphorylation (Fig. 2). This result indicated a role for intimin in Hp150 phosphorylation. Figure 2 also shows another host membrane protein of about 85 kDa

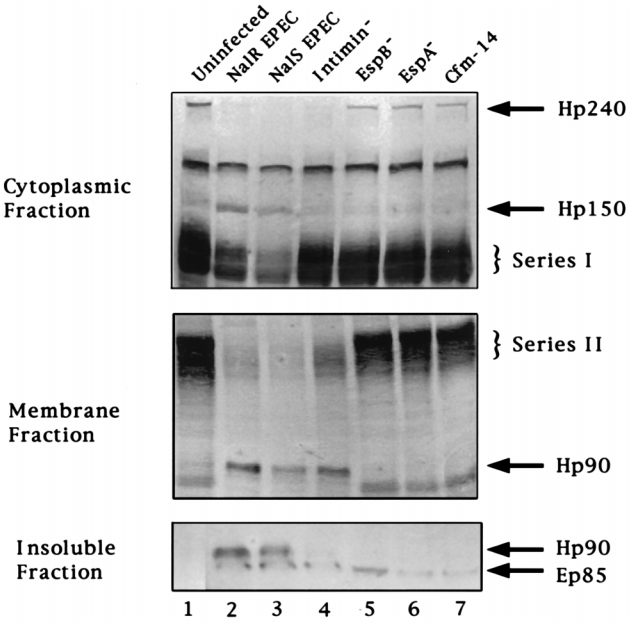

FIG. 2. HeLa cell tyrosine phosphorylation events induced in HeLa cells infected with EPEC mutants. HeLa monolayers were infected with various EPEC strains for 3 h prior to isolation of HeLa cell cytoplasmic, membrane, and insoluble fractions as described in Materials and Methods. Since Cfm-14 was made in a nalidixic acid-sensitive strain while the other EPEC mutants were made in a nalidixic acid-resistant strain, the tyrosine phosphorylation profiles of both parental strains are shown. Samples were resolved by SDS–6% PAGE and transferred to nitrocellulose before probing with antiphosphotyrosine specific antibodies.

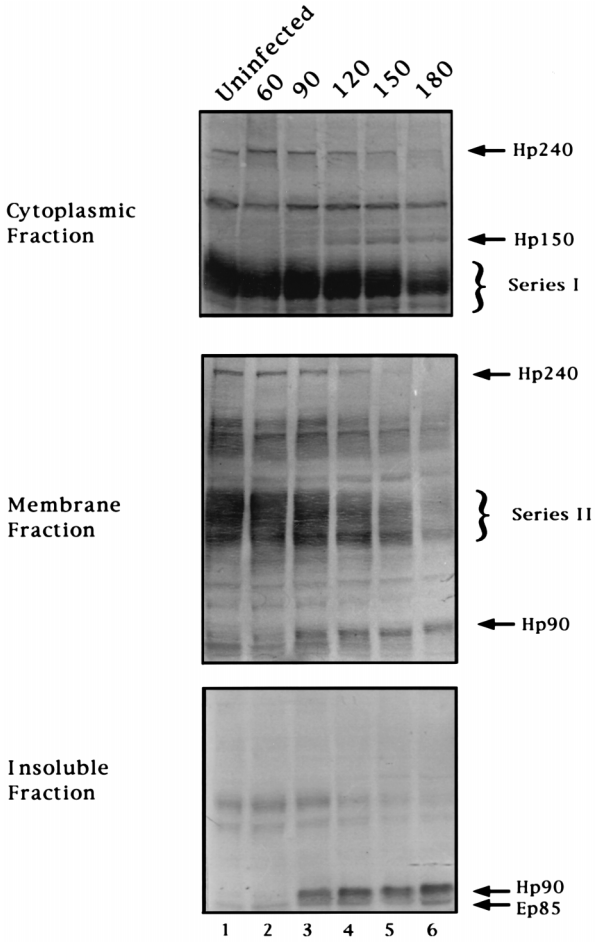

FIG. 3. Alteration in the host tyrosine phosphoprotein profile during the course of a 3-h EPEC infection. HeLa monolayers were left uninfected or infected with wild-type EPEC at time (*t*) 0, 30, 60, 90, or 120 min and incubated until a *t* of 180 min. The monolayers were separated into cytoplasmic, membrane, and insoluble fractions as described in Materials and Methods and resolved by SDS–6% PAGE. After being transferred to nitrocellulose, the blots were probed with antiphosphotyrosine specific antibodies.

which became dephosphorylated following EPEC-induced signaling, although this event was not detected in all experiments.

We examined the sequential order of these EPEC-induced phosphorylation events during the time course of a 3-h infection. Figure 3 shows that the tyrosine phosphorylation of Hp90 was evident after 90 min while Hp150 phosphorylation was readily evident only 120 min following EPEC addition. Tyrosine dephosphorylation of Hp240 and the series of membrane proteins (series II) was not evident until 120 min, while dephosphorylation of the cytoplasmic protein series (series I) was not readily discernible until 180 min. Similar results were found with the intimin mutant except that no phosphorylation of Hp150 was detected (data not shown). A role for tyrosine phosphoprotein dephosphorylation in pedestal formation was tested by pretreating HeLa cells with sodium orthovanadate to inhibit host tyrosine phosphatases. Although this drug inhibited tyrosine dephosphorylation events, it did not prevent actin accumulation beneath the adherent EPEC (data not shown).

Intimin-dependent signaling event. As stated above, the intimin mutant CVD206 induced signaling in host cells as evidenced by Hp90 tyrosine phosphorylation (Fig. 2), IP_3 fluxes, and the accumulation of host cytoskeletal proteins beneath the adherent bacteria (10, 25). However, CVD206 is unable to focus these host cytoskeletal components to form pedestal-like structures (25) or induce Hp150 tyrosine phosphorylation (Fig. 2). Both of these phenotypes are apparently due to the absence of the EPEC protein intimin. The requirement of intimin for Hp150 tyrosine phosphorylation was demonstrated in double-infection experiments. HeLa cells were first infected with the intimin mutant (to induce signaling) and subsequently with the signaling-defective mutant Cfm-14 (which expresses intimin) to induce pedestal formation (25). Western analysis of the tyrosine phosphorylation events induced in these cells doubly infected with the intimin mutant and Cfm-14 demonstrated that the Cfm-14 strain triggered Hp150 tyrosine phosphorylation (Fig. 4) in addition to pedestal formation (25; data not shown). This result indicates an additional EPEC-induced signal transduction event which requires intimin for triggering, unlike the other previously described EPEC-induced signaling events such as tyrosine phosphorylation of Hp90.

Effect of inhibitors on EPEC-induced tyrosine phosphorylation events. Since intimin triggers Hp150 tyrosine phosphorylation (Fig. 4) and pedestal formation (25), it is possible that Hp150 tyrosine phosphorylation is required for pedestal formation. To assess the putative role of this phosphorylated protein, we attempted to block Hp150 tyrosine phosphorylation. We used a general kinase inhibitor (staurosporine), a tyrosine protein kinase inhibitor (genistein), and an inhibitor of actin polymerization (cytochalasin D). As reported previously (25), we observed that both staurosporine and genistein inhibited the tyrosine phosphorylation of Hp90 (Fig. 5A) and pedestal formation (25; data not shown) while also causing a similar reduction in Hp150 phosphorylation (Fig. 5A). Although cytochalasin D did not prevent the accumulation of

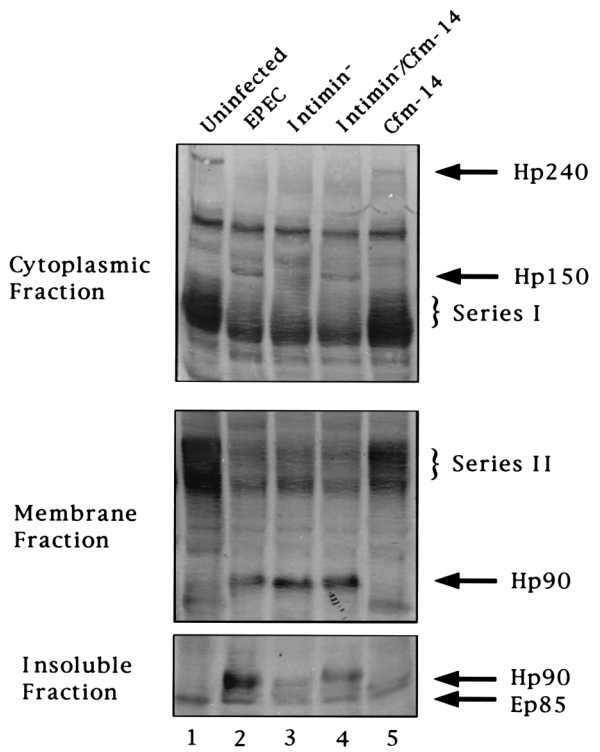

FIG. 4. Intimin-dependent triggering of Hp150 tyrosine phosphorylation. HeLa monolayers were left uninfected or infected with wild-type EPEC, the intimin mutant (CVD206), or the signaling mutant (Cfm-14) for 3 h. In the case of the double intimin mutant–Cfm-14 infections, the monolayers were first incubated with the intimin mutant (CVD206) for 2.5 h and washed with PBS and then the Cfm-14 mutant was added for a further 2.5 h. The HeLa monolayers were then separated into cytoplasmic, membrane, and insoluble fractions as described in Materials and Methods. Samples were resolved by SDS–6% PAGE and transferred to nitrocellulose before probing with antiphosphotyrosine specific antibodies.

tyrosine-phosphorylated Hp90 in the membrane fraction (Fig. 5A) (27), it inhibited Hp150 tyrosine phosphorylation. Cytochalasin D also inhibited the tyrosine dephosphorylation of Hp240 and the series I and II bands (Fig. 5A) as well as preventing pedestal formation (25; data not shown). Fluorescence microscopy studies revealed that cytochalasin D prevented the sequestration and tight focusing of tyrosine phosphoproteins beneath adherent EPEC (Fig. 5B), although the cells exhibited wild-type levels of tyrosine-phosphorylated Hp90 in the membrane fraction (Fig. 5A). Cytochalasin D also inhibited the migration of tyrosine-phosphorylated Hp90 into the insoluble fraction associated with the adherent EPEC (Fig. 5A). This process is usually mediated through intimin interaction with its host receptor, Hp90 (27).

Transient nature of Hp90 tyrosine phosphorylation event. Since the tyrosine kinase inhibitors hindered the tyrosine phosphorylation of both Hp90 and Hp150, we uncoupled these events to investigate the role of tyrosine-phosphorylated Hp150 in cytoskeletal rearrangements. We first preinfected HeLa cells with the intimin-negative mutant CVD206 to induce Hp90 phosphorylation and then added the appropriate inhibitor together with the signaling-defective mutant, Cfm-14, to induce pedestal formation (25). Surprisingly, even under these conditions, both kinase inhibitors led to the dephosphorylation of Hp90, which had already been phosphorylated by the addition of the intimin mutant prior to drug incubation (Fig. 6), as well as the inhibition of intimin-triggered Hp150 phosphorylation (Fig. 6). Fluorescence microscopy studies revealed that both staurosporine and genistein inhibited the accumulation and focusing of tyrosine phosphoproteins and actin beneath the adherent Cfm-14 bacteria (data not shown). Whether this inhibition of pedestal formation was due to the inhibition of Hp90 and/or Hp150 phosphorylation events remains to be determined. In contrast to the effect of the kinase inhibitors, coincubation of the Cfm-14 mutant in the presence of cytochalasin D did not alter the level of membrane-associated tyrosine-phosphorylated Hp90 (already induced by the intimin mutant) although it inhibited Hp150 tyrosine phosphorylation (Fig. 6), as observed with the wild-type strain (Fig. 5A). Fluorescence studies again demonstrated the inhibitory effect of cytochalasin D on the ability of the Cfm-14 bacteria to sequester and focus tyrosine phosphoproteins (data not shown), although the membranes contained normal levels of Hp90 previously induced by the intimin mutant (Fig. 6).

EPEC induces tyrosine phosphorylation of PLC-γ1. The detection of IP_3 and Ca^{2+} fluxes in host cells following EPEC infection has led to the proposed activation of a host phospholipase, such as PLC-γ1 (1, 8, 10). PLC-γ1 is a 150-kDa cytoplasmic protein which, following tyrosine phosphorylation, can interact with and cleave phosphatidylinositol 4,5-biphosphate (PIP_2) (12). Cleavage of PIP_2 releases inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (2), secondary messengers implicated in the release of intracellular Ca^{2+} stores and activation of protein kinase C. Since one of the EPEC-induced tyrosine-phosphorylated proteins had the same apparent molecular mass as PLC-γ1, we investigated whether this protein was equivalent to PLC-γ1. Antiphosphotyrosine specific antibodies were used to immunoprecipitate the cytoplasmic and membrane tyrosine-phosphorylated proteins from uninfected or EPEC-infected HeLa cells, and the immunoprecipitate was probed for PLC-γ1 (Fig. 7). Figure 7 shows that only significant levels of PLC-γ1 can be detected in the phosphotyrosine immunoprecipitate derived from the cytoplasm of EPEC-infected HeLa cells. Similar studies did not detect any tyrosine-phosphorylated PLC-γ1 induced in HeLa cells infected with the intimin or Cfm-14 mutant (data not shown).

Tyrosine-phosphorylated Hp150 is heterogeneous in composition. To confirm that Hp150 was composed of PLC-γ1, we immunoprecipitated PLC-γ1 from EPEC-infected HeLa cells and probed the precipitates with antiphosphotyrosine antibodies. As a control, we treated HeLa cells with EGF to trigger the tyrosine phosphorylation of PLC-γ1. Analysis of the resulting immunoprecipitates demonstrated that the Hp150 tyrosine-phosphorylated band induced by EGF or EPEC stimulation was heterogeneous in composition (Fig. 8A). Estimation of the amount of Hp150 in the various fractions indicates that only about 10 to 20% of the EPEC-induced tyrosine-phosphorylated Hp150 band coimmunoprecipitated with all of the cytoplasmic PLC-γ1 (Fig. 8A). Although a minor amount of PLC-γ1 was not precipitated following EGF stimulation, the heterogeneous nature of the resulting Hp150 band is evident. The tyrosine-phosphorylated Hp150 remaining in the cytoplasm following PLC-γ1 immunoprecipitation appeared to have a slightly lower molecular mass than PLC-γ1 by SDS-PAGE (data not shown). As another isoform of PLC, PLC-γ2 (148 kDa) can be tyrosine phosphorylated in response to receptor signaling events we investigated if this enzyme also became tyrosine phosphorylated (29) following EPEC or EGF stimulation. Analysis of the phosphotyrosine immunoprecipitates from EPEC- or EGF-stimulated HeLa cells with anti-PLC-γ2 specific antibodies revealed that although a small amount of PLC-γ2 was detected in the EGF-stimulated cells, no tyrosine-phosphorylated PLC-γ2 was seen with the EPEC-

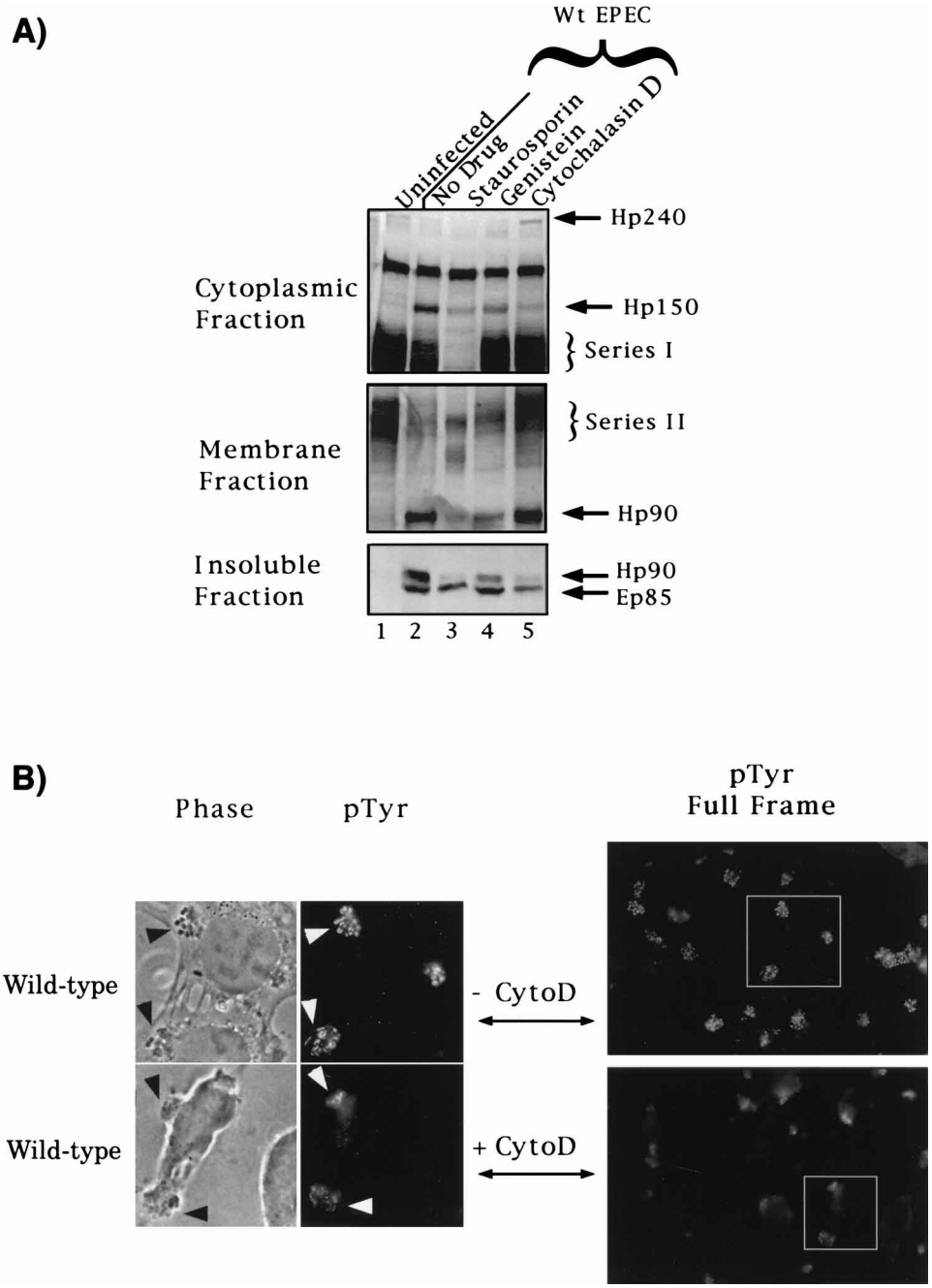

FIG. 5. Effect of inhibitors on EPEC-induced tyrosine phosphorylation events (A) and focusing of phosphotyrosine proteins beneath the adherent bacteria in the presence or absence of cytochalasin D (B). HeLa cells were infected simultaneously with EPEC and the inhibitory drug as described in Materials and Methods. Three hours postinfection, the HeLa cells were fractionated for immunoblot analysis (A) or fixed for fluorescence microscopy studies (B). Immunoblot samples were probed with antiphosphotyrosine specific antibodies, while fixed cells were probed for tyrosine phosphoproteins as described in Materials and Methods. Panel B shows the inhibitory effect of cytochalasin D on the accumulation and organization of tyrosine phosphoproteins beneath the adherent EPEC. Wt, wild type; pTyr, phosphotyrosine; CytoD, cytochalasin D.

induced Hp150 tyrosine-phosphorylated band (data not shown).

Similar immunoprecipitation experiments did not reveal any tyrosine-phosphorylated PLC-γ1 in cells infected with intimin-minus EPEC. In contrast, double infection of HeLa cells with the intimin mutant followed by the Cfm-14 mutant resulted in the induction of Hp150 tyrosine phosphorylation, of which again 10 to 20% was precipitated along with all the cytoplasmic PLC-γ1 (Fig. 8B).

DISCUSSION

A previous study by Rosenshine et al. (25) demonstrated tyrosine phosphorylation of several host proteins (Hp90, Hp72,

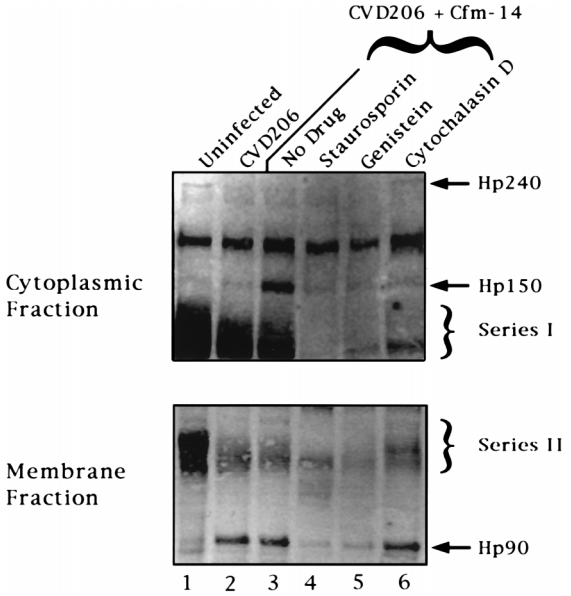

FIG. 6. Effect of inhibitors on intimin-triggered tyrosine phosphorylation events. HeLa cells were first infected with the intimin mutant (CVD206) for 2.5 h. The monolayers were washed and then infected with the signaling mutant, Cfm-14, together with the inhibitory drug as described in Materials and Methods. Three hours postinfection, the HeLa cells were fractionated for immunoblot analysis. Samples were resolved by SDS–6% PAGE and transferred to nitrocellulose before probing with antiphosphotyrosine specific antibodies.

and Hp39) following EPEC infection. An important role for EPEC-induced tyrosine phosphorylation events in host-pathogen interactions was demonstrated in experiments in which inhibitors of tyrosine kinases inhibited EPEC-induced cytoskeletal structures (25). In this paper, further examination of EPEC-infected HeLa cell extracts by Western analysis, after isolation of cytoplasmic, membrane, and insoluble fractions, revealed the tyrosine phosphorylation of an additional protein, Hp150, and novel tyrosine dephosphorylation events.

Although intimin-negative EPEC induces Hp90 tyrosine phosphorylation, it cannot induce pedestal formation (25). Analysis of the tyrosine phosphorylation profile induced in HeLa cells by the intimin mutant CVD206 revealed the absence of the tyrosine-phosphorylated Hp150 band found in cells infected with the wild type. This suggested that Hp150 phosphorylation requires intimin. This was confirmed by demonstrating that tyrosine phosphorylation of Hp150 was triggered in intimin mutant-infected cells by subsequent addition of the Cfm-14 signaling mutant (which expresses intimin). This identifies a second EPEC-induced signaling event which is dependent on the intimin protein and can be differentiated from earlier signaling events, such as Hp90 phosphorylation, by its requirement for intimin.

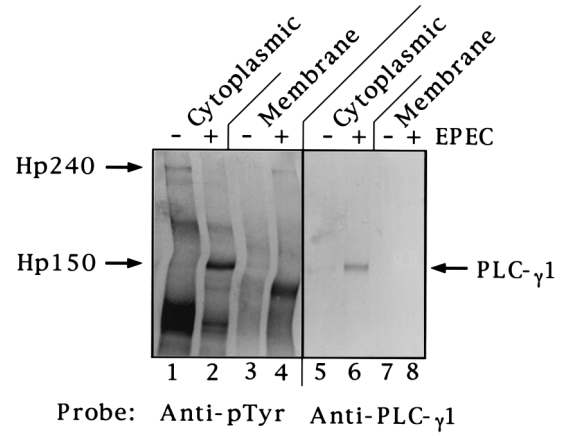

FIG. 7. Tyrosine phosphorylation of PLC-γ1 following EPEC infection. Control uninfected HeLa cells or cells infected with EPEC were fractionated as described in Materials and Methods, and the phosphotyrosine proteins were immunoprecipitated from the cytoplasmic and membrane fractions. Immunoprecipitates were loaded in duplicate and resolved by SDS-PAGE. After transfer to nitrocellulose, the blot was cut in half; each half was probed with antiphosphotyrosine (Anti-pTyr) or anti-PLC-γ1 specific monoclonal antibodies. +, EPEC-infected cells; −, uninfected cells.

Since Hp150 tyrosine phosphorylation was correlated with pedestal formation, we attempted to assess whether this phosphorylation event was required to direct cytoskeletal reorganization. The kinase inhibitors staurosporine and genistein and an inhibitor of actin polymerization, cytochalasin D, all inhibited Hp150 tyrosine phosphorylation and pedestal formation. Since the kinase inhibitors inhibited both Hp90 and Hp150 tyrosine phosphorylation, we first induced Hp90 tyrosine phosphorylation (in the absence of Hp150 tyrosine phosphorylation) by infection with the intimin mutant. We then added the appropriate drugs together with the Cfm-14 signaling mutant to trigger Hp150 tyrosine phosphorylation. Surprisingly, addition of the kinase inhibitors led to the dephosphorylation of the preinduced tyrosine-phosphorylated Hp90, revealing the labile nature of this event. Thus, it was not possible to determine whether one or both of these tyrosine-phosphorylated proteins (Hp90 or Hp150) are essential for pedestal formation by use of these kinase inhibitors.

We were able to specifically inhibit Hp150 tyrosine phosphorylation and uncouple it from Hp90 tyrosine phosphorylation by the addition of cytochalasin D. Fluorescence microscopy studies of cytochalasin D-treated cells demonstrated that adherent wild-type EPEC was unable to efficiently sequester and focus tyrosine phosphoproteins (presumably the intimin receptor, tyrosine-phosphorylated Hp90) (27), unlike the untreated infected cells. Although Rosenshine et al. (27) state that this drug did not prevent the sequestration of tyrosine-phosphorylated proteins beneath EPEC, the resolution of the figure in their report is not sufficient to determine if focusing occurs. In these experiments, tyrosine phosphorylation and accumulation, presumably of Hp90, probably occur directly beneath these bacteria. However, in double infection experiments, the Cfm-14 mutant (which expresses intimin) must sequester tyrosine-phosphorylated proteins from the host membrane, previously induced by the intimin mutant CVD206, and the inhibitory effect of cytochalasin D on this process was readily evident. We propose that cytochalasin D is inhibiting Hp150 tyrosine phosphorylation indirectly by preventing recruitment of the EPEC intimin protein with its host receptor, tyrosine-phosphorylated Hp90 (27). The absence of this interaction would then inhibit triggering of the intimin-dependent Hp150 phosphorylation. This hypothesis would also explain why tyrosine-phosphorylated Hp90 remains almost exclusively in the host membrane fraction during cytochalasin D treatment due to its inability to interact with the bacterial intimin protein and comigrate into the insoluble fraction. An implication of this inhibition of Hp90-intimin interaction by cytochalasin D is that the site of EPEC-induced Hp90 tyrosine phosphorylation is not necessarily beneath the adherent bacteria, and thus an actin-based host transport mechanism is required to deliver tyrosine-phosphorylated Hp90 for interaction with the intimin protein. However, whether the tyrosine phosphorylation of Hp150 is required to initiate pedestal formation or is

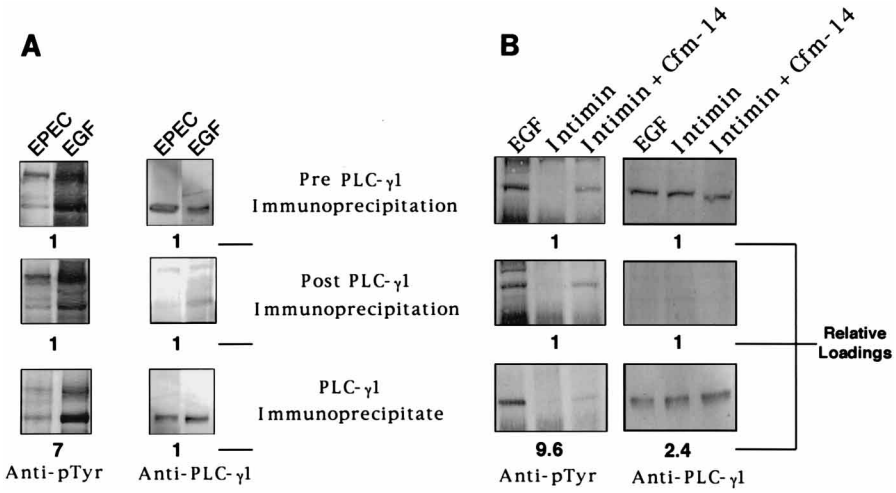

FIG. 8. Heterogeneous nature of Hp150 tyrosine-phosphorylated band. Tyrosine phosphorylation of Hp150 was induced in HeLa cells with EFG (100 ng/ml for 10 min to induce tyrosine phosphorylation of PLC-γ1) or wild-type EPEC (A) and EGF, the intimin-negative mutant, or the intimin and Cfm-14 mutants (B) as described in Materials and Methods. HeLa cells were fractionated and PLC-γ1 immunoprecipitated from the cytoplasmic fractions. Samples were also taken prior to and following PLC-γ1 immunoprecipitation for immunoblot analysis. Samples were resolved by SDS-PAGE and transferred to nitrocellulose, and separate blots were probed with either antiphosphotyrosine (Anti-pTyr) or anti-PLC-γ1 specific monoclonal antibodies. The relative loadings are indicated beneath the panels, where 1 denotes the cytoplasmic extract or immunoprecipitate derived from 3×10^5 HeLa cells. The developed Western blots were scanned into Adobe Photoshop (see Materials and Methods), and the area under each Hp150-related band was determined and used to estimate the percentage of Hp150 in each fraction. The phosphotyrosine and PLC-γ1 profiles in the extracts prior to immunoprecipitation (upper panels), in the extract remaining after immunoprecipitation (middle panels), and in the PLC-γ1 immunoprecipitates (lower panels) are shown.

stimulated as a result of intimin-induced actin polymerization remains to be determined.

The ability of EPEC to induce IP_3 and Ca^{2+} fluxes in host cells led to the hypothesis that a host phospholipase was being activated, possibly PLC-γ1 (1, 8, 10). PLC-γ1 is a 150-kDa cytoplasmic protein which, following tyrosine phosphorylation, can interact with and cleave PIP_2 (12), triggering IP_3 and Ca^{2+} fluxes which have been observed following EPEC infection (8, 10). Immunoprecipitation studies revealed that EPEC induces tyrosine phosphorylation of PLC-γ1 but that this protein comprises only about 10 to 20% of the EPEC-induced tyrosine-phosphorylated Hp150 band. Indeed, stimulation of PLC-γ1 tyrosine phosphorylation with EGF led to the coimmunoprecipitation of less than half of the EGF-induced Hp150 tyrosine phosphorylated with PLC-γ1. Since the Hp150 which was resistant to PLC-γ1 precipitation had a slightly lower molecular mass and another isoform of PLC, 148-kDa PLC-γ2 (29), can also be tyrosine phosphorylated, we tested to determine whether this protein was tyrosine phosphorylated following EPEC or EGF stimulation. Although a small amount of PLC-γ2 appeared to be tyrosine phosphorylated following EGF stimulation, it was not tyrosine phosphorylated in EPEC-infected cells. The identity of the majority of the tyrosine-phosphorylated 150-kDa band remains undetermined.

Figure 8 demonstrates the requirement of intimin to trigger detectable PLC-γ1 tyrosine phosphorylation. These results suggest that PLC-γ1 tyrosine phosphorylation is a late signaling event dependent on intimin-Hp90 interaction and thus may not be responsible for the IP_3 flux detected in intimin mutant-infected cells (8, 10). Although IP_3 fluxes have been reported in intimin mutant-infected cells, this mutant does not induce detectable Ca^{2+} fluxes (8, 10). Although this may be due to the detection limits of the assay employed, the observed differences between mutant- and wild-type-infected host cells, together with our results, suggest that EPEC may trigger two phospholipase activities. The first could be associated with Hp90 tyrosine phosphorylation and could be independent of detectable, or induce a very transient, Ca^{2+} release, while the second could be dependent on intimin-Hp90 interaction to trigger activation of PLC-γ1 and subsequent IP_3 and Ca^{2+} fluxes. The possibility that EPEC triggers two phospholipase activities which can be uncoupled in the absence of the intimin protein is supported by the observation that the level of IP_3 fluxes detected in intimin mutant-infected cells is about half that of wild-type infected cells (8). However, it is also plausible that EPEC triggers a single phospholipase activity but that Hp90-intimin interaction is required to stably activate or phosphorylate PLC-γ1 to stimulate a detectable Ca^{2+} release.

Our results show that EPEC signaling in host cells can be divided into two events, the first being dependent on the EPEC-secreted proteins and resulting in Hp90 phosphorylation (10, 16, 25) and the second being dependent on these earlier events but also requiring Hp90-intimin interactions to trigger Hp150 phosphorylation and pedestal formation (25; present study). Analysis of the sequential order of the EPEC-induced tyrosine phosphorylation events during the course of a 180-min infection showed that tyrosine phosphorylation of Hp90 was evident 90 min after EPEC infection. This time interval is apparently required for EPEC to induce the expression of bacterial proteins required for cell adherence and signaling, since tyrosine phosphorylation of Hp90 can occur almost immediately if the bacteria are preinduced in media prior to infection and centrifuged onto the host cells (26). Tyrosine phosphorylation of Hp150 occurs later, 120 min following infection, suggesting that it follows Hp90 phosphorylation. This is supported by the observation that Hp150 tyrosine phosphorylation is dependent not only on intimin interaction with host cells but on the presence of tyrosine-phosphorylated Hp90 in these membranes. In contrast, EPEC-induced tyrosine dephosphorylation appears to be part of a later signaling event, since dephosphorylation was not apparent until 120 min (Hp240 and the series II set of proteins) or 180 min (series I set of proteins) following EPEC infection. However, similar dephosphorylation profiles were seen with the intimin mutant CVD206, sug-

gesting that these dephosphorylation events are independent of intimin. These results argue that EPEC-induced tyrosine dephosphorylation is triggered as part of the early (intimin-independent) signaling process which is detectable by our assay only at later time points. The ability of cytochalasin D to inhibit these tyrosine dephosphorylation events suggests that they may be linked to cytoskeletal structure and/or reorganization. However, cytochalasin D could not inhibit these dephosphorylation events if added following a 2.5-h infection of host cells with the intimin-negative mutant. Surprisingly, under these conditions, cytochalasin D reversed the dephosphorylation of Hp240 (Fig. 6). This suggests that both EPEC and cytochalasin D can influence the phosphorylation of Hp240 and suggests a possible role for this phosphorylated protein in actin polymerization-depolymerization events. A role for tyrosine dephosphorylation events in pedestal formation was not evident in studies using the tyrosine phosphatase inhibitor sodium vanadate. *Yersinia* spp. induce the tyrosine dephosphorylation of host proteins as a mechanism to avoid host antibacterial processes (3), which contributes to their virulence. Thus, it is possible that EPEC induces dephosphorylation events for avoidance of host defense mechanisms.

We detected similar dephosphorylation events upon infection of other cell lines, including the human colon adenocarcinoma Caco-2 and the human laryngeal carcinoma HEp-2 cell lines (data not shown). Although pedestal formation has been reported for Caco-2 and HEp-2 cell lines (19, 25), we were not able to detect significant tyrosine-phosphorylated Hp90 by Western analysis of protein extracts. This suggests that EPEC-induced signal transduction events are reduced in these cell lines or that these tyrosine phosphorylation events are more labile than they are with HeLa cells. Given the important role of tyrosine-phosphorylated Hp90 in EPEC signal transduction events and that Hp150 phosphorylation is dependent on prior Hp90 tyrosine phosphorylation (present study), our inability to detect Hp150 phosphorylation with these cells lines was not surprising.

In summary, we have identified several new EPEC-induced tyrosine phosphorylation and dephosphorylation events that occur in epithelial cells. We have identified PLC-γ1 as a minor constituent of the EPEC-induced tyrosine-phosphorylated Hp150 band, and this activated enzyme is probably responsible for the IP_3 and Ca^{2+} fluxes detected following EPEC infection. Phosphorylation of PLC-γ1 and the remaining Hp150 protein(s) comprises a second set of signaling events induced by EPEC after activation of other host signals, including Hp90 phosphorylation, and is dependent on intimin–tyrosine-phosphorylated Hp90 interaction. Several tyrosine dephosphorylation events which appear to be part of the early signaling event were also described, although their effect is discernible only at later time points. The identification of these new EPEC-triggered signaling events increases our knowledge of the complexities of EPEC-host interactions and should aid further studies of EPEC-induced signaling events in host cells contributing to pathogenicity.

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REFERENCES

1. **Baldwin, T. J., W. Ward, A. Aitken, S. Knutton, and P. H. Williams.** 1991. Elevation of intracellular free calcium levels in HEp-2 cells infected with enteropathogenic *Escherichia coli*. Infect. Immun. **59:**1599–1604.
2. **Berridge, M. J.** 1993. Inositol triphosphate and calcium signaling. Nature **361:**315–325.
3. **Bliska, J. B., K. Guan, J. E. Dixon, and S. Falkow.** 1991. Tyrosine phosphate hydrolysis of host proteins by an essential Yersinia virulence determinant. Proc. Natl. Acad. Sci. USA **88:**1187–1191.
4. **Donnenberg, M. S., S. B. Calderwood, A. Donohue-Rolfe, G. T. Keusch, and J. B. Kaper.** 1990. Construction and analysis of Tn*phoA* mutants of enteropathogenic *Escherichia coli* unable to invade HEp-2 cells. Infect. Immun. **58:**1565–1571.
5. **Donnenberg, M. S., and J. B. Kaper.** 1991. Construction of an *eae* deletion mutant of enteropathogenic *Escherichia coli* by using a positive-selection suicide vector. Infect. Immun. **59:**4310–4317.
6. **Donnenberg, M. S., J. A. Giron, J. P. Nataro, and J. B. Kaper.** 1992. A plasmid-encoded type IV fimbrial gene of enteropathogenic *Escherichia coli* associated with localized adherence. Mol. Microbiol. **6:**3427–3437.
7. **Donnenberg, M. S., J. Yu, and J. B. Kaper.** 1993. A second chromosomal gene necessary for intimate attachment of enteropathogenic *Escherichia coli* to epithelial cells. J. Bacteriol. **175:**4670–4680.
8. **Dytoc, M., L. Fedorko, and P. M. Sherman.** 1994. Signal transduction in human epithelial cells infected with attaching and effacing *Escherichia coli* in vitro. Gastroenterology **106:**1150–1161.
9. **Finlay, B. B., I. Rosenshine, M. S. Donnenberg, and J. B. Kaper.** 1992. Cytoskeletal composition of attaching and effacing lesions associated with enteropathogenic *Escherichia coli* adherence to HeLa cells. Infect. Immun. **60:**2541–2543.
10. **Foubister, V., I. Rosenshine, and B. B. Finlay.** 1994. A diarrheal pathogen, enteropathogenic *Escherichia coli* (EPEC), triggers a flux of inositol phosphates in infected epithelial cells. J. Exp. Med. **179:**993–998.
11. **Foubister, V., I. Rosenshine, M. S. Donnenberg, and B. B. Finlay.** 1994. The *eaeB* gene of enteropathogenic *Escherichia coli* is necessary for signal transduction in epithelial cells. Infect. Immun. **62:**3038–3040.
12. **Goldschidt-Clermont, P. J., J. K. Kim, S. G. R. Machesky, and T. D. Pollard.** 1991. Regulation of phospholipase C-γ1 by profilin and tyrosine phosphorylation. Science **251:**1231–1233.
13. **Gomez-Duarte, O. G., and J. B. Kaper.** 1995. A plasmid-encoded regulatory region activates chromosomal *eaeA* expression in enteropathogenic *Escherichia coli*. Infect. Immun. **63:**1767–1776.
14. **Jarvis, K. G., J. A. Giron, A. E. Jerse, T. K. McDaniel, M. S. Donnenberg, and J. B. Kaper.** 1995. Enteropathogenic *Escherichia coli* contains a specialized secretion system necessary for the export of proteins involved in attaching and effacing lesion formation. Proc. Natl. Acad. Sci. USA **92:**7996–8000.
15. **Jerse, A. E., J. Yu, B. D. Tall, and J. B. Kaper.** 1990. A genetic locus of enteropathogenic *E. coli* necessary for the production of attaching and effacing lesions on tissue culture cells. Proc. Natl. Acad. Sci. USA **87:**7839–7843.
16. **Kenny, B., and B. B. Finlay.** 1995. Secretion of proteins by enteropathogenic *E. coli* which mediate signaling in host epithelial cells. Proc. Natl. Acad. Sci. USA **97:**7991–7995.
17. **Kenny, B., L.-C. Lai, B. B. Finlay, and M. S. Donnenberg.** 1996. EspA, a protein secreted by enteropathogenic *Escherichia coli* (EPEC), is required to induce signals in epithelial cells. Mol. Microbiol. **20:**313–323.
18. **Knutton, S., D. R. Lloyd, and A. S. McNeish.** 1987. Adhesion of enteropathogenic *Escherichia coli* to human intestinal enterocytes and cultured intestinal mucosa. Infect. Immun. **55:**69–77.
19. **Knutton, S., T. Baldwin, P. H. Williams, and A. S. McNeish.** 1989. Actin accumulation at sites of bacterial adhesion to tissue culture cells: basis of a new diagnostic test for enteropathogenic and enterohemorrhagic *Escherichia coli*. Infect. Immun. **57:**1290–1298.
20. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
21. **Manjarrez-Hernandez, H. A., T. J. Baldwin, A. Aitken, S. Knutton, and P. H. Williams.** 1992. Intestinal epithelial cell protein phosphorylation in enteropathogenic *Escherichia coli* diarrhoae. Lancet **339:**521–523.
22. **Moon, H. W., S. C. Whipp, R. A. Argenzio, M. M. Levine, and R. A. Giannella.** 1983. Attaching and effacing activities of rabbit and human enteropathogenic *Escherichia coli* in pig and rabbit intestines. Infect. Immun. **41:** 1340–1351.
23. **Rijnboutt, S., H. M. Aerts, H. J. Geuze, J. M. Tager, and G. J. Strous.** 1991. Mannose 6-phosphate-independent membrane association of cathepsin D, glucocerebrosidase, and sphingolipid-activated protein in HepG2 cells. J. Biol. Chem. **266:**4862–4868.
24. **Robins-Browne, R. M.** 1987. Traditional enteropathogenic *Escherichia coli* of infantile diarrhea. Rev. Infect. Dis. **9:**28–53.
25. **Rosenshine, I., M. S. Donnenberg, J. B. Kaper, and B. B. Finlay.** 1992. Signal exchange between enteropathogenic *Escherichia coli* (EPEC) and epithelial cells: EPEC induce tyrosine phosphorylation of host cell protein to initiate cytoskeletal rearrangement and bacterial uptake. EMBO J. **11:**3551–3560.
26. **Rosenshine, I., S. Ruschkowski, and B. B. Finlay.** 1996. Expression of at-

taching/effacing activity by enteropathogenic *Escherichia coli* depends on growth phase, temperature, and protein synthesis upon contact with epithelial cells. Infect. Immun. **64:**966–973.
27. **Rosenshine, I., S. Ruschkowski, M. Stein, D. J. Reinscheid, S. D. Mills, and B. B. Finlay.** 1996. A pathogenic bacterium triggers epithelial signals to form a functional bacterial receptor that mediates actin psuedopod formation. EMBO J. **15:**2613–2624.
28. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
29. **Sultzman, L., C. Ellis, L.-L. Lin, T. Pawson, and J. Knopf.** 1991. Platelet-derived growth factor increases the in vivo activity of phospholipase C-γ_1 and phospholipase C-γ_2. Mol. Cell. Biol. **11:**2018–2025.
30. **Vuopio-Varkila, J., and G. K. Schoolnik.** 1991. Localized adherence by enteropathogenic *Escherichia coli* is an inducible phenotype associated with the expression of new outer membrane proteins. J. Exp. Med. **174:**1167–1177.

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