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# Enteropathogenic *Escherichia coli* induces modification of the focal adhesions of infected host cells

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## Summary

**Enteropathogenic *Escherichia coli* (EPEC) is a human-specific pathogen that causes severe diarrhoea in young children. The disease involves intimate interaction between the pathogen and the brush border of enterocytes. During infection, EPEC uses a type III secretion system (TTSS) to inject several proteins into the infected cells, and these effector proteins modify specific processes in the host cell. We show that, upon infection, EPEC induces detachment of the infected host cells from the substratum, modification of focal adhesions (FA) in the infected cells and specific dephosphorylation of focal adhesion kinase (FAK). We also show that EPEC-induced cell detachment is dependent on FAK expression by the infected cells. Finally, we demonstrate that cell detachment, FA modification and FAK dephosphorylation are dependent on functional TTSS in the infecting EPEC. These results suggest that EPEC is using its TTSS to inject protein(s) into the infected cells, which can induce FAK dephosphorylation, as well as FAK-dependent FA modification and cell detachment. These processes are specific and probably play an important role in EPEC virulence.**

## Introduction

Enteropathogenic *Escherichia coli* (EPEC) is a pathogen that adheres to human intestinal epithelial cells and causes severe diarrhoea in infants and young children. Upon infection, EPEC induces effacement of brush border microvilli on the intestinal epithelial surface and assembly of highly organized actin structures, termed 'actin pedestals' or 'attaching and effacing (A/E) lesions' (Frankel *et al*., 1998). The formation of A/E lesions is dependent on a type III secretion system (TTSS) encoded by a unique 35 kbp chromosomal pathogenicity island, termed the locus of enterocyte effacement (LEE; Elliott *et al*., 1998). The TTSS mediates the secretion of proteins to the medium and also the translocation of bacterial virulence proteins (effectors) into the membrane and cytoplasm of infected host cells. The TTSS apparatus is composed of about 20 proteins, including EscV and EscN that are essential for both secretion and translocation (Frankel *et al*., 1998). Three additional proteins, EspA, EspB and EspD, are required for protein translocation into host cells but not for secretion (Frankel *et al*., 1998). Five EPEC effectors were identified that are translocated by TTSS into the host cell: Tir (EspE), EspB, EspF, EspG and Map (Kenny *et al*., 1997; Deibel *et al*., 1998; Wolff *et al*., 1998; Kenny and Jepson, 2000; Crane *et al*., 2001; Elliott *et al*., 2001; McNamara *et al*., 2001). Map appears to interfere with the mitochondria membrane potential (Kenny and Jepson, 2000). EspB, in addition to its role in protein translocation (Wolff *et al*., 1998), has been implicated in enhancing actin polymerization (Taylor *et al*., 1999). Tir is involved in the formation of actin-containing protrusions, termed actin pedestals or attaching and effacing (A/E) lesions (Kenny *et al*., 1997; Deibel *et al*., 1998). EspF has been implicated in the disruption of tight junction structure and in the induction of apoptosis (Crane *et al*., 2001; McNamara *et al*., 2001). No apparent phenotype was identified in *espG* knock-out (Elliott *et al*., 2001). In addition to the local assembly of actin pedestals, EPEC exerts more global effects on the host cell cytoskeleton. These include effacement of brush border microvilli (Staley *et al*., 1969), rounding-up of infected cells and cell detachment (Baldwin *et al*., 1993; our unpublished observations). These global effects are still poorly defined, and the specific effectors involved are yet to be identified.

Epithelial cells and fibroblasts attach to the extracellular matrix (ECM) via adhesive structures termed focal adhesions (FAs). The FAs contain a high density of integrin receptors associated with cytoskeletal proteins, such as talin, paxillin, vinculin, and actin filaments. Together, these proteins form adhesive complexes (Zamir *et al*., 2000). These adhesive complexes play a key role

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in recruiting cytoskeletal components and protein kinases (PTKs) involved in integrin signalling, among which is the focal adhesion kinase (FAK) (Howe *et al*., 1998). FAK is localized in vinculin-containing FAs and becomes activated upon integrin interaction with ECM proteins (Schlaepfer *et al*., 1999). Under these conditions, FAK autophosphorylation at Tyr-397 induces the transient association and further tyrosine phosphorylation of FAK by Src family PTKs and the formation of a signalling complex (Schlaepfer *et al*., 1999). These events may promote the phosphorylation of proteins such as paxillin and the recruitment to FAs of additional proteins, such as Grb2 and Cas, which serve as adaptors linking FAK to multiple signalling pathways, among them MAPK and Rho family GTPases cascades (Schlaepfer *et al*., 1999). Activation of these signalling pathways leads to cytoskeletal rearrangements that are essential for cell growth, prevention of apoptosis, cell motility and maintenance of cell shape (Sastry and Burridge, 2000).

In this study, we characterized the process of cell detachment upon EPEC infection. We report that EPEC-induced cell detachment is associated with FA modification and a specific dephosphorylation of FAK. We also show that cell detachment is dependent on FAK expression by the infected cells. Finally, we demonstrated that cell detachment, FA modification and FAK dephosphorylation are dependent on functional TTSS in the infecting EPEC. Based on these results, we propose that EPEC uses its TTSS to inject an effector into the host cell, which induces specific dephosphorylation of FAK leading to cell round-up and detachment.

## Results

### *EPEC is using its TTSS to induce the detachment of infected cells*

While processing cells infected with EPEC for microscopic observation, we noticed an extensive detachment

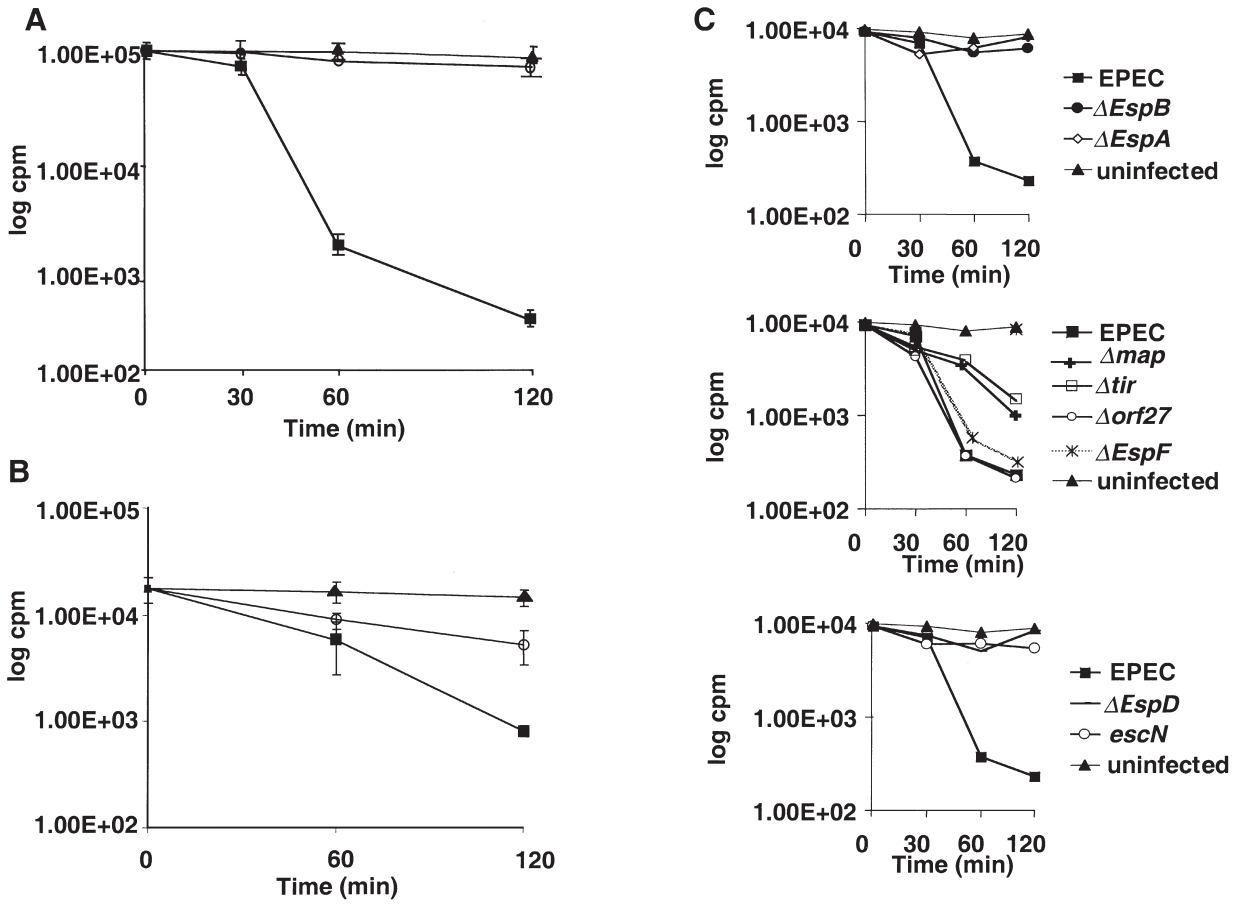



**Fig. 1.** EPEC-induced detachment of infected cells. DU17 cells (A) or HeLa cells (B) were labelled with [$^3$H]-adenine and infected with activated cultures of EPEC wild type (black squares), EPEC *escN*::Tn*phoA* (empty circles) or not at all (black triangles).
C. The DU17 cells were infected with activated cultures of wild-type EPEC or with mutants including *escN*, *espA*, *espB*, *EspD*, *espF*, *orf27*, *tir*, *map* and (*eae*) or not at all. At different time points, the detached cells were washed, and the remaining cell-associated radioactivity was determined. The standard errors obtained in the experiments described in (C) are not shown to simplify the figure but were similar to those in (A) and (B).

of the infected cells from the substratum. This was observed with several cell lines including epithelial HeLa cells and DU17 fibroblasts (data not shown). Similar observations were reported by Crane *et al*. (2001). In that case, EPEC induced the detachment of T84 polarized epithelial cells. To characterize cell detachment further, we developed a simple detachment assay (see *Experimental procedures*). We used this assay to quantify the detachment of HeLa epithelial cells and DU17 fibroblasts upon EPEC infection. In agreement with the microscopic observation, EPEC caused the detachment of both cell lines. The detachment was faster and more severe in DU17 fibroblasts (Fig. 1A) than in HeLa cells (Fig. 1B). We next compared the detachment levels of DU17 fibroblasts infected with EPEC wild type or with EPEC mutated in genes encoding TTSS components (Fig. 1C). In contrast to EPEC wild type, mutants known to be deficient in protein translocation did not cause any detachment. These included mutants in *escN*, *espA*, *espB*, *espD* (Fig. 1C) and mutants in *escV*, *orf4*, *ler* and *ihfA* (data not shown). Mutants in *tir* and *map* still caused detachment, but at a slower rate. Mutants in *espF* and *orf27* were similar to the wild-type strain in causing detachment of DU17 cells (Fig. 1C).

### *EPEC is using its TTSS to induce modification of FAs in infected cells*

We examined the effect of EPEC on the composition and distribution of FAs. HeLa cells were infected with wild-type EPEC or EPEC *escN* mutant for 4.5 h, and the FAs were labelled with antiphosphotyrosine antibody (Fig. 2A). We also carried out a double immunolabelling of HeLa cells infected with wild-type EPEC or EPEC *escN* mutant and fixed at 3.5 and 4.5 h after infection, using an antiphosphotyrosine polyclonal antiserum and antivinculin, antipaxillin and anti-FAK antibodies respectively (Fig. 2B; data not shown). In uninfected cells, the antiphosphotyrosine antibody strongly labelled the FAs, which are rich in tyrosine-phosphorylated proteins. In cells infected with wild-type EPEC, FAs were reduced in number, smaller in size and redistributed to the cell periphery (Fig. 2). Similar results were obtained when we used CaCo2 colon carcinoma cells or DU17 fibroblasts (data not shown). We next used computerized immunofluorescence microscopy (Zamir *et al*., 1999) to quantify the relative amount of FA-associated proteins in the remaining FAs that were larger than 50 pixels ($\approx 0.9\,\mu m^2$). After 4.5 h infection of HeLa cells with wild-type EPEC, the labelling of the remaining FAs by antiphosphotyrosine was reduced to 46% (Table 1). The levels of vinculin, paxillin and FAK in these FAs remained almost constant under all examined circumstances (Table 1).

Interestingly, we detected a smaller reduction, to 64%, in the levels of tyrosine-phosphorylated proteins in FAs of cells infected for 4.5 h with the EPEC *escN* mutant. Nevertheless, in cells infected with EPEC *escN*, we did not observe any reduction in the FA number, or FA shrinking or redistribution of FAs to the cell periphery (Fig. 2). Similarly, we did not detect these changes in FAs in cells infected with other mutants that, like *escN*, are unable to use the TTSS for protein translocation. These include mutants in *espA*, *espB*, *espD*, *orf4*, *escV*, *ler* and *ihfA* (data not shown). Longer infection periods or increased multiplicity of infection (MOI) could not restore the capacity of these mutants to induce the above modifications in FAs. Mutants in *eae* or in genes encoding potential effector proteins, including *tir*, *map*, *espF* and *orf27*, were tested as well. These mutants still induce the modification of FAs (data not shown), but the mutants in *tir* and *map* induce the modification of FAs at slower kinetics (data not shown). Taken together, these results suggest that EPEC use the TTSS to induce modification of FAs in infected cells.

The decay of antiphosphotyrosine labelling of FAs in infected cells coincided with a strong labelling of the tyrosine-phosphorylated Tir localized to the A/E lesions beneath the attached EPEC at the cells' apical side. In contrast, we could not detect any specific labelling of the A/E lesions with antivinculin, antipaxillin or anti-FAK antibodies (Fig. 2; data not shown).

### *EPEC induces dephosphorylation of FAK but not of paxillin*

Focal adhesion kinase (FAK) is a major FA-associated tyrosine-phosphorylated protein. The reduction in FA-associated labelling with antiphosphotyrosine upon EPEC infection may suggest that EPEC causes dephosphorylation of FAK. To test this hypothesis, we used DU17 fibroblasts, which express high levels of FAK. DU17 cells were infected with EPEC wild type or with the EPEC *escN*

**Table 1.** Average intensities of staining for different FA compounds (control levels normalized to 100%).

| | Labelling with anti | | | |
|---|---|---|---|---|
| Cells infected with | Phospho-Y | Paxillin | Vinculin | FAK |
| Uninfected | 100 (33) | 100 (21) | 100 (23) | 100 (21) |
| EPEC *escN* 4.5 h | 64 (21) | 100 (28) | 91 (19) | 91 (25) |
| EPEC wt 3.5 h | 63 (18) | 102 (20) | 109 (23) | 112 (27) |
| EPEC wt 4.5 h | 46 (18) | 102 (21) | 102 (22) | 100 (23) |

Average intensity of immunostaining with antiphosphotyrosine (phospho-Y), antivinculin, antipaxillin and anti-FAK before and after EPEC infection. The data shown represent the average of five (phosphotyrosine) or two (vinculin, paxillin, FAK) independent stainings out of two different experiments. For each staining, all focal adhesions larger than 50 pixels of 12 individual cells were used for the calculation. Although phosphotyrosine is drastically reduced, all the other components remain essentially unchanged.

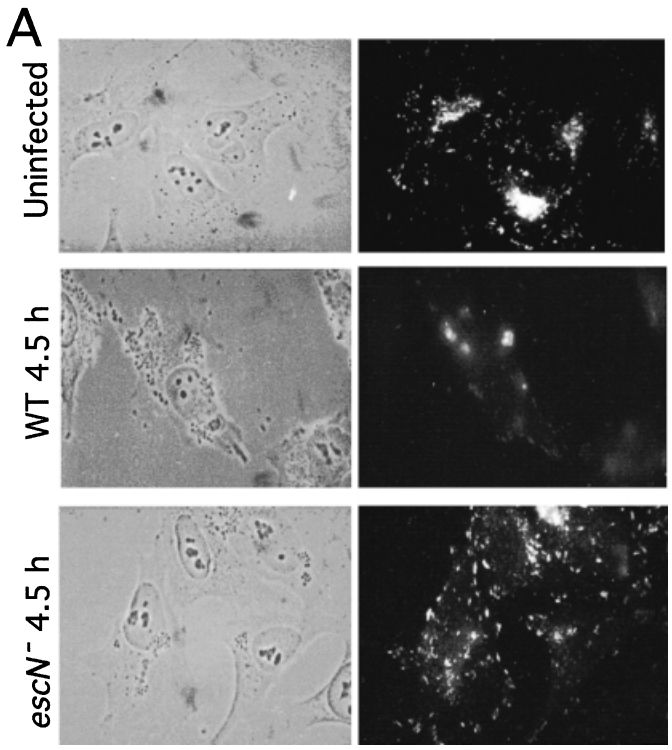
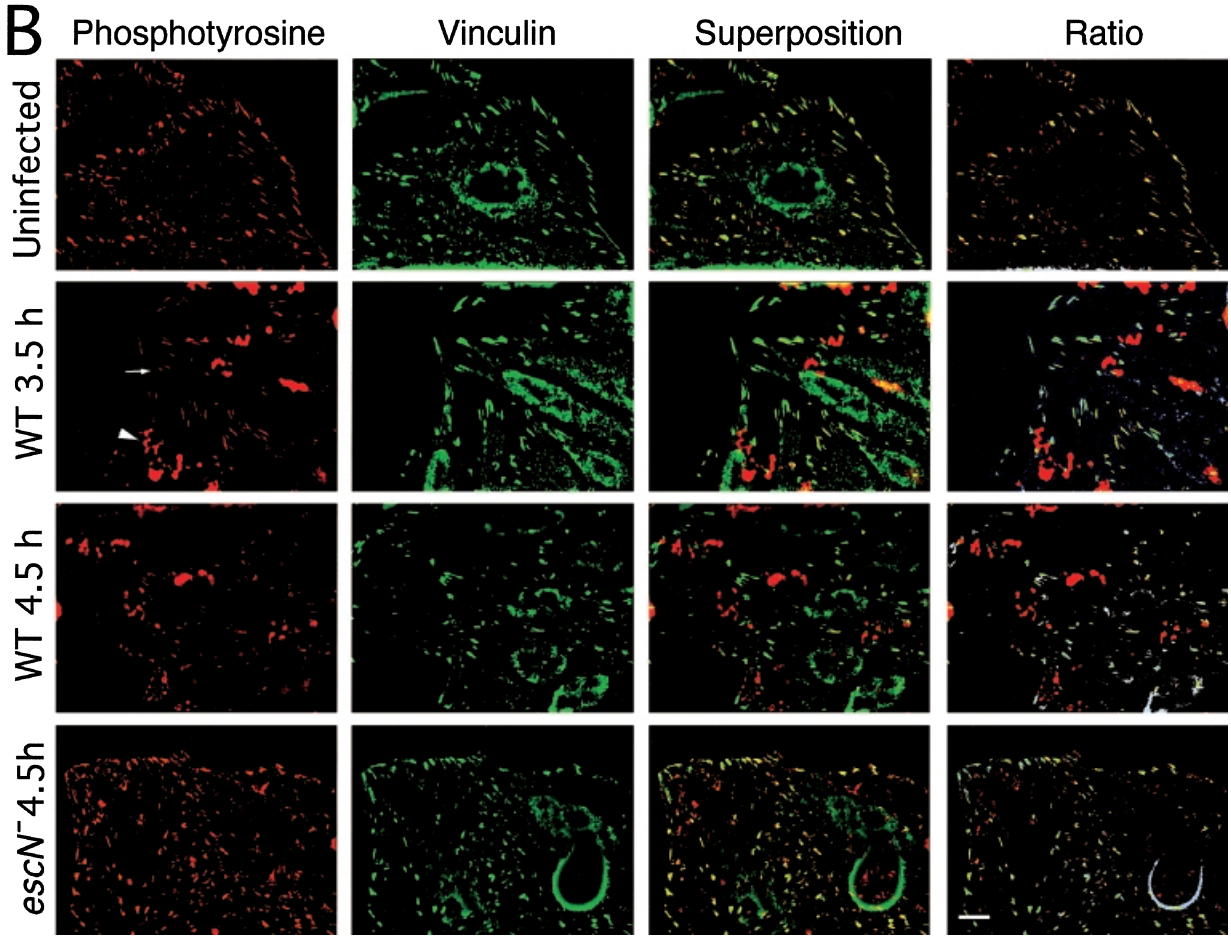



**Fig. 2.** Immunostaining of HeLa cells before and after EPEC infection.
A. HeLa cells were infected for 4.5 h with wild-type EPEC, *escN* mutant, which carry inactivated TTSS, or not at all, and the effect of the infection on focal adhesions was visualized using an antiphosphotyrosine antibody.
B. For double immunostaining, HeLa cells were incubated with EPEC for 3.5 or 4.5 h, respectively, as well as with the *escN* mutant. Staining was performed with an anti-phosphotyrosine antiserum (shown in red) and antivinculin (shown in green). Similar staining was done using antipaxillin and anti-FAK antibodies, instead of antivinculin antibody (data not shown). In the ratio image, the ratio of the two intensities is displayed on a spectrum scale. Red indicates a 10-fold excess of the first label (phosphotyrosine), blue a 10-fold excess of the second label, and yellow represents equal distribution between the two labels. Note the difference in size and distribution of focal adhesions occurring after EPEC infection. Bar = 10 μm. The antiphosphotyrosine antibody labelled both Tir accumulated beneath attached EPEC (an example is indicated by the arrowhead) and FA (an example is indicated by the small arrow).



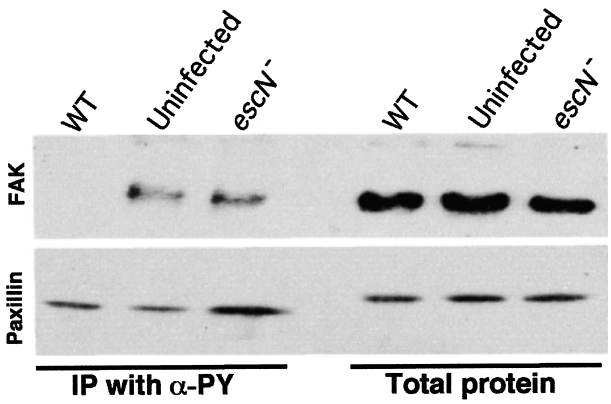



**Fig. 3.** EPEC induces dephosphorylation of FAK but not of paxillin. Proteins were extracted from DU17 fibroblasts infected with EPEC wild type or *escN* mutant or uninfected cells. Tyrosine-phosphorylated proteins were immunoprecipitated from the extracts. The levels of FAK and paxillin in the crude extract and in the immunoprecipitate, representing tyrosine-phosphorylated FAK and paxillin, were determined by immunoblot analysis with anti-FAK and antipaxillin antibodies.

mutant that carry inactivated TTSS. After 4.5 h infection, the fibroblasts were harvested, extracted, and tyrosine-phosphorylated proteins were immunoprecipitated with antiphosphotyrosine antibody. The precipitated proteins were resolved by SDS–PAGE and used for immunoblot analysis with anti-FAK antibody and antipaxillin antibody. The level of phosphorylated FAK was reduced in the EPEC-infected cells but not in cells infected with the *escN* mutant or in the uninfected cells (Fig. 3). In contrast, the levels of tyrosine-phosphorylated paxillin remained constant in all samples (Fig. 3).

### *Inhibition of inositol-3-phosphate kinase (PI3K) is not involved in EPEC-induced cell detachment*

EPEC appear to inhibit directly or indirectly the activation of PI3K in infected macrophages (Celli *et al*., 2001). We have checked whether this EPEC-associated activity is involved in causing FA deterioration and cell detachment. DU17 cells were infected with EPEC or remained uninfected in the presence or absence of 25 μM PI3K inhibitor LY294002 and were subjected to a detachment assay. Cell detachment was induced by EPEC infection but was not affected by LY294002 (Fig. 4A). Similarly, LY294002 did not affect the EPEC-induced FA modification (data not shown). To validate that LY294002 was active under our experimental conditions, we tested whether it inhibited invasion mediated by invasin of *Yersinia pseudotuberculosis*, as reported by Schulte *et al*. (1998). Indeed, treatment with 25 μM LY294002 reduced the efficiency of invasion of *E. coli* HB101 expressing invasin of *Y. pseudotuberculosis* by two orders of magnitude (data not shown), confirming that the inhibitor is active. Thus, inhibition of PI3K does not appear to play an important role in EPEC-induced cell detachment or FA modification.

### *EPEC-induced detachment is FAK dependent*

Recent studies have indicated that FAK plays a role in FA turnover rather than in their assembly (Ilic *et al*., 1995). Disassembly and the dynamics of focal adhesion complexes are essential for cell migration. In agreement, FAK knock-out fibroblasts (DU3) are deficient in motility but not in attachment and assembly of FAs (Ilic *et al*., 1995). We hypothesized that EPEC-induced cell detachment may be dependent on FAK-mediated FA turnover. To test this hypothesis, we used EPEC wild type to infect knock-out FAK (–/–) fibroblasts (DU3) and wild-type fibroblasts FAK (+/+) (DU17). The cell lines were labelled with [$^3$H]-adenine, infected, and the detachment levels of the FAK (–/–) and FAK (+/+) cells were compared (Figs 5A and B). In the FAK (–/–) cells, no detachment could be detected (Fig. 5B).

An additional possible explanation for the inability of EPEC to induce detachment of DU3 cells is that all aspects of EPEC infection, including invasion and the formation of actin pedestals, are FAK dependent. To test this possibility, we compared the ability of EPEC to form actin pedestals in infected DU3, DU17 and HeLa cells. In all cases, EPEC exhibited similar efficiency in inducing the formation of actin pedestals (data not shown). We next compared the efficiency of invasion of HeLa and DU3 by EPEC (DU17 cells were not used here because the massive detachment interferes with the invasion assay). The results indicate that EPEC invade DU3 cells at high

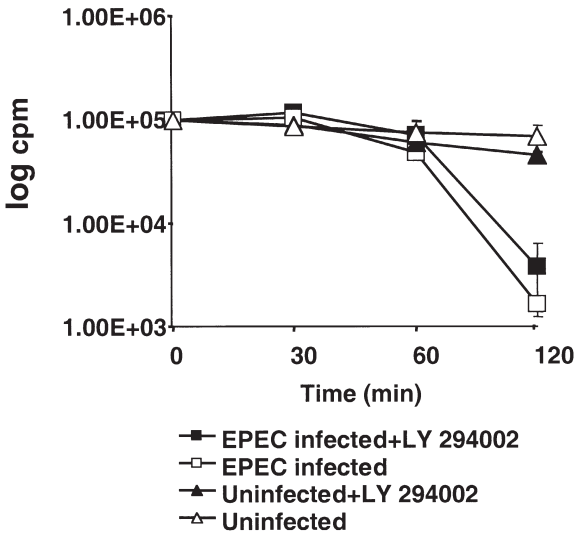



**Fig. 4.** Inhibition of PI3K is not involved in EPEC-induced host cell detachment. DU17 fibroblasts were infected with EPEC or not infected in the presence or absence of 25 μM LY294002, and subjected to a detachment assay.

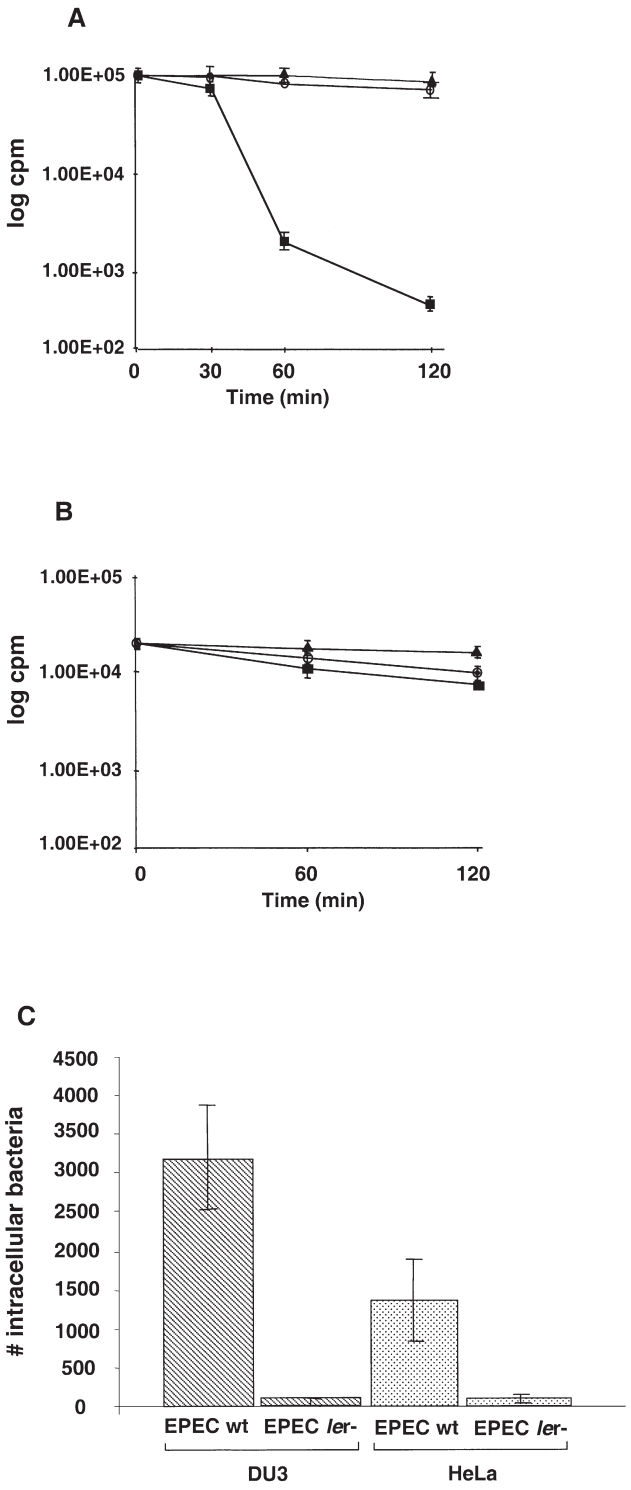



**Fig. 5.** EPEC-induced detachment is FAK dependent. DU17 cells expressing FAK (A) and DU3 FAK knock-out cells (B) were infected with EPEC wild type (black squares), *escN* mutant (open circles) or not at all (black triangles). A detachment assay was carried out at the indicated time points. The c.p.m. levels reflect the levels of cell attachment. The data in (C) indicate that EPEC attachment is FAK independent. DU3 FAK knock-out cells and HeLa cells (FAK+/+) were infected with EPEC wild type or EPEC *ler* mutant, and the efficiency of invasion was determined.

efficiency (Fig. 5C). These results indicate that (i) EPEC can infect DU3 cells and use the TTSS to translocate effectors into DU3 cells; and (ii) although induction of cell detachment is FAK dependent, both invasion and the formation of actin pedestals are FAK independent.

## Discussion

Early studies of EPEC infection indicated that EPEC cause cell round-up, detachment and death (Staley *et al*., 1969; Baldwin *et al*., 1993). We used a very simple assay to quantify cell detachment upon EPEC infection. We showed that EPEC induced the detachment of all the examined cell lines including epithelial cell lines (HeLa, Caco2) and fibroblasts (DU17). Detachment of other epithelial cell lines including T84 was noticed by others but not quantified (Crane *et al*., 2001). We demonstrated that detachment of infected cells was initiated early after infection and was particularly dramatic in the DU17 fibroblast cell line. The detachment was associated with changes in FA morphology. These include an apparent reduction in the number of FAs as well as a redistribution of FAs to the cell periphery. In addition, we noticed a strong reduction in the levels of FA-associated tyrosine phosphorylation. FAK is the major FA-associated tyrosine-phosphorylated protein and, therefore, the microscopy data suggested that FAKs undergo dephosphorylation upon EPEC infection. Indeed, we demonstrated that EPEC specifically induced the dephosphorylation of FAK but not of paxillin, another FA-associated protein. These results suggest that, by inducing FAK dephosphorylation, EPEC manipulate the FAs to cause cell detachment.

To test the role of FAK in EPEC-induced cell detachment further, we compared the infection outcome on isogenic FAK (–/–) and FAK (+/+) cell lines. The results indicate that FAK is required for the induced detachment and support the hypothesis that, by manipulating FAK, EPEC causes cell detachment. In addition, these results demonstrated that FAK is not required for EPEC invasion or the formation of EPEC-induced actin pedestals.

EPEC use the TTSS to deliver toxic effector proteins into the host cells. We demonstrated that EPEC-induced detachment, FA modification and FAK dephosphorylation are all dependent on the capability of EPEC to use the TTSS for protein translocation. However, EspF, Orf27 and intimin are not involved in the induction of cell detachment, whereas Tir and Map appear to enhance the efficiency of the EPEC-induced modification of FAs and cell detachment, but are not absolutely required.

Previous reports indicated that EPEC uses the TTSS to inhibit phagocytosis by macrophages (Goosney *et al*., 1999). This was correlated with EPEC-induced tyrosine dephosphorylation of several unidentified proteins (Kenny

and Finlay, 1997; Goosney *et al*., 1999). Our data suggest that at least one of these proteins is FAK. Interestingly, *Yersinia* inhibits phagocytosis by translocation of YopH, a protein tyrosine phosphatase (PTP) that mediates FAK dephosphorylation (Persson *et al*., 1997).

The EPEC antiphagocytic activity was also correlated with EPEC-mediated inhibition of PI3K (Celli *et al*., 2001). This raises the possibility that PI3K inhibition and FAK dephosphorylation are different stages in the same signalling cascade that leads to antiphagocytic activity in macrophages and cell detachment in epithelial cells. However, our data suggest that inhibition of PI3K is not involved in EPEC-induced cell detachment or in FA modification.

In conclusion, our results suggest that EPEC encode for a putative effector(s) that specifically cause FAK dephosphorylation and FA modification and consequently cell detachment. This hypothesis is also supported by the finding that cells that do not express FAK are specifically immune to the EPEC-induced cell detachment but not to the EPEC-induced formation of actin pedestals or EPEC invasion. It appears that a concerted action of the putative effector(s) that cause detachment from the substratum, and EspF that disrupts tight junction integrity, damages the intestinal epithelial monolayer. It has yet to be seen whether EPEC possess additional effector(s) that specifically disrupt the adhesion belt. Damaging the epithelial integrity may allow the bacteria access to nutrients from the lamina propria and may result in local inflammation.

## Experimental procedures

### *Bacterial strains, tissue cultures, growth and infection conditions*

Bacterial strains used in this study are listed in Table 2. All strains were grown in Luria–Burtani (LB) broth without shaking at 37°C unless otherwise stated. Where appropriate, media were supplemented with ampicillin (100 μg ml$^{-1}$), chloramphenicol (25 μg ml$^{-1}$) or kanamycin (50 μg ml$^{-1}$). The cell lines HeLa, DU17 and DU3 were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 units ml$^{-1}$ penicillin and 0.1 mg ml$^{-1}$ streptomycin at 37°C, 5% $CO_2$. Before infections, the cells were washed with PBS to remove antibiotics. For extraction and fractionation of cells and protein analysis, cells were seeded on 100 mm tissue culture dishes at a density of $4 \times 10^6$ cells per plate. In some cases, EPEC cultures were activated before infection: the overnight cultures, grown without shaking in LB broth at 37°C, were diluted 1:100 into DMEM without FCS and grown for 3.5 h without shaking at 37°C, 5% $CO_2$.

### *Immunofluorescence microscopy*

HeLa cells were grown in 6 cm dishes and 5 ml of DMEM and 10% FCS to 80% confluency. These cultures were subsequently infected (1 day after plating) with 50 μl of overnight bacterial culture. Cells were permeabilized for 2 min in 0.5% Triton X-100, 3% paraformaldehyde in PBS and then fixed for 45 min in 3% paraformaldehyde in PBS. They were washed twice in PBS and incubated for 1 h with the primary antibody. After $3 \times 10$ min washing in PBS, the secondary antibody was administered for 40 min. The samples were washed again for $3 \times 10$ min in PBS and mounted on a slide embedded in elvanol (Moviol 4-88; Hoechst). The primary antibodies used were a polyclonal antiserum against phosphotyrosine (PT 40, kindly provided by Professors I. Pecht and A. Licht, The Weizmann Institute, Israel) and monoclonal antibodies against vinculin (anti-HVin; Sigma Chemical), paxillin and FAK (both from Transduction Laboratories). Secondary antibodies (all from Jackson Immuno-

**Table 2.** List of bacterial strains.

| Strain | Genotype | Source |
|---|---|---|
| E2348/69 | Parental wild-type EPEC | J. Kaper |
| Mutants with inactivated TTSS[a] | | |
| 27-3-2(1) | *escN*::Tn*pho* | J. Kaper |
| 14-2-1(1) | *orf4*::Tn*phoA* | J. Kaper |
| CVD452 | *escV*::*Kn* | J. Kaper |
| Mutants that do not express the TTSS[b] | | |
| DF1 | *lhfA*::*Kn* | Friedberg *et al*. (1999) |
| DF2 | *ler*::*Kn* | Friedberg *et al*. (1999) |
| Mutants deficient in protein translocation[c] | | |
| UMD782 | *espA*::*Kn* | J. Kaper |
| UMD864 | Δ*espB* | J. Kaper |
| UMD870 | Δ*espD* | J. Kaper |
| Mutants in intimin and Tir[d] | | |
| | Δ*tir* | B. Kenny |
| CVD 206 | Δ*eaeA* | J. Kaper |
| 10-5-1(1) | *eaeA*::Tn*phoA* | J. Kaper |
| Mutants in genes encoding effectors or putative effectors[e] | | |
| | Δ*map* | B. Kenny |
| | Δ*orf27* | B. Kenny |
| | Δ*EspF* | B. Kenny |

**a.** The mutated genes encode essential components of the TTSS. These mutants cannot secrete proteins nor can they translocate them into the target cells. Therefore, these mutants cannot perform any activity associated with the TTSS, including the induction of actin pedestal formation, intimate attachment and invasion.
**b.** The mutated genes encode positive regulators that are required for transcription of the genes encoding the TTSS. These mutants do not express the TTSS and exhibit phenotypes similar to mutants with inactivated TTSS.
**c.** The mutated genes encode an extension of the TTSS that is not needed for protein secretion but is essential for protein translocation into infected cells. Therefore, these mutants cannot perform any activity associated with TTSS-mediated protein translocation, including induction of actin pedestal formation, intimate attachment and invasion.
**d.** The mutated genes encode Tir and intimin. These mutants cannot perform activities associated with Tir–intimin interaction, including induction of actin pedestal formation, intimate attachment and invasion, but their TTSS is still intact, and they can translocate EspB and effectors such as EspF and Map into infected cells.
**e.** The mutated genes encode specific effectors (EspF and Map) or a putative effector (Orf27). These effectors play a subtle role in EPEC–host cell interaction. These mutants carry a fully functional TTSS and exhibit a wild-type phenotype with respect to attachment, invasiveness and induction of the formation of actin pedestals.

Research Laboratories) were Cy-3-conjugated $F(ab')_2$ fragment goat anti-mouse antibody and Alexa-conjugated $F(ab')_2$ fragment goat anti-rabbit antibody. The computerized immunofluorescence microscopy, image filtration, segmentation, quantification and ratio imaging were performed as described previously (Zamir *et al*., 1999; 2000). For each experiment, focal adhesions larger than 50 pixels from 12 different cells were analysed. The data were then normalized (control levels were set to 100%), and the data from two different experiments were used to calculate the numbers in Table 1.

### *Detachment assay*

Cells were grown overnight on round coverslips in a 24-well plate, washed and incubated with DMEM supplemented with [$^3$H]-adenine (0.6 μCi per well) for 3 h. The cells were then washed three times with PBS to remove antibiotics and unincorporated [$^3$H]-adenine and infected with activated EPEC cultures. When necessary, cells were incubated for 30 min with 25 μM PI3K inhibitor LY 294002 (Sigma) before infection. At different time points, coverslips were washed three times with PBS to remove detached cells and placed in scintillation vials. The remaining coverslip-associated radioactivity was measured in a scintillation counter.

### *Immunoprecipitation and immunoblotting*

Cells grown overnight on 100 mm tissue culture dishes were infected with an overnight bacterial culture, diluted 1:100, for 4.5 h. The infected attached cells were washed and scraped in 1 ml of cold PBS. In addition, the medium containing the detached cells was collected. Both the attached and the detached cells were combined and recovered by 2 min centrifugation at 200 *g*. The cells were lysed in 1.0 ml of lysis solution [1% Triton X-100, 50 mM Tris-HCl, pH 7.6, 0.4 mM $NaVO_4$, 0.1 mg ml$^{-1}$ phenylmethylsulphonyl fluoride (PMSF) and 10 mg ml$^{-1}$ leupeptine], and lysates were cleared by centrifugation. The clear lysates (150 μl) were saved, and the tyrosinephosphorylated proteins in the remaining 850 μl were precipitated with 50 μl of agarose beads conjugated to antiphosphotyrosine antibody (PT-66; Sigma) at 4°C for 60 min. The beads were then washed three times with lysis solution and mixed with 50 μl of 2.5× loading buffer. The samples were subsequently subjected to SDS–PAGE and transferred to nitrocellulose membranes (Optitran BA-S 83; Schleicher and Schuell) using a NovaBlot electrophoretic transfer unit. The membrane was blocked by 5% bovine serum albumin (BSA) in Tris-buffered saline (TBS) and incubated with anti-FAK or anti-paxillin antibodies and secondary anti-mouse IgG or anti-rabbit IgG conjugated to horseradish peroxidase (Sigma). Detection was carried out with SuperSignal substrate (Pierce).

### *Invasion assay*

Invasion assays with HB101 encoding invasin and with EPEC were carried out as described previously (Rosenshine *et al*., 1992a, b).

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