

Internalization of *Escherichia coli* by Human Renal Epithelial Cells Is Associated with Tyrosine Phosphorylation of Specific Host Cell Proteins

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Human renal epithelial cells are capable of internalizing *Escherichia coli* regardless of whether the bacteria are isolated from individuals with pyelonephritis or from healthy volunteers. In this study, we investigated the role of host cell tyrosine kinase activity in internalization. We found that internalization of both fecal and pyelonephritis isolates is blocked by tyrosine kinase inhibitors. We found increased intensity of two tyrosine-phosphorylated proteins, with relative mobilities of approximately 123,000 and 110,000, in Western blots of extracts from human renal epithelial cells infected with *E. coli*. The increased intensity of these tyrosine-phosphorylated proteins was observed only in the Triton X-100-insoluble fraction, suggesting that these proteins could be associated with the cytoskeleton. Increased tyrosine phosphorylation of these proteins upon *E. coli* infection was observed in both transformed and primary human renal epithelial cells and in cells infected with several different strains of *E. coli* isolated from the feces of healthy individuals or from the blood or urine of patients with pyelonephritis. The increased tyrosine phosphorylation of these proteins required live bacteria and was blocked by tyrosine kinase inhibition but not by protein synthesis inhibitors or cytochalasin D. These experiments establish a strong link between *E. coli* internalization and host cell signaling through tyrosine kinases in human kidney cells and provide evidence that specific proteins are involved in these processes.

Escherichia coli is the most common cause of urinary tract infections (UTIs), responsible for 75 to 80% of all community-acquired and a substantial minority of complicated UTIs (27). It is estimated that 40 to 50% of adult women will have a UTI during their lifetimes (14). In some cases, cystitis or urethritis can progress to pyelonephritis, the most serious UTI since infection of the kidney can lead to sepsis. In fact, the most common source of *E. coli* bacteremia is the urinary tract (9). The mechanisms by which bacteria breach the uroepithelial barrier and gain access to the bloodstream are not known. One potential pathway to initiate tissue invasion, supported by several lines of evidence, is the internalization of *E. coli* by renal epithelial cells. Bacteria have been observed in membrane-bound vesicles of both phagocytic and epithelial cells in renal biopsies from patients with acute pyelonephritis (11). In addition, it was shown by electron microscopy that human renal epithelial cells can internalize *E. coli* strains in vitro, whether the strains are of fecal origin or from the urine or blood of patients with acute pyelonephritis (28). These morphological observations are supported by quantitative data obtained by using the gentamicin protection assay, which confirmed that primary human renal epithelial cells in tissue culture are capable of internalizing *E. coli* of both fecal and UTI origin in similar numbers (7).

Bacterial entry into host cells is an important aspect of

several infectious diseases. Many pathogenic bacteria have the capacity to enter nonphagocytic host cells such as epithelial cells by a process of bacterium-induced phagocytosis. Cell invasion often involves triggering host cell signal transduction mechanisms to induce rearrangement of the cytoskeleton at the site of attachment, which facilitates uptake of the bacteria into the cells. The specific cytoskeletal components and signaling mechanisms vary from pathogen to pathogen. Some pathogens subvert host cell tyrosine kinase signaling pathways for bacterium-induced phagocytosis. Inhibitors of tyrosine kinases block invasion by *Listeria monocytogenes*, enteropathogenic *E. coli* (EPEC), *Campylobacter jejuni*, and *E. coli* expressing the invasin of *Yersinia pseudotuberculosis* (17, 18, 23, 30). One group has reported that invasion by *Salmonella typhimurium* is accompanied by tyrosine phosphorylation of the epidermal growth factor receptor (8), but other investigators, unable to reproduce this finding, reported that internalization of this pathogen is not blocked by tyrosine kinase inhibitors (19). In host cells, tyrosine kinases function in diverse pathways of signal transduction, activated by hormones or growth factors through transmembrane receptor molecules or by cell-to-cell contact through integrins (10, 22, 31). Proteins phosphorylated by tyrosine kinases may transmit the phosphorylation signal to other molecules in a cascade which activates secondary signals such as G proteins, inositol phosphates, calcium, arachidonic acid metabolism, and transcriptional factors (2, 13, 16, 26).

Experiments to assess the role of tyrosine kinases in internalization of bacteria by human renal cells have not been reported. To test the hypothesis that signaling through tyrosine phosphorylation of specific host cell proteins is required for entry of *E. coli* into human kidney cells, we measured internalization in the presence and absence of tyrosine kinase inhibitors. In addition, we used Western blotting with phosphotyrosine antibodies to gain insight into the tyrosine kinase

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substrates that are associated with this signal transduction response.

MATERIALS AND METHODS

Bacterial strains. *E. coli* CFT191, CFT205, CFT325, and E44324, which are nonhemolytic, P-fimbriated isolates from the blood or urine of patients with acute pyelonephritis, and fecal strains EFC1, EFC2, and FN414, which were isolated from the feces of normal volunteers, were described previously (7). EPEC strains 2087-77 and E2348/69 are also clinical isolates that have been described elsewhere (6). All strains were stored at -70°C in 50% glycerol-50% LB broth (vol/vol) and grown on LB agar plates and in LB broth with aeration at 37°C . To kill bacteria, an overnight culture was centrifuged, resuspended in sterile phosphate-buffered saline (PBS) to an optical density at 600 nm of 0.5, transferred into a 96-well plate, and exposed to 40 min of UV irradiation (FisherBiotech 312-nm transilluminator, 3 to 6 mm from source).

Cell lines. Primary cultures of human renal proximal tubule epithelial cell (HRPTEC) lines 539, 535, 630, and 518 were obtained from A. Trifillis (25). These primary cells and A-498 cells, a human kidney carcinoma cell line (American Type Culture Collection HTB 44), were maintained in Eagle's minimal essential medium (EMEM) supplemented with 10% fetal bovine serum (inactivated by heating at 56°C for 30 min), penicillin (50 U/ml), and streptomycin (50 $\mu\text{g}/\text{ml}$). The cell cultures were incubated in an atmosphere of 95% air-5% CO_2 at 37°C .

Internalization assays. Internalization of *E. coli* by human renal epithelial cells was measured by using the gentamicin protection assay as described previously (7). When indicated, the tyrosine kinase inhibitors genistein (ICN, Costa Mesa, Calif.) and tyrphostin (Gibco BRL, Gaithersburg, Md.) were added at 250 μM and included for the entire 3-h incubation. The rapid reversibility of genistein was explored by including the inhibitor for the 3-h incubation, washing, and incubating the cells for an additional 30 min in medium lacking inhibitors prior to the addition of gentamicin. Each experiment was performed in duplicate and repeated a total of four times. The geometric mean number of survivors for each condition was determined and compared by using two-way analysis of variance. *P* values less than or equal to 0.05 were considered significant.

Immunoblot analysis. HRPTEC and A-498 cells were seeded into six-well plates at densities of 4×10^5 to 1×10^6 cells per well in 4 ml of supplemented EMEM. After an overnight incubation, cells were washed three times with 3 ml of sterile PBS and incubated at 37°C for 30 min in unsupplemented EMEM containing 0.04% NaHCO_3 . Specific inhibitors such as genistein (250 μM), cytochalasin D (2 μM), cycloheximide (20 $\mu\text{g}/\text{ml}$), and chloramphenicol (50 $\mu\text{g}/\text{ml}$) were diluted in unsupplemented EMEM to the final concentrations indicated and were added 30 min prior to infection. Overnight bacterial cultures were resuspended in sterile PBS to an optical density at 600 nm of 0.2, and 100 μl was added to each well. After incubation for 3, 4.5, or 6 h at 37°C in an atmosphere of 95% air-5% CO_2 , the plates were placed on ice, and all subsequent steps were performed at 4°C . Tissue culture medium containing nonadherent bacteria was removed from wells and discarded. The adherent cells were washed three times with 3 ml of PBS containing 0.4 mM Na_3VO_4 , 1 mM NaF, and 0.1 mg of phenylmethylsulfonyl fluoride per ml and removed from the plate by scraping into 1.5 ml of the same buffer. The resuspended cells were pelleted by centrifugation at $10,000 \times g$ for 1 min. The pellet was resuspended and incubated with gentle rocking in 100 μl of lysis buffer (50 mM Tris-HCl [pH 7.6] containing 0.4 mM Na_3VO_4 , 1 mM NaF, 0.1 mg of phenylmethylsulfonyl fluoride per ml, 0.01 mg of leupeptin per ml, and 1% Triton X-100). The lysate was then subjected to centrifugation at $14,000 \times g$ for 30 min. After removal of the Triton X-100-soluble supernatant, the pellet was resuspended in 100 μl of lysis buffer. For identification of tyrosine-phosphorylated proteins of *E. coli*, bacteria were grown for 4.5 h in EMEM containing 0.04% NaHCO_3 , pelleted, and processed as described above. In preliminary experiments, equivalent volumes of the Triton X-100 fractions were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (10% T [total monomer], 0.65% C [cross-linker]), using a modification of the method of Laemmli (15). In later experiments, equivalent amounts of protein (10 to 20 μg) were mixed with 5 \times SDS-PAGE loading buffer (0.313 M Tris-HCl [pH 6.8], 10% [vol/vol] SDS, 25% [vol/vol] 2-mercaptoethanol, 50% [vol/vol] glycerol, 0.01% [wt/vol] bromophenol blue), boiled for 10 min, and subjected to SDS-PAGE. Protein concentrations were determined by using a microtiter well bicinchoninic acid assay as described by the manufacturer (Pierce, Rockford, Ill.) with bovine serum albumin diluted into lysis buffer as a standard. The proteins were transferred to polyvinylidene difluoride membranes (Immobilon P; Millipore, Bedford, Mass.), using a Transblot system (Hofer, San Francisco, Calif.) at 300 mA for 3 h at 4°C (24). Membranes were blocked in PBS containing 0.5% Tween 20 for 1 h. A two-step detection method was used for identification of phosphotyrosine-containing proteins in preliminary experiments. Blots were first incubated with antiphosphotyrosine monoclonal antibody 4G10 (Upstate Biotechnology, Inc., Lake Placid, N.Y.) diluted 1:2,500 in PBS containing 0.1% Tween 20, followed by an incubation with sheep anti-mouse immunoglobulin G (1:50,000) conjugated to horseradish peroxidase (HRP) (Boehringer Mannheim, Indianapolis, Ind.). In subsequent experiments, phosphotyrosine-containing proteins were identified by using an antiphosphotyrosine immunoglobulin G-HRP conjugate (PY20; Pierce) diluted 1:25,000 in PBS containing 0.1% Tween 20 for 1 h and washed three times in 3

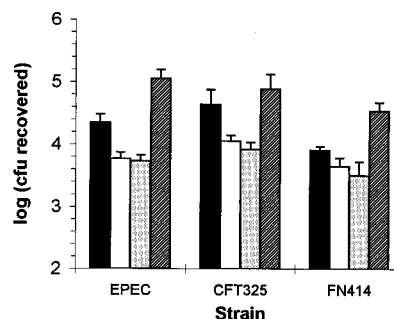


FIG. 1. Effects of tyrosine kinase inhibitors on internalization of *E. coli* by primary HRPTEC. Cells were infected with EPEC strain E2348/69, pyelonephritis strain CFT325, or fecal strain FN414 for 3 h in the absence of inhibitors (black bars) or in the presence of tyrosine kinase inhibitor tyrphostin (white bars) or genistein (stippled bars). Alternatively, cells were incubated for 3 h in the presence of genistein and then for 0.5 h in the absence of inhibitors (hatched bars). After the infection period, cells were incubated for 1 h in the presence of gentamicin (100 $\mu\text{g}/\text{ml}$) to kill extracellular bacteria. The \log_{10} CFU recovered after lysis of the cells with Triton X-100 was measured by serial dilution and plate counting. Bars represent standard errors of the geometric means.

volumes of dilution buffer. Phosphotyrosine-containing proteins were detected by using enhanced chemiluminescence reagents (Amersham) as instructed by the manufacturer. Blots were analyzed on a pdi (Huntington Station, N.Y.) 420oe densitometer, using Quantity One (version 2.7) software.

RESULTS

Internalization of *E. coli* into HRPTEC is dependent on tyrosine kinase activity. We performed internalization assays in the presence and absence of tyrosine kinase inhibitors. These inhibitors had no effect on the viability of the bacteria (data not shown). We used two different HRPTEC lines, a prototype nonhemolytic *E. coli* isolate from a patient with pyelonephritis (CFT325) and a fecal *E. coli* strain (FN414). As a control, we included an EPEC strain because invasion by EPEC is known to be dependent on host cell tyrosine kinase activity (17). We observed a significant reduction in internalization by HRPTEC of all bacterial strains in the presence of genistein compared to control values ($P < 0.001$ for EPEC, $P = 0.002$ for CFT325, and $P = 0.015$ for FN414) (Fig. 1). Internalization of both the pyelonephritis isolate and the EPEC strain was also significantly inhibited by tyrphostin ($P = 0.011$ and $P < 0.001$, respectively). For the fecal strain, the difference between tyrphostin and control values was not significant ($P = 0.115$). Interestingly, when the rapidly reversible inhibitor genistein was removed, we observed a significant increase in internalization of the EPEC and fecal strains in comparison to control values in the absence of inhibitor ($P < 0.001$ for both). A similar increase in internalization of the pyelonephritis isolate upon removal of genistein was noted but was not statistically significant compared with control values ($P = 0.25$). These results indicate that internalization of *E. coli* by human renal epithelial cells is dependent on host cell tyrosine kinase activity.

Induction of tyrosine-phosphorylated proteins in human kidney cells infected with uropathogenic *E. coli*. In preliminary experiments, infection of A-498 cells with the uropathogenic *E. coli* strain CFT325 resulted in a marked increase in the level of at least two Triton X-100-insoluble tyrosine phosphate-containing proteins, as detected with the mouse monoclonal antibody and HRP-conjugated secondary antibody (Fig. 2). The molecular weights of these proteins (hereafter referred to as pY123 and pY110) were estimated to be 123,000 and 110,000 (standard deviation, $<5\%$) when compared in 10, 11, and 12%

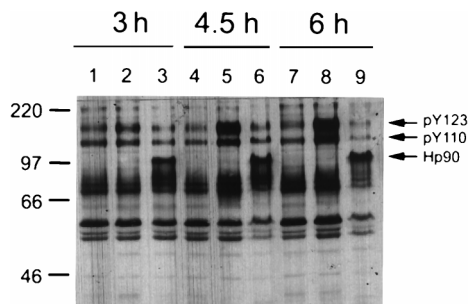


FIG. 2. Effect of *E. coli* infection on tyrosine phosphorylation of Triton X-100-insoluble proteins in human kidney carcinoma A-498 cells. A-498 cells were incubated for 3 (lanes 1 to 3), 4.5 (lanes 4 to 6), or 6 (lanes 7 to 9) h in the absence of bacteria (lanes 1, 4, and 7), in the presence of pyelonephritis isolate CFT325 (lanes 2, 5, and 8), or in the presence of EPEC strain E2348/69 (lanes 3, 6, and 9). Equivalent volumes of Triton X-100-insoluble protein samples were resolved by SDS-PAGE (10% gel) and analyzed by immunoblotting with antiphosphotyrosine antibody 4G10. The blot was developed with enhanced chemiluminescence reagents and protocol as recommended by the manufacturer (Amersham). The sizes (10^3) and migration of Rainbow molecular weight markers (Amersham) are indicated. The arrows indicate the positions of prominent tyrosine-phosphorylated proteins, as labeled.

polyacrylamide gels to the mobility of Perfect Protein Markers (Novagen; Madison, Wis.). No such change in the levels of these or any other tyrosine phosphate-containing proteins was observed in the Triton X-100-soluble fraction (data not shown). Infection of these cells with EPEC strain E2348/69 did not consistently elicit increased levels of either pY123 or pY110 but did stimulate induction of a ~90-kDa tyrosine phosphate-containing protein (Hp90) similar to that observed upon EPEC infection of HeLa cells (17). Results from time course experiments showed that the levels of pY123 and pY110 increased from 3 h postinfection to become the prominent phosphotyrosine-containing proteins at 4.5 and 6 h postinfection (Fig. 2). The effect was also found at these latter time points when the medium was replaced after 3 h of infection. After infection with *E. coli*, an increase in the intensity of tyrosine-phosphorylated proteins of similar relative mobility was also observed in primary HRPTEC grown from two different decedents (Fig. 3). Although an increase in intensity of pY123 and pY110 was difficult to detect in A498 cells in this experiment, this result confirms that the effect of *E. coli* infection on the intensity of these tyrosine-phosphorylated proteins is demonstrable in primary cells as well. All subsequent experiments were carried out with A-498 cells.

The proteins pY123 and pY110 are distinct from the Hp90, Hp72, and Hp39 proteins induced by EPEC in HeLa cells and are of host cell origin. We tested whether the uropathogenic *E. coli* strain CFT325 or EPEC strains produced proteins that were reactive to the antiphosphotyrosine antibodies under the conditions of infection. Like other investigators (17), we found bacterial proteins that reacted with both 4G10 and PY20. However, the molecular weights of these proteins (80,000, 45,000, and 35,000) were clearly distinct from those of pY123 and pY110 (data not shown).

The observed higher levels of pY123 and pY110 in infected cells than in uninfected cells persisted in subsequent experiments using the HRP-conjugated monoclonal antibody PY20. The increase in pY123 and pY110 was noted after adjustment of each sample to contain equivalent protein concentrations. The increase in pY123 was noted in cells infected with all pyelonephritis and fecal strains tested (Fig. 4). In the experiment shown in Fig. 4, the intensity of pY123 was significantly greater in the eight infected samples than in the four unin-

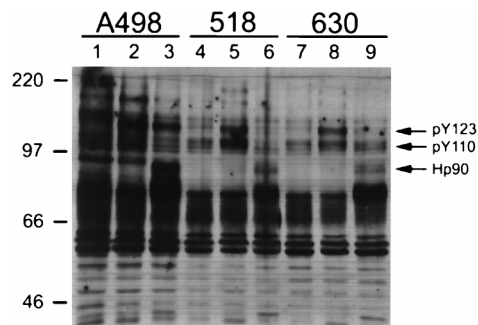


FIG. 3. Comparison of Triton X-100-insoluble phosphotyrosine-containing proteins from transformed and primary renal epithelial cell lines after *E. coli* infection. Human renal carcinoma A-498 cells (lanes 1 to 3), primary HRPTEC line 518 (lanes 4 to 6), and primary HRPTEC cell line 630 (lanes 7 to 9) were incubated for 4.5 h in the absence of bacteria (lanes 1, 4, and 7), in the presence of pyelonephritis isolate CFT325 (lanes 2, 5, and 8), or in the presence of EPEC strain E2348/69 (lanes 3, 6, and 9). Equivalent volumes of Triton X-100-insoluble protein samples were resolved by SDS-PAGE (10% gel) and analyzed by immunoblotting with antiphosphotyrosine antibody 4G10. The sizes (10^3) and migration of Rainbow molecular weight markers (Amersham) are indicated. The arrows indicate the positions of prominent tyrosine-phosphorylated proteins, as labeled.

ected samples ($P = 0.004$, Wilcoxon rank sum test). In this experiment, the intensities of pY110 in uninfected and infected samples were not significantly different. However, paired comparison of all uninfected and infected samples from subsequent experiments indicated that the intensity of pY110 in infected cells was significantly greater than that in uninfected cells ($P = 0.03$, signed rank test).

Live bacteria were required to stimulate the increase of pY123 and pY110, as nonviable (UV-irradiated) *E. coli* resulted in no appreciable increase in the intensities of the two proteins (Fig. 5). Since live bacteria have the opportunity to multiply during the course of the experiment, we also added killed bacteria at 20 times the usual inoculating dose to approximate the number of viable bacteria present at the end of the experiment (data not shown). Even at this higher dose, the killed bacteria failed to increase the levels of pY123 and pY110 (Fig. 5).

The increase in intensity of pY123 and pY110 was dependent on the activity of protein tyrosine kinases, as genistein (250 μ M) inhibited this effect (Fig. 6). In contrast, inclusion of the cytoskeletal inhibitor cytochalasin D (2 μ M) had little

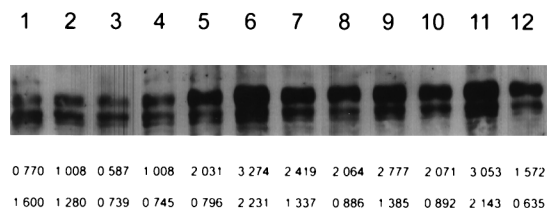


FIG. 4. Induction of tyrosine phosphorylation in Triton X-100-insoluble proteins of A-498 cells infected with various *E. coli* strains. A-498 cells were left uninfected (lanes 1 to 4) or infected for 4.5 h with *E. coli* strains, as follows: lane 5, enteropathogenic strain 2087-77; lanes 6 to 8, fecal strains EFC1 (lane 6), EFC2 (lane 7), and FN414 (lane 8); lanes 9 to 12, pyelonephritis strains CFT191 (lane 9), CFT205 (lane 10), CFT325 (lane 11), and E44324 (lane 12). Equivalent amounts of protein were separated by SDS-PAGE (10% gel) and analyzed for phosphotyrosine content by immunoblotting with antiphosphotyrosine antibody PY20. The exposed film was scanned and subjected to densitometry. Only the relevant portion of the gel is shown. The upper number below each lane indicates the relative density of pY123; the lower number indicates the relative density of pY110.

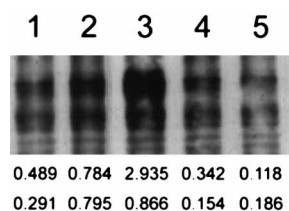


FIG. 5. Ability of nonviable *E. coli* to induce an increase of Triton X-100-insoluble phosphotyrosine-containing proteins of A-498 cells. A-498 cells were left uninfected (lanes 1 and 2), infected with viable *E. coli* pyelonephritis strain CFT191 (lane 3), infected with UV-irradiated *E. coli* CFT191 (lane 4), or infected with UV-irradiated *E. coli* CFT191 at 20 times the usual inoculating dose (lane 5). Equivalent amounts of protein were separated by SDS-PAGE (10% gel) and analyzed for phosphotyrosine content by immunoblotting with antiphosphotyrosine antibody PY20. The exposed film was scanned and subjected to densitometry. Only the relevant portion of the gel is shown. The upper number below each lane indicates the relative density of pY123; the lower number indicates the relative density of pY110.

effect on the *E. coli*-induced increase of pY123 and pY110 (Fig. 7). Since this concentration of cytochalasin D blocks internalization by >90% (7), this result suggests that internalization of bacteria is not a requirement for the increased intensity of these tyrosine-phosphorylated proteins.

Inhibitors of either eukaryotic (cycloheximide [20 μ g/ml]) or prokaryotic (chloramphenicol [50 μ g/ml]) protein synthesis had no detectable effect on the increased intensities of these proteins (Fig. 8). These results suggest that the increase in pY123 and pY110 is not due to de novo synthesis of these proteins by the host cells postinfection but rather that there is an increase in phosphorylation of preexisting proteins. Furthermore, the bacterial trigger of this response, if a protein, is present prior to infection.

DISCUSSION

We have previously demonstrated that human renal epithelial cells are capable of internalizing *E. coli*, that internalization requires actin polymerization, and that the ability to enter these cells is not dependent on whether the bacteria originate from clinical cases of pyelonephritis or from the stool of healthy volunteers (7). In this report, we focus on the role of host cell tyrosine kinase activity in uptake of *E. coli* by human

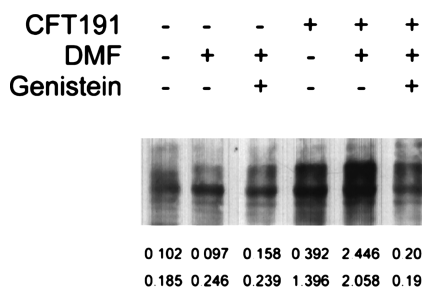


FIG. 6. Effect of genistein on *E. coli* induction of Triton X-100-insoluble phosphotyrosine-containing proteins of A-498 cells. Uninfected A-498 cells or cells infected with pyelonephritis *E. coli* strain CFT191 were incubated for 4.5 h in unsupplemented medium, in medium containing dimethyl formamide (DMF) solvent alone, or in medium containing genistein solubilized in dimethyl formamide. Plus and minus signs indicate inclusion and exclusion, respectively, of the indicated substance. Equivalent amounts of protein were separated by SDS-PAGE (10% gel) and analyzed for phosphotyrosine content by immunoblotting with antiphosphotyrosine antibody PY20. The exposed film was scanned and subjected to densitometry. Only the relevant portion of the gel is shown. The upper number below each lane indicates the relative density of pY123; the lower number indicates the relative density of pY110.

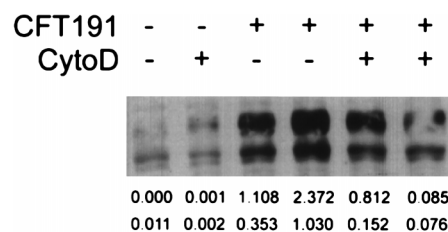


FIG. 7. Effect of cytochalasin D on *E. coli* induction of Triton X-100-insoluble phosphotyrosine-containing proteins of A-498 cells. Uninfected A-498 cells or cells infected with pyelonephritis *E. coli* strain CFT191 were incubated for 4.5 h in unsupplemented medium or in medium containing cytochalasin D (CytoD; 2 μ M). Plus and minus signs indicate inclusion and exclusion, respectively, of the indicated substance. Equivalent amounts of protein were separated by SDS-PAGE (10% gel) and analyzed for phosphotyrosine content by immunoblotting with antiphosphotyrosine antibody PY20. The exposed film was scanned and subjected to densitometry. Only the relevant portion of the gel is shown. The upper number below each lane indicates the relative density of pY123; the lower number indicates the relative density of pY110.

renal epithelial cells. We demonstrate that internalization of both a fecal and a pyelonephritis isolate by primary human renal cells is reduced by tyrosine kinase inhibitors. We demonstrate that upon infection with *E. coli*, two Triton X-100-insoluble host cell tyrosine-phosphorylated proteins become more prominent. We found this effect both in human renal carcinoma epithelial cells and in two primary HRPTEC lines. Increased tyrosine phosphorylation of these proteins was observed in human renal epithelial cells infected with a variety of *E. coli* isolated from the stool of healthy volunteers or from the blood or urine of patients with acute pyelonephritis. Genistein completely inhibited the increase in tyrosine phosphorylation of these proteins, while cytochalasin D did not. The increased tyrosine phosphorylation of these proteins required live bacteria but persisted in the presence of inhibitors of eukaryotic and prokaryotic protein synthesis. These observations demonstrate a close association between internalization of *E. coli* by human renal epithelial cells and tyrosine phosphorylation of specific host cell proteins.

The fact that internalization of *E. coli* by human renal epithelial cells is dramatically reduced by tyrosine kinase inhibitors provides strong evidence that internalization is an active

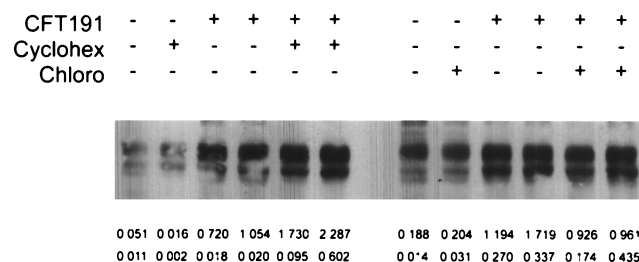


FIG. 8. Effects of protein synthesis inhibitors on *E. coli* induction of Triton X-100-insoluble phosphotyrosine-containing proteins of A-498 cells. Uninfected A-498 cells or cells infected with pyelonephritis *E. coli* strain CFT191 were incubated for 4.5 h in unsupplemented medium or in medium containing cycloheximide (Cyclohex) or chloramphenicol (Chloro). The inoculating dose in the presence of chloramphenicol was 20-fold higher than other conditions to compensate for the lack of bacterial multiplication in the presence of this inhibitor. Plus and minus signs indicate inclusion and exclusion, respectively, of the indicated substance. Equivalent amounts of protein were separated by SDS-PAGE (10% gel) and analyzed for phosphotyrosine content by immunoblotting with antiphosphotyrosine antibody PY20. The exposed film was scanned and subjected to densitometry. Only the relevant portion of the gel is shown. The upper number below each lane indicates the relative density of pY123; the lower number indicates the relative density of pY110.

process on the part of the epithelial cell involving specific signal transduction pathways. The observed increase in bacterial uptake upon removal of the rapidly reversible inhibitor genistein suggests that a burst of tyrosine kinase activity is associated with an increase in bacterial internalization. These results are consistent with those of assays using several other bacterial strains and species, including *L. monocytogenes*, *C. jejuni*, EPEC, and *E. coli* expressing invasins from *Y. pseudotuberculosis* (17, 18, 23, 30), which also depend on host cell tyrosine kinase activity for entry. Several host cell signaling pathways that link tyrosine kinases with cytoskeletal proteins provide plausible explanations for this phenomenon (4, 10, 16, 31).

In an attempt to provide information on tyrosine kinase substrates associated with entry, we identified two tyrosine-phosphorylated proteins that become more prominent after infection with *E. coli*. The association between increased tyrosine phosphorylation of these two proteins and bacterial internalization is supported by the fact that both processes are inhibited by genistein. That entry of *E. coli* is almost completely blocked by cytochalasin D, but tyrosine phosphorylation of these two proteins is not, indicates that internalization is not required for phosphorylation of these proteins but leaves open the possibility that tyrosine phosphorylation of these proteins is required for entry.

Increased tyrosine phosphorylation of pY123 and pY110 appears to be a general phenomenon that occurs upon infection of renal epithelial cells with *E. coli*, as it is observed with several different fecal and urinary tract isolates and in both primary and transformed human renal epithelial cells. In many cases, we noted the effect even in cells infected with EPEC strains, which characteristically induce more prominent tyrosine phosphorylation of a 90-kDa protein. We have no information on the nature of the bacterial factor that is recognized by the host cell to initiate the response, except that the response requires live organisms but not new protein synthesis on the part of the bacteria. Thus, the factor, if a protein, must be synthesized prior to the interaction with host cells. Identification of the bacterial trigger of the signal transduction response might be amenable to mutational analysis if it is not an essential product. We would predict that the trigger would be common among *E. coli*, as both uptake and the increased tyrosine phosphorylation of pY123 and pY110 are not limited to the pyelonephritis isolates among the strains that we examined.

We observed increased tyrosine phosphorylation of pY123 and pY110 only in the Triton X-100-insoluble fraction of the infected cells. Proteins with identical mobility were present in the soluble fraction as well, but the density of these proteins neither increased nor decreased with infection. Thus, we are unsure as to whether the detergent-soluble and -insoluble proteins are similar molecules. One intriguing possibility is that tyrosine phosphorylation of these proteins at new or additional sites, triggered by bacterial infection, targets the proteins to the cytoskeleton, where they participate in actin rearrangement to allow bacterial entry. This possibility is consistent with the increase in levels in the Triton X-100-insoluble fraction, which includes cytoskeletal proteins, and with the observation that the process is not inhibited by cycloheximide and therefore does not require the synthesis of new proteins. That we did not observe a compensatory decrease in the levels of proteins with similar mobility in the tyrosine-soluble fraction could be due several factors. If the fraction of the proteins present in the soluble pool is much greater than the fraction in the insoluble pool, then a substantial increase in the amount in the insoluble pool may not be accompanied by a detectable decrease in the

soluble pool. Alternatively, since our only probe for these proteins is a phosphotyrosine antibody, it could be that both the soluble and the insoluble fractions arise by tyrosine phosphorylation at different sites of a non-tyrosine-phosphorylated form of the protein. If this is the case, then the interaction with bacteria increases the degree of phosphorylation at specific tyrosine residues that result in targeting to a Triton X-100-insoluble site. Another explanation for the lack of a compensatory change in the Triton X-100-soluble proteins is that the soluble and insoluble proteins are not related.

The tyrosine phosphorylation of specific proteins upon bacterial infection has been noted with other bacteria and cell types. Interaction of EPEC with HeLa cells, MDCK cells, Caco-2 cells, and, as demonstrated in this study, human renal epithelial cells results in the tyrosine phosphorylation of a 90-kDa host cell protein (17). The tyrosine-phosphorylated form of this protein was recently shown to be a receptor for the EPEC outer membrane protein intimin (20), which is required for intimate attachment of the bacteria to the host cell (12). Invasion of HeLa cells by *Shigella flexneri* is associated with tyrosine phosphorylation of cortactin through the activity of pp60^{c-src} (5), while invasion of CHO cells by *S. flexneri* is associated with tyrosine phosphorylation of focal adhesion kinase pp125^{FAK} and paxillin (29). Both of these observations provide plausible links to the cytoskeleton that could result in bacterial entry. Interaction of *L. monocytogenes* with Henle-407, HeLa, and Caco-2 cells results in the tyrosine phosphorylation of mitogen-activated kinases (23). *Mycoplasma penetrans* infection of HeLa cells results in the tyrosine phosphorylation of a 145-kDa protein (1), and *Helicobacter pylori* infection of human gastric adenocarcinoma cells causes an increase in tyrosine phosphorylation of a 145-kDa protein, and a moderate increase in tyrosine phosphorylation of a 105-kDa protein (21). Similarly, *Chlamydia trachomatis* invasion of HeLa and Henle-407 cells results in tyrosine phosphorylation of a 140-kDa protein among others (3). As we observed with uropathogenic *E. coli*, cytochalasin D inhibited uptake of *C. trachomatis* but did not change the profile of tyrosine-phosphorylated proteins, suggesting that intracellular bacteria are not required to trigger signal transduction events. It is not clear whether these diverse pathogens signal cells to cause tyrosine phosphorylation of the same proteins in a common pathway for pathogenesis. Few of these proteins, with the exception of pp125^{FAK}, are similar in size to pY123 or pY110. However, we found that the estimated molecular weights of pY123 and pY110 varied widely, depending on the molecular weight standards used for determination. Thus, it is possible that some of the tyrosine-phosphorylated proteins identified upon interactions of other bacteria with host cells are, in fact, identical to pY123 or pY110. This possibility will be addressed only when the proteins involved are identified. The identification of pY123 and pY110 will also make possible experiments to test whether the tyrosine phosphorylation of these proteins is required for bacterial internalization. Until then, the relationship that we observed between internalization of *E. coli* by human renal epithelial cells and tyrosine phosphorylation of two host cell proteins remains correlative rather than causal.

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