Actin Pedestal Formation on Mammalian Cells by Enteropathogenic *Escherichia coli*: A Dissertation

Kenneth Geno Campellone

*University of Massachusetts Medical School*

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ACTIN PEDESTAL FORMATION ON MAMMALIAN CELLS
BY ENTEROPATHOGENIC ESCHERICHIA COLI

A Dissertation Presented

By

KENNETH GENO CAMPELLONE

Submitted to the Faculty of the
University of Massachusetts Medical School, Worcester
Graduate School of Biomedical Sciences
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 22, 2003

Molecular Genetics and Microbiology
ACTIN PEDESTAL FORMATION ON MAMMALIAN CELLS
BY ENTEROPATHOGENIC ESCHERICHIA COLI

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May 22, 2003
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Life goes by faster than a summer vacation.
Abstract

Enteropathogenic *Escherichia coli* (EPEC) and enterohemorrhagic *E. coli* O157:H7 (EHEC) form characteristic lesions on infected mammalian cells called actin pedestals. Each of these two pathogens injects its own translocated intimin receptor (Tir) molecule into the plasma membranes of host cells. Interaction of translocated Tir with the bacterial outer membrane protein intimin is required to trigger the assembly of actin into focused pedestals beneath bound bacteria. Despite similarities between the Tir molecules and the host components that associate with pedestals, recent work indicates that EPEC and EHEC Tir are not functionally interchangeable. For EPEC, Tir-mediated binding of Nck, a host adaptor protein implicated in actin signaling, is both necessary and sufficient to initiate actin assembly. In contrast, for EHEC, pedestals are formed independently of Nck, and require translocation of bacterial factors in addition to Tir to trigger actin signaling.
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CHAPTER I

INTRODUCTION

Enteropathogenic Escherichia coli (EPEC) and enterohemorrhagic E. coli O157:H7 (EHEC) are diarrheagenic human pathogens belonging to a distinct family of enteric bacteria that form unique structures called attaching and effacing (AE) lesions on the surfaces of intestinal epithelial cells. AE lesions are characterized by the loss of microvilli, an intimate adherence of bacteria adjacent to the host cell membrane, and the generation of organized cytoskeletal structures containing filamentous actin beneath sites of bacterial attachment, termed actin pedestals (Figure 1a). The formation of actin pedestals by EPEC and EHEC is recapitulated on cultured mammalian cells (Figure 1b–d), and the ability of AE organisms to form pedestals on cultured cells correlates with their ability to colonize the intestine and cause disease in human and other mammalian hosts.

EPEC and EHEC each contain a highly homologous 35 kb chromosomal pathogenicity island called the locus of enterocyte effacement (LEE) (Elliott et al., 1998; Perna et al., 1998), which contains genes critical for pedestal formation. The LEE encodes a type III protein secretion system for the contact-dependent translocation of bacterial proteins into host cells. Effector proteins that are transported through the type III secretion apparatus are also encoded within the LEE. Translocated proteins that have been identified to date are the E. coli secreted proteins EspA, EspB, EspD, EspF and EspG, Map (mitochondria-associated protein), and the translocated intimin receptor (Tir/EspE) (Figure 2a). EspA forms a filamentous structure on the
bacterial surface to contact host cells, through which other LEE-encoded effectors are secreted, including EspB and EspD (Ebel et al., 1998a; Knutton et al., 1998; Sekiya et al., 2001). EspB and EspD are thought to transit through the EspA filament to form a pore, or translocon, in the host plasma membrane, to deliver other virulence factors into the cell (Ide et al., 2001; Kresse et al., 1999; Wachter et al., 1999; Warawa et al., 1999; Wolff et al., 1998) and perhaps act as translocated effectors themselves (Kodama et al., 2002; Taylor et al., 1999; Taylor et al., 1998). EspF, EspG and Map affect other host cell processes but do not play an observable role in actin pedestal formation (Elliott et al., 2001; Kenny et al., 2002; Kenny and Jepson, 2000; McNamara et al., 2001). A functional EspA conduit and EspB/D translocon are required for translocation of Tir, the best-characterized bacterial effector and the one that plays the central role in triggering actin pedestal formation by the host cell.

Our conception of how actin pedestals are formed was transformed by the discovery that EPEC and EHEC inject Tir into the host plasma membrane, where it functions as a bacterial receptor (Deibel et al., 1998; Kenny et al., 1997b). In the plasma membrane, Tir adopts a hairpin-loop structure featuring a central extracellular domain that binds to the LEE-encoded outer membrane protein intimin (Batchelor et al., 2000; Liu et al., 2002; Luo et al., 2000) (Figure 2b). Intimin may also promote initial adherence by binding to endogenous host cell receptors (Reece et al., 2001; Sinclair and O'Brien, 2002), but this aspect of intimin will not be discussed here. The amino- and carboxy-terminal domains of Tir reside in the host cell cytoplasm (de Grado et al., 1999; Hartland et al., 1999; Kenny, 1999), where they are capable of interacting with host cytoskeletal and signaling components (Figure 2b). After translocation of Tir and other EspS, the interaction between Tir and intimin is sufficient to initiate actin assembly, because intimin-coated beads form pedestals on mammalian cells pre-infected with an EPEC strain that does not
express intimin but does translocate Tir and other effectors (Liu et al., 1999; Rosenshine et al. 1996). The key role of this interaction is emphasized by the observation that intimin point mutations that reduce Tir binding activity in vitro exhibit similarly diminished abilities to trigger pedestal formation (Liu et al., 2002). Thus, in addition to serving as a bacterial adhesion receptor, Tir accomplishes the added function of exploiting actin signaling cascades within host cells upon its interaction with intimin. In this review we discuss the mechanisms by which EPEC and EHEC Tir exploit the mammalian actin assembly machinery.

**Microbial pathogens target the Arp2/3 pathway of actin assembly**

A critical controller of actin polymerization at the eukaryotic plasma membrane is the heptameric actin-related protein 2/3 complex (Arp2/3 complex) that is capable of promoting actin nucleation (Bear et al., 2001; Welch and Mullins, 2002). The actin nucleating activity of the Arp2/3 complex can be stimulated by its interaction with Wiskott–Aldrich syndrome protein (WASP) family members, such as neuronal (N-)WASP, the most widely expressed member of this family of proteins. The ability of N-WASP to stimulate the Arp2/3 complex can, in turn, be activated by Rho-family GTPases (e.g. Rho, Cdc42) and by several adaptor proteins containing Src homology 2 (SH2) and Src homology 3 (SH3) domains. One such adaptor protein, Nck, binds to phosphotyrosine residues via its SH2 domain (Buday et al., 2002) and stimulates N-WASP to trigger actin polymerization with its SH3 domains (Rohatgi et al., 2001).

Several microbial pathogens have evolved unique strategies to exploit the Arp2/3 pathway to promote actin-based motility within host cells (Frischknecht and Way, 2001; Goldberg, 2001). For example, the ActA protein of *Listeria* binds and activates the Arp2/3 complex directly, to initiate actin tail formation, whereas the *Shigella* IcsA surface protein...
contacts N-WASP. Vaccinia virus utilizes a tyrosine phosphorylated membrane protein, A36R, to recruit the adaptor proteins Nck and Grb2, thereby indirectly activating N-WASP (Frischknecht et al., 1999; Scaplehorn et al., 2002). Given this precedent, it was no surprise that the N-WASP–Arp2/3 pathway is also involved in controlling actin assembly within pedestals. The Arp2/3 complex is recruited to sites of pedestal formation by both EPEC and EHEC in a manner that depends on N-WASP (Table 1). This recruitment is required for actin focusing by EPEC and is also likely to be essential for EHEC (Kalman et al., 1999). N-WASP, in turn, is recruited to sites of EPEC and EHEC actin assembly in a Tir-dependent fashion (Table 1) and is absolutely required for pedestal formation, because cells that lack N-WASP are resistant to actin pedestal formation by EPEC (Lommel et al., 2001) and EHEC (S. Snapper, personal communication). N-WASP does not seem to be activated by the well-characterized Rho-family GTPases during pedestal formation, because inhibitors of Rac, Rho and Cdc42 do not inhibit actin assembly by EPEC (Ben-Ami et al., 1998; Ebel et al., 1998b) or EHEC (K. Campellone, unpublished data).

Divergence in Tir-based actin signaling

EPEC and EHEC each use highly homologous gene products encoded on their LEE elements to generate pedestals that appear morphologically similar. However, in part owing to its ability to form pedestals at a higher efficiency in vitro (Cantey and Moseley, 1991), EPEC rather than EHEC has been preferentially used in studies on actin assembly, and in many ways has been regarded as a model for EHEC pedestal formation. Nevertheless, detailed analyses of the processes that EPEC and EHEC employ to assemble actin have demonstrated differences in the mechanisms used to trigger actin polymerization. Notably, the carboxy-terminal cytoplasmic
portions of EPEC and EHEC Tir, domains capable of interacting with the intracellular signaling machinery, are only 44% identical. Furthermore, while each Tir appears to be modified by serine/threonine phosphorylation upon entry into the host cell (DeVinney et al., 1999; Kenny, 1999; Warawa and Kenny, 2001), only EPEC Tir is additionally phosphorylated on a tyrosine residue, Tyr474, located in its carboxy-terminal cytoplasmic region (Ismaili et al., 1995; Kenny, 1999). This phosphorylated tyrosine residue is critical for actin signaling and pedestal formation by EPEC (Goosney et al., 2000; Kenny, 1999). A clue about the possible function of the phosphorylated tyrosine came from a study by Goosney et al. (Goosney et al., 2001), who observed that the mammalian adaptor proteins Grb2 and CrkII localize to EPEC pedestals, but not EHEC pedestals (Table 1). Recruitment of these proteins is dependent upon EPEC Tir tyrosine phosphorylation, suggesting that disparities in adaptor recruitment by Tir may underlie differences in the actin signaling mechanisms of EPEC and EHEC. In fact, several laboratories have now demonstrated that these differences in Tir have profound actin signaling consequences. Whereas EHEC 0157:H7 strains can use either Tir homolog to trigger actin assembly, EPEC strains engineered to express EHEC Tir are unable to form actin pedestals (Campellone et al., 2002; DeVinney et al., 2001; Kenny, 2001).
Table 1. Host protein composition of EHEC/EPEC actin pedestals.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
<th>Presence in EPEC</th>
<th>Presence in EHEC</th>
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<td></td>
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<tr>
<td>Arp2/3</td>
<td>Nucleates actin</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>[37,46]</td>
</tr>
<tr>
<td>Nck</td>
<td>SH2/SH3 adaptor</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>[49,50]</td>
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<tr>
<td>N-WASP</td>
<td>Activates Arp2/3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>[37,38,46]</td>
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<tr>
<td>WIP</td>
<td>Interacts with WASP</td>
<td>+</td>
<td>Unknown</td>
<td></td>
<td>[S Snapper, personal communication]</td>
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<td><strong>Actin regulatory proteins with undefined roles in pedestal formation</strong></td>
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<tr>
<td>Cofilin</td>
<td>Depolymerizes actin</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Cortactin</td>
<td>Activates Arp2/3</td>
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<tr>
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<td>Regulates filamentation rates</td>
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<td>+</td>
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<td>α-Actinin</td>
<td>Crosslinks actin</td>
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<td>+</td>
<td>+</td>
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<tr>
<td>Talin</td>
<td>Links integrins to actin</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Vinculin</td>
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* Nck is required for pedestal formation by EPEC, but not by EHEC. GPI, glycosylphosphatidylinositol.

Figure 1. Actin pedestals of EPEC and EHEC. (a) EPEC generates AE lesions on the intestinal epithelium after infection of gnotobiotic piglets. Note the pedestal-like structures on host cells beneath attached bacteria. (Transmission electron micrograph courtesy of S. Tzipori.) (b) Actin pedestals that resemble AE lesions formed in vivo are also generated on cultured epithelial (HeLa) cells. (Scanning electron micrograph courtesy of S. Knutton) (c) The host adaptor protein Nck localizes to the tips of actin pedestals generated by EPEC. Cultured HeLa cells were infected with EPEC and examined by immunofluorescence microscopy. F-actin is shown in red, bacterial DNA in blue, and the host protein Nck in green. Co-localization of Nck and F-actin beneath bacteria is depicted in yellow. (d) Nck is not recruited to sites of EHEC pedestal formation. Cultured HeLa cells were infected with EHEC and examined by immunofluorescence microscopy as in (c).
Figure 2. Model of translocation of bacterial effectors into host cells. (a) Upon contact, EPEC and EHEC use a type III translocation apparatus to inject bacterial effector proteins into mammalian cells. These bacteria translocate a number of proteins: EspB and EspD, which form a translocon in the plasma membrane; the cytoplasmic proteins EspF, EspG and Map (there is also a cytoplasmic pool of EspB); the translocated intimin receptor Tir, which inserts into the plasma membrane; and other unidentified effectors. (b) Membrane-localized Tir contains a central extracellular domain that binds to the bacterial outer membrane protein intimin, and amino- and carboxy-terminal cytoplasmic domains that interact with cytoskeletal elements. The interaction between Tir and intimin is the final bacterial signal to trigger the assembly of actin into pedestals within host cells.
CHAPTER II

A TYROSINE-PHOSPHORYLATED 12-AMINO-ACID SEQUENCE OF ENTEROPATHOGENIC ESCHERICHIA COLI TIR BINDS THE HOST ADAPTOR PROTEIN NCK AND IS REQUIRED FOR NCK LOCALIZATION TO ACTIN PEDESTALS

Summary

Enteropathogenic Escherichia coli (EPEC) and enterohaemorrhagic E. coli (EHEC) each promote the reorganization of actin into filamentous pedestal structures beneath attached bacteria during colonization of the intestinal epithelium. Central to this process is the translocation of the protein Tir (translocated intimin receptor) into the plasma membrane of host cells, where it interacts with the bacterial outer membrane protein intimin and triggers cellular signalling events that lead to actin rearrangement. Actin signalling by EPEC Tir requires a tyrosine residue, Y474, which is phosphorylated in the host cell. In contrast, EHEC Tir lacks this residue and generates pedestals independently of tyrosine phosphorylation. Consistent with this difference, recent work indicates that EHEC Tir cannot functionally replace EPEC Tir. To identify the role that tyrosine phosphorylation of EPEC Tir plays in actin signalling, we generated chimeric EHEC/EPEC Tir proteins and identified a 12-residue sequence of EPEC Tir containing Y474 that confers actin-signalling capabilities to EHEC Tir when the chimera is expressed in EPEC. Nck, a mammalian adaptor protein that has been implicated in the initiation of actin signalling, binds to this sequence in a Y474 phosphorylation-dependent manner and is recruited to the pedestals of EPEC, but not of EHEC.
Introduction

Two closely related diarrheagenic microorganisms, enteropathogenic *Escherichia coli* (EPEC) and enterohaemorrhagic *E. coli* (EHEC), belong to the attaching and effacing (AE) family of Gram-negative enteric pathogens that produce specific histopathological alterations of intestinal enterocytes, called AE lesions (Nataro and Kaper, 1998). AE lesions are characterized by microvillar effacement, close adherence of the bacterium to the host cell membrane and the generation of a filamentous (F-) actin structure beneath the site of bacterial attachment. These distinctive cytoskeletal structures that AE pathogens produce are called actin pedestals. The pedestal-forming capability of these pathogens correlates with their ability to colonize the intestine and cause disease in mammalian hosts (Donnenberg et al., 1993b; Schauer and Falkow, 1993; Tzipori et al., 1995).

Gene products required for EPEC and EHEC to form actin pedestals are contained on a 35 kb chromosomal pathogenicity island called the locus of enterocyte effacement (LEE) that is highly conserved between these two strains (Perna et al., 1998). The LEE encodes a type III protein secretion system for the injection of bacterial proteins into host cells. One of the LEE-encoded *E. coli* proteins injected, Tir (translocated intimin receptor), becomes localized in the host plasma membrane with its N- and C-terminal domains cytoplasmic and its central domain extracellular (de Grado et al., 1999; Deibel et al., 1998; Hartland et al., 1999; Kenny, 1999; Kenny et al., 1997b). The extracellular domain of Tir binds to the bacterial outer membrane protein intimin, encoded by the *eae* gene present in the LEE, and functional Tir-intimin interaction is the final step in triggering actin pedestal formation (Liu et al., 1999; Rosenshine et al., 1996).
Although the LEE pathogenicity islands of EHEC and EPEC are highly conserved, the pedestals generated by the two strains are not identical. For example, the host proteins Grb2 and CrkII are found in lesions formed by EPEC but not by EHEC (Goosney et al., 2001). These differences apparently reflect mechanistic differences in the function of the respective Tir proteins. Further, tyrosine 474 of EPEC Tir becomes phosphorylated after Tir translocation into the host cell and is critical for Tir's actin signalling function (Kenny, 1999). In contrast, EHEC Tir does not contain an equivalent tyrosine and does not become tyrosine phosphorylated (DeVinney et al., 1999; Ismaili et al., 1995). Recently, Kenny demonstrated that plasmid-encoded EHEC tir does not complement an EPEC tir mutant strain, and that differences in the Tir C-terminal cytoplasmic domains (which are only 44% identical; Perna et al., 1998) are responsible for the inability of EHEC Tir to replace EPEC Tir functionally (Kenny, 2001).

The mammalian actin polymerization pathways identified thus far converge at the step of actin nucleation promoted by the Arp2/3 complex (Pantaloni et al., 2001). This actin nucleation activity can be triggered by binding of WASP family proteins (e.g. N-WASP; Mullins, 2000). N-WASP and Arp2/3 localize to both EPEC and EHEC pedestals (Goosney et al., 2001; Kalman et al., 1999), and N-WASP is essential for the local recruitment of Arp2/3 and for pedestal formation by EPEC (Kalman et al., 1999; Lommel et al., 2001; S. Snapper, personal communication). The ability of N-WASP to stimulate the Arp2/3 complex can be activated by Rho-family GTPases (Mullins, 2000), but inhibitors of these GTPases do not inhibit pedestal formation by EPEC (Ben-Ami et al., 1998; Ebel et al., 1998b). It has been shown recently that the adapter protein Nck, which binds to phosphotyrosine residues via a Src homology 2 (SH2) domain, can functionally substitute for Cdc42 in stimulating N-WASP to trigger actin polymerization in vitro (Rohatgi et al., 2001). Nck is known to be used by at least one microbial
pathogen to mediate actin nucleation, as the vaccinia virus envelope protein A36R has been demonstrated to become tyrosine phosphorylated in the host cell, to recruit Nck and to promote actin-based intracellular movement by the virus (Frischknecht et al., 1999).

In this study, we have examined differences in the mechanisms by which EHEC and EPEC Tir trigger actin pedestal formation on mammalian cells. We show that a 12-residue sequence of EPEC Tir that contains the critical tyrosine Y474 is sufficient to confer upon EHEC Tir the ability to function in an EPEC strain background. Further, we demonstrate that the host protein Nck binds to this 12-amino-acid sequence in a phosphotyrosine-dependent manner and is recruited to EPEC but not EHEC pedestals.

**Experimental procedures**

*Bacterial and mammalian cell culture*

For routine passage, all *E. coli* strains (Table 2) were cultured in LB media at 37°C. For EPEC strains, media were supplemented with 100μg ml⁻¹ ampicillin for retention of pMAR7. For maintenance of pK184-based *tir* plasmids (Table 3), media were supplemented with 25μg ml⁻¹ kanamycin. Before infections of HEp-2 cells, EPEC and EHEC were cultured in DMEM + 100 mM HEPES, pH 7.4, in 5% CO₂, growth conditions shown previously to maximize type III secretion (Beltrametti et al., 1999; Kenny et al., 1997a). HEp-2 cells were cultured in RPMI-1640 supplemented with 7% fetal bovine serum (FBS), 100 U ml⁻¹ penicillin, 100μg ml⁻¹ streptomycin and 2 mM L-glutamine at 37°C in 5% CO₂.

*Strain construction*

The EHEC and EPEC strains used in this study are listed in Table 2. All EPEC derivatives were constructed in a JPN15/pMAR7 (O127:H6) background, whereas the EHECΔ*tir* strain was
derived from TUV93-0. All plasmids harbouring fragments used to create these strains are listed in Table 3, along with descriptions of the plasmids and listings of the DNA templates and primers used for polymerase chain reaction (PCR) cloning of plasmid inserts. All primer sequences are detailed in Table 4. Each EHEC or EPEC mutant described below was generated using bacteriophage lambda 'Red' recombination functions to promote homologous recombination between the bacterial chromosomes and electroporated linear DNA substrates in a method similar to that described for E. coli K-12 (Murphy et al., 2000). Strain EPECΔtir (KC14) was constructed using a PCR-generated DNA fragment containing the selectable marker cat, flanked by 50 bp of tir sequences. Primers 1 and 2, which were tailed with 50 bp of EPEC sequence from the 5' and 3' ends of tir, respectively, were used to amplify the cat gene from pTP883 (Murphy et al., 2000). This linear PCR product was gel purified and electroporated into JPN15/pMAR7 (carrying the Red-expressing plasmid pTP223) to generate EPECΔtir.

Strain EHECΔtir (KC5) was constructed similarly using Red-based techniques and will be described elsewhere (K. Murphy and K. Campellone, manuscript in preparation). EPEC strain KC12 was derived as follows. First, a 1.5 kb PCR product was generated using primers 3 and 4 to amplify the sequence immediately upstream of EPEC tir. A haemagglutinin (HA) tag was incorporated in primer 4 at the putative translational start of tir in this PCR product, which was cloned as a SacI-HindIII fragment into the vector pGEM7zf(+) to generate pKC13. Next, primers 8 and 9 were used to clone the 3' region of EPEC eae and sequence downstream of EPEC eae as a HindIII-XbaI fragment (with a NotI site incorporated 3' to the HindIII site) into pKC13 to generate pKC22. A NotI fragment containing the cat-sacB cassette from pKM154 (Murphy et al., 2000) was then cloned into the NotI site of pKC22 to create pKC24. A SacI-XbaI fragment from pKC24 was electroporated into JPN15/pMAR7 to produce strain KC8. EHEC tir-cesT and EPEC
tir-cesT were PCR amplified using primers 5 and 7 and primers 6 and 7, respectively, and cloned as HindIII-XbaI fragments into pKC13 in frame with the sequence encoding the HA tag to yield pKC14 and pKC15 respectively. Primers 10 and 11 and primers 8 and 12 were used in a two-piece over-lapping PCR to amplify a fragment carrying sequence downstream of EHEC tir featuring a precise junction of the 3' end of EHEC eae with the region immediately downstream of EPEC eae. This PCR product was cloned as an XhoI-XbaI fragment into pKC14 (XhoI is a unique site that lies within EHEC cesT) to generate pKC32. pKC32 thus contains EPEC sequence 1.5 kb immediately upstream of the ATG start of tir and 1.5 kb immediately downstream of the TAA terminator in eae flanking the sequence encoding the EHEC versions of HA-tagged Tir, CesT and intimin. pKC32 was digested with SpeI and KpnI (sites within the 5' and 3' flanking regions respectively) to prepare the linear substrate, which was electroporated into strain KC8 to yield strain KC12. Strain KC12Δtir (KC26) was generated as follows. A SacI-XbaI fragment from pKC14 was cloned into the vector pK184 to create pKC16. Inverse PCR was performed with primers 13 and 14, using pKC16 as template, to generate an in frame deletion in EHEC tir, incorporating a NotI site at the junction point. The cat-sacB cassette derived from pKM154 (see above) was cloned into this NotI site to produce pKC117. An ApaLI-XhoI fragment from pKC117 was electroporated into KC12 to generate strain KC13. The cat-sacB marker was removed from pKC117 by NotI digestion and then recircularized to yield pKC181. pKC181 was digested with ApaLI and XhoI and electroporated into KC13 to generate KC12Δtir (KC26). Correct plasmid sequences were confirmed by DNA sequencing, and proper chromosomal recombinants were verified by PCR analysis.
Construction of tir complementation plasmids

The plasmids pEHEC tir and pEPEC tir (Table 3) were constructed by subcloning SacI-XbaI fragments from pKC14 and pKC15, respectively, into pK184. The templates and primers used in the construction of plasmids encoding Tir chimeras and point mutants are listed in Table 3. Primer sequences are given in Table 4. The Tir chimera plasmids pHtir505/Ptir54, pHtir461/Ptir93, and pHtir402/Ptir149 were constructed by first performing inverse PCR using pEHEC tir as a template and primers designed to generate deletions in EHEC tir sequence that incorporate a unique KpnI site at the deletion junction. PCR products were gel purified, digested with KpnI, ligated and cloned in E. coli DH5α. PCR products encoding sequence from the 3' region of EPEC tir were generated with KpnI tails and cloned into the KpnI sites of the above EHEC tir deletion plasmids to generate the desired chimeras. Correct sequences of plasmids were confirmed by DNA sequencing. All short tir chimeras (Fig. 3) and point mutants (Table 1) were constructed by PCR using EHEC or EPEC tir plasmids as templates and self-complementary oligonucleotides as primers. Briefly, oligos designed to generate the desired mutations were used to amplify the relevant tir plasmid, and PCR products were gel purified and transformed into DH5α. The nucleotide changes within the resulting tir plasmids were verified by DNA sequencing.

Bacterial infections and immunofluorescence microscopy

To assess actin pedestal formation by EHEC and EPEC, subconfluent monolayers of HEp-2 cells grown on 12 mm glass coverslips were infected with 5x10^5 bacteria in RHFM medium (RPMI supplemented with 20 mM HEPES, pH 7.4, 0.5% D-mannose, 2% FBS and 2 mM L-glutamine) for 3 h, washed twice with PBS and incubated for a further 3 h in fresh media. Monolayers were washed five times with PBS, fixed for 30 min with PBS + 2.5% paraformaldehyde,
permeabilized for 5 min with PBS + 0.1% Triton X-100, washed three times with PBS and treated with mouse antiphosphotyrosine monoclonal antibody (mAb) PY99 (Santa Cruz) diluted 1:100 or mouse anti-HA mAb HA.11 (Covance) diluted 1:500 for 30 min. Monolayers were then washed three times, incubated with Alexa 488-conjugated anti-mouse IgG (Molecular Probes) at a dilution of 1:200 and 1μg ml\(^{-1}\) TRITC-phalloidin (Sigma) for 30 min, washed three times with PBS and examined by fluorescent and light microscopy. For Nck immunolocalization, rabbit anti-Nck (Upstate Biotechnology) was used at a dilution of 1:200 and visualized with Alexa 488-conjugated anti-rabbit IgG (Molecular Probes).

Nck-binding assays

The plasmid used to overexpress the GST-Nck fusion protein (Rohatgi et al., 2001) was a gift from S. Rankin and H. Ho. GST and GST-Nck proteins were purified on glutathione cross-linked agarose beads according to the manufacturer's instructions (Sigma). Infected HEp-2 cell lysates were prepared as follows. Ninety per cent confluent monolayers of HEp-2 cells grown in six-well plates were either left uninected or infected in RHFM with 2.5x10\(^6\) bacteria per well of KC12Δtir harbouring pEPEC tir, pH/P-tir-12aa, pH/P-tir-12aa-Y474F or H/P-tir-7aa for 3.5 h. Monolayers were then washed twice with PBS and incubated for a further 1.5 h in fresh media. Monolayers were again washed twice and incubated for one additional hour in RHFM supplemented with 50μg ml\(^{-1}\) gentamicin to kill bacteria. Monolayers were lifted with PBS + 0.2 mM EDTA, washed once with PBS and lysed in 50 mM HEPES, pH 7.4, 0.5% glycerol, 50 mM NaCl, 1% Triton X-100, 1 mM Na\(_3\)VO\(_4\), 1 mM phenylmethylsulphonyl fluoride (PMSF) and 10 μg ml\(^{-1}\) each of aprotinin, leupeptin and pepstatin (Sigma). To assess the ability of full-length Tir molecules to interact with Nck, 1μg per lane of purified recombinant GST-Nck or GST was subjected to SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes.
Membranes were then blocked in PBS + 5% milk (PBSM) for 30 min before treatment with HEP-2 lysate probes. Equal amounts of lysate (the equivalent of one infected well) were diluted into 1 ml of PBS + 0.2% BSA + 1 mM Na₃VO₄ and incubated with membranes for 3 h. Lysates were removed, and membranes were blocked in PBS + 5% milk (PBSM) for 1 h, followed by treatment with anti-HA antiserum (diluted 1:1000 in PBSM) at 4°C overnight. Membranes were then washed twice with PBS and three times with PBS + 0.5% Tween-20 before treatment with anti-mouse IgG antiserum conjugated to alkaline phosphatase (diluted 1:5000 in PBSM; Promega) for 2 h. Membranes were washed three times with PBS + 0.5% Tween-20, soaked in Tris buffer (100 mM Tris, pH 9.5, 100 mM NaCl, 5 mM MgCl₂) for 10 min and developed with BCIP/NBT. Each binding assay was performed three independent times and yielded indistinguishable results.

High-performance liquid chromatography (HPLC)-purified Tir peptides were purchased from the Tufts University School of Medicine Microsequencing Core Facility. Each peptide contained an amino-terminal biotin, an AHA spacer and a Tir amino acid sequence. The sequence of the tyrosine-phosphorylated peptide H/P-Tir-12aa-PO₄ was QVPEEHI(Y-PO₄)DEVAADPG. Peptide H/P-Tir-12aa consisted of the identical sequence, but without the phosphate group added to the tyrosine. Peptide H/P-Tir-7aa-PO₄ contained the tyrosine-phosphorylated sequence QVPEEHI(Y-PO₄)DEVQNMG. To analyse Tir peptides for direct interaction with Nck by gel overlay assay, 1µg per lane of purified recombinant GST-Nck or GST was subjected to SDS-PAGE and transferred to PVDF membranes. Membranes were blocked in PBS + 5% milk (PBSM) for 30 min, followed by a 2 h incubation with 5µg ml⁻¹ Tir peptide in PBS + 0.2% BSA + 0.5 mM Na₃VO₄. Membranes were washed three times with PBS, blocked with PBSM for 30 min and incubated with antibiotin antiserum conjugated to alkaline
phosphatase (diluted 1:5000; Sigma) for 2 h, then washed and developed as described above.
Each binding assay was performed three independent times and yielded indistinguishable results.

**Results**

*Precise replacement of EPEC tir-cesT-aae with EHEC tir-cesT-aae results in an EPEC strain incapable of triggering actin pedestal formation*

To begin a functional comparison between EPEC and EHEC Tir, EPEC and EHEC strains were constructed with chromosomal deletions in *tir*. As expected from previous studies (DeVinney et al., 1999; Kenny et al., 1997b), EPECΔ*tir* and EHECΔ*tir* were each incapable of triggering actin polymerization in cultured mammalian cells, as visualized by a lack of high-intensity tetramethylrhodamine isothiocyanate (TRITC)-phalloidin staining (actin focusing) at points of bacterial contact (data not shown). These strains were then transformed with low-copy plasmids encoding HA epitope-tagged EHEC Tir or EPEC Tir, as well as the cognate Tir chaperone, CesT (Abe et al., 1999; Elliott et al., 1999a), and tested for the ability to focus actin after infection of HEp-2 cells. As reported recently (Kenny, 2001), EHECΔ*tir* was complemented by either EHEC or EPEC *tir*, whereas EPECΔ*tir* was complemented only by EPEC *tir* and not by EHEC *tir* (Fig. 1A). The functional defect of EHEC Tir expressed in EPEC was not caused by a defect in translocation by the EPEC type III secretion apparatus, because translocated (HA-tagged) EHEC Tir was detected by anti-HA antibody staining (Fig. 1A); in contrast, no HA staining was evident when EHEC Tir was expressed by an EPEC strain defective for type III secretion (data not shown).

Recombinant EHEC Tir recognizes EPEC intimin with reduced efficiency compared with EHEC intimin, whereas EPEC Tir apparently recognizes both EHEC and EPEC intimin with
high efficiency (DeVinney et al., 1999). Additionally, co-infection of mammalian cells with one bacterial strain delivering EHEC Tir and a second strain expressing EPEC intimin resulted in a reduced efficiency of pedestal formation by the latter strain (DeVinney et al., 1999). It was therefore necessary to test whether the expression of EHEC Tir with its cognate (EHEC) intimin in an EPEC strain background could restore actin signalling to EPEC. To exclude potential artifacts resulting from plasmid-borne protein expression, we precisely replaced the EPEC *tir-cesT-eae* operon from the start codon of *tir* to the stop codon of *eae* with the homologous *tir-cesT-eae* operon of EHEC (see Experimental procedures). To allow for an assessment of Tir translocation in this strain, termed KC12, the EHEC Tir was HA tagged (Fig. 1B). In fact, anti-HA staining demonstrated that Tir expressed by KC12 was translocated and localized beneath bacteria bound to HEp-2 cells (Fig. 1C). Nevertheless, KC12 was unable to focus actin (Fig. 1C), indicating that the EHEC *tir-cesT-eae* operon is not capable of promoting actin pedestal formation in an otherwise EPEC background. Pedestal formation could be restored to KC12 by introducing a plasmid-borne copy of EPEC *tir*, but not EHEC *tir* (Fig. 1C), indicating that the defect in pedestal formation by KC12 is indeed the result of differences between the Tir molecules and not between the intimin homologues, as this strain expresses EHEC intimin.

*Substitution of EPEC Tir Y474 with phenylalanine, aspartate or glutamate results in loss of Tir function in EPEC but not EHEC*

It was shown previously that Y474 in the C-terminal cytoplasmic domain of EPEC Tir becomes phosphorylated in the host cell, and that substitution of Y474 with either phenylalanine or serine results in loss of Tir function (Goosney et al., 2000; Kenny, 1999). We generated a phenylalanine substitution of Y474 (Y474F), confirmed that it did not function for actin focusing...
when expressed in EPECΔtir and also found that it was not functional when expressed in a tir
deletion mutant of the EPEC-derived strain KC12 (i.e. KC12Δtir, which differs from EPECΔtir
by virtue of carrying EHEC cesT and eae in place of EPEC cesT and eae) (Table 1). If
phosphorylation of Y474 functions only to provide a negative charge at this site in Tir, then
function might be retained upon substitution with a negatively charged residue. Thus, we
replaced Y474 with either aspartate (Y474D) or glutamate (Y474E) and expressed the mutants in
both EPECΔtir and KC12Δtir. However, neither mutant promoted actin focusing by either strain
(Table 1).

EHEC Tir does not contain a tyrosine residue equivalent to Y474 of EPEC Tir,
suggesting that whatever role Y474 plays in promoting actin focusing by EPEC, it is not required
for actin focusing by EHEC. Consistent with this hypothesis, the EPEC Tir mutants Y474F,
Y474D and Y474E each retained actin signalling function when expressed in EHECΔtir (Table
1).

A region in the C-terminal cytoplasmic domain of EPEC Tir that contains Y474 is critical for
actin focusing by EPEC

The C-terminal cytoplasmic domain is the least conserved component of EHEC and EPEC Tir,
sharing only 44% identity, compared with 66% identity for the rest of the molecules (Perna et al.,
1998). Kenny (2001) recently demonstrated that an EHEC/EPEC Tir chimera that contained the
entire C-terminal cytoplasmic domain of EPEC Tir maintained actin signalling function when
expressed in EPEC. To localize further the region of EPEC Tir capable of restoring actin
signalling function to EHEC Tir when expressed in an EPEC strain background, we introduced
into KC12 plasmids encoding chimeric EHEC/EPEC Tir molecules possessing 54, 93 or 149
amino acids of the EPEC C-terminal cytoplasmic domain (Fig. 2A). Among the three Tir chimeras tested, only the two that contained EPEC Y474 restored to KC12 the ability to focus actin upon infection of HEp-2 cells (Fig. 2B).

**Tyrosine 474 and nearby flanking sequences are required for actin signalling by EPEC**

Given the apparent critical role of Y474 in actin signalling by EPEC, we generated HA-tagged EHEC/EPEC Tir chimeras that contained this residue or short sequences of EPEC Tir that encompass this residue, replacing the equivalent amino acids of EHEC Tir (Fig. 3A). To retain cognate EHEC Tir and intimin binding partners, and to avoid co-expression of chimeric and wild-type EHEC Tir proteins, these chimeras were expressed in KC12Δtir and tested by fluorescent antibody staining for translocation and tyrosine phosphorylation, and by phalloidin staining for the ability to trigger actin focusing. CLUSTALW alignment of conserved residues within the EPEC and EHEC Tir C-termini identified EHEC residue S478 (the C-terminal-most serine shown in Fig. 3A) as the equivalent of EPEC Y474. H/P-Tir-1aa, in which S478 is replaced by Y, was translocated (data not shown), but not phosphorylated, and did not confer to KC12Δtir the ability to trigger actin focusing (Table 1). H/P-Tir-7aa (Fig. 3A), a chimera with a 7-residue sequence from EPEC Tir, was translocated, phosphorylated and elicited a diffuse F-actin signal beneath bound bacteria, but did not promote efficient actin condensation into focused pedestals (Table 1, Fig. 3B). H/P-Tir-12aa (Fig. 3A), which contains a 12-residue EPEC Tir sequence, was also translocated and phosphorylated. In addition, this chimera clearly promoted actin focusing by KC12Δtir (Table 1, Fig. 3B). Identical results were obtained when the chimeras were expressed in EPECΔtir (Table 1), indicating that EPEC and EHEC intimin are apparently functionally interchangeable in this strain background. These results indicate that the
seven-amino-acid region encompassing Y474 contains sufficient sequence information to direct phosphorylation of Y474 in the host cell, but insufficient information to confer to EHEC Tir the ability to promote effective actin signalling when expressed by EPEC. In contrast, the 12 amino acids encompassing Y474 are not only tyrosine phosphorylated, but are also sufficient to confer EPEC Tir function upon the otherwise non-functional EHEC Tir.

To confirm that Y474 was critical for actin signalling by H/P-Tir-12aa, we tested a derivative of H/P-Tir-12aa with a phenylalanine substitution. As predicted, the H/P-Tir-12aa-Y474F mutant chimera was not functional in KC12Δtir or EPECΔtir (Table 1; Fig. 3B). In contrast, H/P-Tir-12aa-Y474F as well as H/P-Tir-1aa and H/P-Tir-7aa were fully functional when expressed in EHECΔtir, consistent with our previous observation that this region is apparently not critical for Tir function in EHEC (Table 1).

The 12-amino-acid sequence of EPEC Tir that encompasses Y474 binds to the mammalian protein Nck in a phosphotyrosine-dependent manner

The ability of the H/P-Tir-12aa chimera containing Y474 to function in an EPEC strain background and the apparent requirement for phosphorylation of Y474 are consistent with a role for this small region in the recruitment of a host adaptor protein, presumably one that encodes an SH2 phosphotyrosine-binding domain (Pawson and Scott, 1997; Songyang and Cantley, 1995). In fact, the EPEC sequence immediately flanking Y474 is highly homologous to a region of the vaccinia virus A36R protein (Fig. 3A) that becomes tyrosine phosphorylated and recruits the SH2-containing adaptor protein Nck to promote actin polymerization (Frischknecht et al., 1999; Frischknecht and Way, 2001). We therefore tested whether Tir could interact with Nck. In gel overlay assays, purified glutathione S-transferase (GST)-Nck was probed with extracts from
uninfected HEp-2 cells or from HEp-2 cells infected with KC12Δtir expressing either HA-tagged wild-type EPEC Tir, H/P-Tir-12aa, H/P-Tir-12aa-Y474F or H/P-Tir-7aa. Bound Tir was detected by antibody directed against the HA epitope, and only the Tir molecules previously shown to be capable of focusing actin, i.e. EPEC Tir and H/P-Tir-12aa, interacted with GST-Nck (Fig. 4A). This binding was specific for Nck, as these proteins did not bind the control GST protein (Fig. 4A). Further, H/P-Tir-12aa-Y474F and H/P-Tir-7aa did not recognize Nck in these assays (Fig. 4A), suggesting that the actin signalling defect of these chimeras results from a deficiency in Nck binding.

To determine whether Nck binding was mediated by the 12-residue Y474-containing sequence, three peptides were tested for the ability to bind GST-Nck in gel overlay assays: (i) H/P-Tir-12-PO₄, a tyrosine-phosphorylated peptide that encompasses the 12-residue EPEC Tir sequence of chimera H/P-Tir-12aa; (ii) H/P-Tir-12, an identical peptide that lacks the phosphate group; and (iii) H/P-Tir-7-PO₄, which contains the equivalent tyrosine-phosphorylated sequence from the H/P-Tir-7aa chimera (Fig. 3A). As predicted, peptide H/P-Tir-12-PO₄ bound directly to purified GST-Nck in this gel overlay assay (Fig. 4B). Tyrosine phosphorylation was required for Nck binding, because peptide H/P-Tir-12 demonstrated no significant Nck binding (Fig. 4B). Lastly, peptide H/P-Tir-7-PO₄ bound Nck only weakly (Fig. 4B), further indicating that the defect in the H/P-Tir-7aa chimera in actin signalling is likely to result from a defect in Nck binding. These results are consistent with the known recognition specificity of phosphotyrosine-containing motifs by SH2 domains, which commonly depend on residues C-terminal to the phosphotyrosine (Pawson and Scott, 1997; Songyang and Cantley, 1995); the H/P-Tir-7aa chimera contains only three EPEC residues C-terminal to Y474, whereas the H/P-Tir-12aa chimera contains eight (Fig. 3A).
**EPEC, but not EHEC, efficiently recruits Nck to its actin pedestals**

Given the presence of a Nck-binding sequence in EPEC Tir but not in EHEC Tir, we tested whether Nck localized to actin pedestals generated specifically by EPEC. Anti-Nck antibody intensely stained EPEC pedestals but not EHEC pedestals (Fig. 5A), consistent with the absence of Y474 and the Nck-binding sequence in EHEC Tir (Fig. 3A). An intact Nck-binding sequence was necessary for this recruitment, because Nck localized beneath bound EPEC expressing the functional H/P-Tir-12aa chimera (Fig. 5A), but not EPEC expressing either H/P-Tir-12aa-Y474F or H/P-Tir-7aa (Fig. 5B), the Tir chimeras defective for Nck binding (Figs 4A and B).

**Discussion**

The inability of EHEC Tir to function in an EPEC background with either EPEC or EHEC intimin provided an opportunity to identify those features of EPEC Tir critical for function in this background. We found that an EPEC strain in which the endogenous tir-cesT-eae region was precisely replaced by the tir-cesT-eae of EHEC failed to generate actin pedestals, consistent with recent plasmid-based complementation studies (DeVinney et al., 2001; Kenny, 2001). In contrast, an EHEC/EPEC Tir chimera that contained only 12 amino acids from EPEC could function for actin signalling in this background. This 12-residue sequence contained Y474, an amino acid critical to EPEC Tir function that becomes phosphorylated after Tir is translocated to the host cell (Kenny, 1999). Tir molecules with this sequence, present in extracts from infected mammalian cells, interacted with the host adaptor protein Nck in vitro. Furthermore, a peptide encompassing this sequence was shown to bind directly to Nck in a phosphotyrosine-dependent manner, whereas a corresponding peptide derived from the non-functional chimera that
contained only seven amino acids from EPEC Tir bound Nck much less efficiently. Finally, the presence of the 12-residue Nck-binding sequence in Tir resulted in the recruitment of Nck to actin pedestals during infection of mammalian cells. Recently, Gruenheid et al. (2001) similarly demonstrated that Nck bound EPEC Tir, and also showed that EPEC was incapable of triggering actin focusing on Nck1/Nck2-deficient mouse embryonic fibroblasts.

The binding of Nck by EPEC Tir suggests several models by which the N-WASP/Arp2/3 complex actin nucleation machinery may become localized and active at sites of bacterial adherence. Nck directly stimulates N-WASP to activate the Arp2/3 complex in vitro (Rohatgi et al., 2001), and a simple model is that Nck, after recruitment to the plasma membrane by tyrosine-phosphorylated Tir, binds and activates N-WASP to trigger actin condensation. Nck and the phosphoinositide PIP2 synergistically activate N-WASP in vitro (Rohatgi et al., 2001), and one can also speculate that Tir-mediated recruitment of Nck further activates bound N-WASP by bringing it into proximity to PIP2 in the membrane. This model does not require stimulation of N-WASP by Rho-GTPases, as Nck and the Rho-family GTPase Cdc42 stimulate N-WASP in a functionally redundant manner in vitro (Rohatgi et al., 2001). Thus, this model is consistent with studies suggesting that neither Rho GTPases (Ben-Ami et al., 1998; Ebel et al., 1998b) nor the GTPase-binding (CRIB) domain of N-WASP (Lommel et al., 2001) are required for EPEC pedestal formation. It is not clear, however, how to reconcile this simple model with the observation that the polyproline region of N-WASP, which has been suggested to bind Nck (Rivero-Lezcano et al., 1995; Rohatgi et al., 2001), is also not required for N-WASP recruitment (Kalman et al., 1999) and pedestal formation (Lommel et al., 2001).

In alternative models, Nck may bind EPEC Tir, but may not bind directly to N-WASP during actin pedestal formation. For example, Nck bound to the vaccinia virus A36R protein is
thought to recruit N-WASP indirectly by binding WIP (WASP interacting protein; (Moreau et al., 2000)), a protein that interacts with both Nck (Anton et al., 1998) and WASP family proteins (Ramesh et al., 1997). Nck also binds to other proteins that regulate the actin cytoskeleton, such as p21-activated kinase, Abl and Cbl (Buday, 1999; McCarty, 1998). Thus, the signalling effector(s) immediately downstream of Nck during pedestal formation remain to be identified. Nonetheless, the results presented here suggest that Nck binding is one of the proximal events in actin signalling by EPEC Tir.

It is not known whether Nck clustering at the membrane by EPEC Tir is sufficient to generate the signal for actin condensation, or whether Tir has other activities that promote pedestal formation. For example, although the Nck SH2 domain is not required for maximal stimulation of N-WASP in vitro (Rohatgi et al., 2001), the experiments described here do not rule out the possibility that Tir binding by this domain might activate Nck in some fashion. In addition, although H/P-Tir-12aa, the Tir chimera with 12 residues from EPEC Tir, was clearly competent for actin signalling, the pedestals it generated appeared to stain slightly less intensely with phalloidin than those generated by wild-type EPEC Tir (K. Campellone, unpublished observations). This apparent difference is consistent with the hypothesis that other regions of Tir have important functions in AE lesion formation. In fact, the N-terminus of EPEC Tir has been shown to bind to a number of proteins associated with focal adhesions, such as the actin-binding protein α-actinin (Freeman et al., 2000; Goosney et al., 2000). Recruitment of these proteins might stabilize the actin pedestal and the link between the bound bacterium and the host cytoskeleton.

Our observations that EHEC tir-cesT-eae could not function in place of the EPEC tir-cesT-eae operon, even when located at the same site on the chromosome and presumably under
endogenous (EPEC) regulatory control, and that Nck localized to EPEC pedestals but not to EHEC pedestals, fully support previous suggestions that EHEC and EPEC have evolved somewhat different mechanisms to activate the actin polymerization machinery (DeVinney et al., 1999; Goosney et al., 2001; Ismaili et al., 1995; Kenny, 2001). One attractive model is that EHEC encodes an additional factor or factors, not present in EPEC, that are required for EHEC Tir to trigger actin pedestal formation. DeVinney et al. (2001) recently demonstrated that, although the Y474-containing region of EPEC Tir was essential for actin signalling specifically by EPEC, pedestal formation by EHEC (which lacks Y474) apparently requires the delivery of an additional factor (or factors) by the type III secretion system. This putative EHEC actin signalling effector might be a LEE-encoded molecule that has co-evolved with EHEC Tir to allow phosphotyrosine (and presumably Nck)-independent pedestal formation. Conversely, this factor might be encoded outside the LEE, as suggested by the observation that a cosmid encoding the EPEC LEE can confer the pedestal-forming phenotype to non-pathogenic K-12 E. coli (McDaniel and Kaper, 1997), whereas an equivalent EHEC LEE cosmid cannot (Elliott et al., 1999b). Just as the current study of EPEC Tir has highlighted a role for Nck in the control of actin polymerization, the characterization of EHEC Tir and additional EHEC effectors is likely to provide further insight into pathways of actin signalling.
Table 1. Summary of plasmid complementation of EHEC and EPEC tir deletions

<table>
<thead>
<tr>
<th>Plasminic</th>
<th>Tyrosine phosphorylation</th>
<th>EHECΔtir</th>
<th>EPECΔtir</th>
<th>KCI2Δtir</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type Tir proteins</td>
<td>-</td>
<td>+d</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pEHEC tir</td>
<td>+</td>
<td>+d</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pEPEC tir</td>
<td>-</td>
<td>+d</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>EPEC Tir Y474 mutants</td>
<td>NO</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pEPEC tir-Y474D</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pEPEC tir-Y474E</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pEPEC tir-Y474F</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>EHEC/EPEC Tir chimeras</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pHPl-tir-1aa</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pHPl-tir-7aa</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+d</td>
</tr>
<tr>
<td>pHPl-tir-12aa</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pHPl-tir-12aa-Y474F</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

a. +/-, actin pedestals were/were not generated upon infection of HEp-2 cells as determined by phalloidin staining. Each result is the clear consensus of at least three independent experiments.
b. All plasmids express HA-tagged Tir of the indicated type.
c. The tyrosine phosphorylation state of Tir when expressed by EPEC was determined by immunofluorescence microscopy. +, Tir was phosphorylated. - , Tir was not phosphorylated. ND, not determined.
d. See also Fig. 1A. Data are included here for ease of comparison.
e. Each substitution mutant is designated by a single letter amino acid code.
f. The number in each plasmid name reflects the number of residues of EPEC Tir used to replace EHEC Tir sequence in the chimera. All plasmids except pHPl-tir-12aa-Y474F encode a Tir containing Y474.
g. See also Fig. 3B. Data are included here for ease of comparison.
Table 2. Strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDL933</td>
<td>EHEC O157:H7 prototype</td>
<td>Riley et al. (1983)</td>
</tr>
<tr>
<td>TUV68-9</td>
<td>Stx derivative of EDL933</td>
<td>A. Donohue-Rolfe</td>
</tr>
<tr>
<td>JPN15pMAR7</td>
<td>Amp' derivative of EPEC E2348/69 O127:H6 prototype</td>
<td>Jerse et al. (1980)</td>
</tr>
<tr>
<td>KC5</td>
<td>EHECΔtir (in frame removal of codons 24–245)</td>
<td>K. Murphy and K. G. Campellone (manuscript in preparation)</td>
</tr>
<tr>
<td>KC3</td>
<td>EPECΔΔr−oesT−eae:cat−eaeB</td>
<td>This study</td>
</tr>
<tr>
<td>KC12</td>
<td>EPECΔΔr−oesT−eae:EHEC−HA−tir−oesT−eae</td>
<td>This study</td>
</tr>
<tr>
<td>KC13</td>
<td>KC12ΔΔr−cat−eaeB</td>
<td>This study</td>
</tr>
<tr>
<td>KC14</td>
<td>EPECΔΔr (replacement of codons 16–535 with cat gene)</td>
<td>This study</td>
</tr>
<tr>
<td>KC26</td>
<td>KC12ΔΔr (in frame removal of codons 24–245)</td>
<td>This study</td>
</tr>
</tbody>
</table>
Table 3. Plasmids used in this study.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Inserted PCR product</th>
<th>DNA template(s)</th>
<th>Primer pair*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vectors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pK1B4</td>
<td>Low-copy-number cloning vector (Jobling and Holmes, 1990)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>pGEM7zf(+)</td>
<td>High-copy-number cloning vector (Promega)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Allelic exchange plasmids [derived from pGEM7zf(+) with the exceptions of pKC117 and pKC181]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pKC13</td>
<td>Carries 1.5 kb upstream of EPEC tir + HA tag in pGEM7zf(+)</td>
<td>JPN15 genomic DNA</td>
<td>3 + 4</td>
<td></td>
</tr>
<tr>
<td>pKC14</td>
<td>Carries 1.5 kb upstream of EPEC tir + HA + EHEC tir-casT</td>
<td>EDL933 genomic DNA</td>
<td>5 + 7</td>
<td></td>
</tr>
<tr>
<td>pKC15</td>
<td>Carries 1.5 kb upstream of EPEC tir + HA + EPEC tir-casT</td>
<td>JPN15 genomic DNA</td>
<td>6 + 7</td>
<td></td>
</tr>
<tr>
<td>pKC22</td>
<td>Carries 1.5 kb upstream of EPEC tir + HA + cas-casB + 1.5 kb downstream of EPEC eae</td>
<td>JPN15 genomic DNA</td>
<td>8 + 9</td>
<td></td>
</tr>
<tr>
<td>pKC24</td>
<td>Carries 1.5 kb upstream of EPEC tir + HA + EHEC tir-casT-eae + 1.5 kb downstream of EPEC eae (fragment used to generate strain K12)</td>
<td>EDL933 genomic DNA; JPN15 genomic DNA</td>
<td>10 + 11; 8 + 12</td>
<td></td>
</tr>
<tr>
<td>pKC17</td>
<td>Carries 1.5 kb upstream of EPEC tir + HA + EHEC tir-in-frame + casT (fragment used to generate strain K12)</td>
<td>pKC16</td>
<td>13 + 14</td>
<td></td>
</tr>
<tr>
<td>pKC181</td>
<td>Carries 1.5 kb upstream of EPEC tir + HA + EHEC tir-cas-casB + casT</td>
<td>pKC16</td>
<td>13 + 14</td>
<td></td>
</tr>
</tbody>
</table>

**Tir complementation plasmids (all derived from pK1B4)**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Inserted PCR product</th>
<th>DNA template(s)</th>
<th>Primer pair*</th>
</tr>
</thead>
<tbody>
<tr>
<td>pKC13 (pEHEC tir)</td>
<td>Produces EHEC Tir and CasT</td>
<td>EHEC Tir and CasT</td>
<td>pKC17; JPN15 genomic DNA</td>
<td>15 + 16; 25 + 28</td>
</tr>
<tr>
<td>pKC17 (pEPEC tir)</td>
<td>Produces EPEC Tir and CasT</td>
<td>JPN15 genomic DNA</td>
<td>17 + 18; 21 + 22; 29 + 30</td>
<td></td>
</tr>
<tr>
<td>pKC181 (pEPEC tir-Y474D)</td>
<td>Produces EPEC Tir-Y474D and CasT</td>
<td>JPN15 genomic DNA</td>
<td>19 + 20; 21 + 22; 29 + 30</td>
<td></td>
</tr>
<tr>
<td>pKC133 (pEPEC tir-Y474E)</td>
<td>Produces EPEC Tir-Y474E and CasT</td>
<td>EHEC Tir and CasT</td>
<td>pKC16; JPN15 genomic DNA</td>
<td>19 + 20; 21 + 22; 29 + 30</td>
</tr>
<tr>
<td>pKC142 (pEPEC tir-Y474F)</td>
<td>Produces EPEC Tir-Y474F and CasT</td>
<td>JPN15 genomic DNA</td>
<td>19 + 20; 21 + 22; 29 + 30</td>
<td></td>
</tr>
<tr>
<td>pKC121 (pH6505/P654)</td>
<td>Produces chimeric Tir: EHEC Tir + 1-680 + EPEC Tir 457-550 and EHEC CasT</td>
<td>EHEC Tir and CasT</td>
<td>pKC16; JPN15 genomic DNA</td>
<td>19 + 20; 21 + 22; 29 + 30</td>
</tr>
<tr>
<td>pKC123 (pH6461/P669)</td>
<td>Produces chimeric Tir: EHEC Tir + 1-481 + EPEC Tir 489-550 and EHEC CasT</td>
<td>EHEC Tir and CasT</td>
<td>pKC16; JPN15 genomic DNA</td>
<td>14 + 15; 19 + 20; 21 + 22; 29 + 30</td>
</tr>
<tr>
<td>pKC125 (pHP402/P4149)</td>
<td>Produces chimeric Tir: EIEC Tir</td>
<td>EHEC Tir and CasT</td>
<td>pKC16; JPN15 genomic DNA</td>
<td>14 + 15; 19 + 20; 21 + 22; 29 + 30</td>
</tr>
<tr>
<td>pKC136 (pHP tir-1ea)</td>
<td>Produces EHEC Tir S475Y and EHEC CasT</td>
<td>EHEC Tir and CasT</td>
<td>pKC16</td>
<td>14 + 15; 19 + 20; 21 + 22; 29 + 30</td>
</tr>
<tr>
<td>pKC127 (pHP tir-7ea)</td>
<td>Produces chimeric HP-Tir-7ea: EHEC Tir w residues 472-478 (TSNSNTS) replaced with EPEC Tir residues 470-476 (EHHYDE) and EHEC CasT</td>
<td>EHEC Tir and CasT</td>
<td>pKC16</td>
<td>31 + 32; 33 + 34; 35 + 36</td>
</tr>
<tr>
<td>pKC154 (pHP tir-12ea)</td>
<td>Produces chimeric HP-Tir-12ea: EHEC Tir w residues 472-482 (TSNSNTSVQONM) replaced with EPEC Tir residues 470-481 (EHHYDEVAADP) and EHEC CasT</td>
<td>EHEC Tir and CasT</td>
<td>pKC127</td>
<td>33 + 34; 35 + 36</td>
</tr>
<tr>
<td>pKC165 (pHP tir-12ea-Y474F)</td>
<td>Produces chimeric HP-Tir-12ea-Y474F and EHEC CasT</td>
<td>EHEC Tir and CasT</td>
<td>pKC154</td>
<td>33 + 34; 35 + 36</td>
</tr>
</tbody>
</table>

a. Primer numbers refer to oligonucleotides listed in Table 4.
b. NA, not applicable.
c. Six residues (NTDSVY) of EHEC Tir downstream of G433 were deleted to conserve proper downstream alignments of the Tir molecules.
Figure 1. An EPEC strain expressing EHEC Tir and intimin does not trigger actin pedestal formation because of a defect in actin signalling by EHEC Tir.

A. HEp-2 cells were infected with EHECΔtir or EPECΔtir carrying plasmids for the expression of either EHEC Tir or EPEC Tir. Cell-associated bacteria were detected by differential interference contrast (DIC) microscopy. Tir localization was determined by antibody staining for the HA epitope tag incorporated at the N-termini of the Tir molecules. F-actin was visualized by staining with TRITC–phalloidin.
Figure 1. An EPEC strain expressing EHEC Tir and intimin does not trigger actin pedestal formation because of a defect in actin signalling by EHEC Tir.
B. The tir–cesT–eae operons of EPEC, EHEC and the hybrid strain KC12 are depicted. EPEC sequence is shown in shaded boxes, whereas EHEC sequence is displayed in open boxes. KC12 is an EPEC strain in which the chromosomal EPEC tir–cesT–eae operon was precisely replaced with the EHEC tir–cesT–eae operon with an HA-encoding sequence incorporated at the 5′ end of tir (see Experimental procedures).
C. HEp-2 cells were infected with KC12 or KC12 additionally expressing plasmid-encoded EHEC or EPEC Tir. Tir and F-actin were detected as described above.
Figure 2. A region in the C-terminal cytoplasmic domain of EPEC Tir that contains Y474 is critical for actin focusing by EPEC.
A. EHEC Tir and the EPEC portions of EHEC/EPEC Tir chimeras are represented. On the left, the membrane topology of EHEC Tir is depicted, with its N- and C-termini cytoplasmic, and its central intimin-binding domain (IBD) extracellular. Numbers listed at the C-terminus of EHEC Tir correspond to the residue number at the fusion junction for each chimera. On the right, the EPEC Tir segments present in each chimera are depicted, with numbers representing the number of EPEC Tir residues present in each chimera. Arrows indicate the position of Y474.
B. HEp-2 cells were infected with KC12 harbouring plasmids encoding the depicted EHEC/EPEC Tir chimeras. HTir402/PTir149, HTir461/PTir93 and HTir505/PTir54 represent Tir chimeras containing the C-terminal 149, 93 and 54 amino acids of EPEC Tir, respectively, as indicated in shaded boxes. The number of N-terminal amino acids derived from EHEC Tir is shown in open boxes. Arrows indicate the position of Y474. F-actin was visualized as described in the legend to Fig. 1. Tir localization was determined by staining with anti-HA or antiphosphotyrosine monoclonal antibodies, as indicated. (Anti-HA staining of monolayers infected with KC12 expressing chimeric Tir molecules possessing Y474 gave patterns indistinguishable from antiphosphotyrosine staining; data not shown.)
**Figure 3.** Tyrosine 474 and nearby flanking residues are required for actin signalling by EPEC.

A. Amino acid alignment of EPEC Tir (Elliott et al., 1998), EHEC Tir (Perna et al., 1998), H/P-Tir-12aa, H/P-Tir-7aa and vaccinia virus A36R (Goebel et al., 1990). Phosphorylated tyrosines (Y) appear in bold. Underlined sequences indicate EPEC amino acids used to replace EHEC Tir sequence in chimeric molecules.

B. HEp-2 cells were infected with EPEC strain KC12Δtir harbouring plasmids encoding the depicted EHEC/EPEC Tir chimeras. The number listed in each chimera represents the number of residues of EPEC Tir that were incorporated into EHEC Tir. Arrows indicate the position of Y474. Y474F represents a tyrosine to phenylalanine substitution in the expressed chimera. F-actin was visualized as described in the legend to Fig. 1. Tir localization was revealed by staining with anti-HA or antiphosphotyrosine monoclonal antibodies, as indicated. (Anti-HA staining of monolayers infected with KC12Δtir expressing chimeric Tir molecules possessing Y474 gave patterns indistinguishable from antiphosphotyrosine staining; data not shown.)
**Figure 4.** The 12 amino acid sequence of EPEC Tir that encompasses Y474 binds to the mammalian protein Nck in a phosphotyrosine dependent manner.

A. Equal amounts of purified GST (‘GST’) and GST–Nck (‘Nck’) were subjected to SDS–PAGE, transferred to PVDF and probed with lysates of HEp-2 cells, which were either uninfected (‘No Tir’) or previously infected with KC12Δtir expressing HA-tagged EPEC Tir, H/P-Tir-12aa, H/P-Tir-12aa-Y474F or H/P-Tir-7aa. Bound Tir was detected using anti-HA monoclonal antibody (see Experimental procedures).

B. Purified GST (‘GST’) and GST–Nck (‘Nck’) were blotted as above and probed with biotinylated peptides corresponding to sequences shown in Fig. 3A. H/P-Tir-12aa was either unmodified (‘Tir-12’) or tyrosine phosphorylated (‘Tir-12-PO4’). H/P-Tir-7aa was tyrosine phosphorylated (‘Tir-7-PO4’). Bound peptide was detected using anti-biotin monoclonal antibody (see Experimental procedures). A Coomassie-stained version of the PVDF membrane is shown to demonstrate equal loading.
**Figure 5.** EPEC, but not EHEC, recruits Nck efficiently to its actin pedestals.

A. HEp-2 cells were infected with EPEC (JPN15/pMAR7), EHEC (TUV93-0) or KC12Δtir harbouring pH/P-tir-12aa. Box diagrams depict the Tir molecule expressed in each strain. Arrows indicate the position of Y474. Nck was visualized by anti-Nck immunostaining, and F-actin was visualized with TRITC-phalloidin.

B. HEp-2 cells were infected with KC12Δtir harbouring pH/P-tir-12aa-Y474F or pH/P-tir-7aa. Box diagrams depict the Tir molecule expressed in each strain. Arrows indicate the position of Y474. Nck was visualized by anti-Nck immunostaining.
CHAPTER III

CLUSTERING OF NCK BY A TIR PHOSPHOPEPTIDE IS SUFFICIENT TO TRIGGER LOCALIZED ACTIN ASSEMBLY

Abstract

Enteropathogenic *Escherichia coli* (EPEC) is an extracellular pathogen that forms actin pedestals on mammalian cells. To stimulate pedestal formation, EPEC injects a number of proteins into host cells. Among these proteins is Tir, which inserts into the plasma membrane, acts as a receptor for the bacterial surface protein intimin, and reorganizes the actin cytoskeleton. The N-terminal cytoplasmic domain of Tir binds several focal adhesion proteins, while a region within Tir’s C-terminus is tyrosine phosphorylated and binds the host protein Nck, an activator of actin assembly necessary for pedestal formation. In this study, Tir derivatives were ectopically expressed in cultured epithelial cells in the absence of all other EPEC proteins. We demonstrate that clustering of the C-terminus of Tir is both necessary and sufficient to trigger pedestal formation in a phosphotyrosine-dependent manner. In addition, we find that clustering of Nck by a small Tir phosphopeptide is sufficient to trigger actin tail formation in *Xenopus* egg extracts.
Introduction

Enteropathogenic *Escherichia coli* (EPEC), a major cause of infantile diarrhea in developing countries, induces the formation of unique structures called attaching and effacing (AE) lesions on the intestinal epithelium (Campellone and Leong, 2003; Celli et al., 2000; Frankel et al., 1998; Nataro and Kaper, 1998). AE lesions are characterized by effacement of microvilli, intimate adherence of bacteria to the host cell, and the generation of filamentous-actin (F-actin) rich pedestal structures beneath attached bacteria. The formation of actin pedestals by EPEC is recapitulated on cultured mammalian cells (Knutton et al., 1989), and its ability to form pedestals on cultured cells correlates with its ability to cause disease in mammalian hosts (Donnenberg et al., 1993a). In addition to its role in disease, pedestal formation by EPEC serves as a model system for understanding mechanisms by which mammalian cells regulate actin assembly at the plasma membrane.

All of the gene products required for EPEC to form actin pedestals are contained on a 35.6 kilobase pathogenicity island called the locus of enterocyte effacement (LEE) (Elliott et al., 1998; McDaniel and Kaper, 1997). The LEE encodes a type III protein secretion apparatus that translocates bacterial effector proteins into host cells. Effectors, as well as chaperones that facilitate their delivery, are also encoded within the LEE. Proteins known to be secreted by the type III system include the *E. coli* secreted proteins EspA, EspB, EspD, EspF, EspG, EspH, Map (mitochondria associated protein), and the translocated intimin receptor, Tir (EspE).

EspA is a critical component of a filamentous structure that extends from the bacterial surface to contact host cells (Ebel et al., 1998a; Knutton et al., 1998; Sekiya et al., 2001), through which other translocated molecules are delivered to the host cell surface. EspB and EspD form a pore in the plasma membrane to allow the delivery of effectors into the cell (Ide et al., 2001;
Kresse et al., 1999; Wachter et al., 1999; Warawa et al., 1999; Wolff et al., 1998). Among these effectors are EspF, EspG, and Map, which affect several host cell processes but do not play obvious roles in actin pedestal formation (Elliott et al., 2001; Kenny et al., 2002; Kenny and Jepson, 2000; McNamara et al., 2001). EspH also does not appear to be required for actin assembly, but does seem to regulate the kinetics of pedestal formation and pedestal elongation (Tu et al., 2003). Lastly, the translocated intimin receptor, Tir, is absolutely essential for the assembly of pedestals.

Translocation of Tir into host cells is facilitated by its chaperone, CesT, which binds to the amino terminus of Tir but is not itself secreted (Abe et al., 1999; Crawford and Kaper, 2002; Elliott et al., 1999a). Translocated Tir integrates into the plasma membrane in a hairpin-loop conformation featuring a central extracellular domain that binds to intimin (Batchelor et al., 2000; Luo et al., 2000), a bacterial surface adhesin encoded by the eae gene in the LEE. Intimin is required for pedestal formation by EPEC and, after translocation of Tir and other Esps, the interaction of intimin-coated particles with host cells triggers actin pedestal formation (Liu et al., 1999; Rosenshine et al., 1996). Intimin may also bind to endogenous mammalian receptors (Frankel et al., 2001), but the ability of intimin mutants to bind to Tir generally correlates with their ability to promote actin pedestal formation (Liu et al., 2002).

While the extracellular domain of Tir serves as a bacterial receptor (Deibel et al., 1998; Kenny et al., 1997b), its cytoplasmic domains interact with components involved in mammalian actin assembly. The C-terminal domain of Tir contains tyrosine 474, which is phosphorylated following entry into the host cell and is critical for actin pedestal formation (Goosney et al., 2000; Kenny, 1999). Phosphorylated Y474 and flanking residues bind the host adaptor protein Nck (Campellone et al., 2002; Gruenheid et al., 2001), an initiator of the N-WASP-Arp2/3
pathway of actin assembly in mammalian cells (Rohatgi et al., 2001; Welch and Mullins, 2002). Nck is required for EPEC to trigger N-WASP activation and pedestal formation (Gruenheid et al., 2001), indicating that recruitment of Nck by Tir is an essential step in pedestal formation.

Recombinant derivatives of the N- and C-terminal cytoplasmic domains of Tir bind additional host cytoskeletal components found within pedestals, such as the actin binding protein cortactin (Cantarelli et al., 2002) and the focal adhesion proteins α-actinin, talin, and vinculin (Cantarelli et al., 2001; Freeman et al., 2000; Goosney et al., 2001; Goosney et al., 2000; Huang et al., 2002). It has been suggested that cortactin and talin are required for pedestal formation (Cantarelli et al., 2001; Cantarelli et al., 2002), and it has been postulated that binding of α-actinin to the N-terminus of Tir may stabilize the pedestal by anchoring EPEC to the cytoskeleton (Freeman et al., 2000; Goosney et al., 2000). However, it has not been formally determined if the ability of Tir to bind these molecules is necessary for localized actin assembly.

The roles that type III-secreted proteins other than Tir play during actin assembly have been difficult to define for several reasons. First, the complete repertoire of EPEC secreted proteins is unknown, so other unidentified effectors may be required for pedestal formation. In fact, it has been proposed that other Esps must be co-delivered with Tir in order for Tir to be tyrosine phosphorylated (Kenny and Warawa, 2001). Second, secreted proteins with known roles in the translocation of other molecules cannot easily be evaluated for additional effector functions. For example, EspB is involved in actin cytoskeletal rearrangements (Kodama et al., 2002; Taylor et al., 1999), but the analysis of EPEC espB mutants is complicated by the fact that such mutants are incapable of translocating effectors such as Tir. Similarly, it is difficult to assess the roles of EspA and EspD in localized actin assembly, because they are also necessary
for translocation of Tir and other EspS. Hence, current models for how EPEC exploits mammalian signaling cascades to initiate pedestal formation are extremely complex.

In order to define the minimal requirements for EPEC-mediated actin assembly, we have bypassed the bacterial mechanism for Tir delivery by directly expressing Tir derivatives within mammalian cells. Our results indicate that among the entire repertoire of EPEC proteins, only Tir is involved in signaling host cells to generate actin pedestals. In fact, clustering of just the C-terminus of Tir beneath the plasma membrane is entirely adequate for triggering actin polymerization into pedestals. Lastly, using particles coated with a small Nck-binding phosphopeptide derived from this domain of Tir, we demonstrate that clustering of Nck is sufficient to promote actin tail formation \textit{in vitro}.

**Materials and methods**

**Bacterial strains**

All EPEC strains used in this study were derived from the O127:H6 wild type strain JPN15/pMAR7 (Jerse, 1990). EPEC$\Delta$tir (KC14) and EPEC$\Delta$tir + pTir (pKC17) have been described previously (Campellone et al., 2002). EPEC$\Delta$tir$\Delta$cesT$\Delta$eae (KC10) was generated from EPEC$\Delta$tir$\Delta$cesT$\Delta$eae::cat-sacB (KC8) following electroporation of a DNA fragment which contains sequence flanking tir and eae but lacks cat-sacB (Campellone et al., 2002). EPEC$\Delta$tir$\Delta$cesT$\Delta$eae was transformed with pTir (which also encodes the chaperone CesT) to generate a host cell priming strain. MC1061 is a nonpathogenic laboratory \textit{E. coli} strain.

**Plasmid construction**

The intimin expressing plasmid pIntimin contains the EPEC eae gene cloned into the HindIII and BamHI sites of pUC19 and was generated by PCR using primers
CAAAAGCTTCA TTCTAACTCA TTGTGGTG and
GCAGATCTCAACTCGAAAGGTGCTAATA. To generate pTirFL (pKC6), sequence
encoding amino acids 1-550 of Tir was inserted as a HindIII-SacII fragment downstream of HA
sequence within the transfection vector pHM6 (Roche) after PCR-amplification of EPEC
genomic DNA using primers GGGAAGCTTTATGCCTATTGGTAACCTTGG and
GCGTCTAGACCGCGGATATATTTAAACGAAACGTACTG (“ODT401”). To construct a
plasma membrane-targeted Tir derivative, sequence encoding amino acids 1-66 (26 N-terminal
cytoplasmic residues, a 22 amino acid transmembrane domain, and 18 extracellular residues) of
the Newcastle Disease Virus HN surface protein was PCR-amplified from pSVL-HN (a gift from
T. Morrison) using primers GCGGAAGCTTCAGTCATGAACCGCGGC and
GGGCTCGAGGCCTGCTGATGGACATCGTATGGGTATGCCCTAGAGATCGCAGTTG
and cloned into the HindIII and XhoI sites of pCDNA3 (Invitrogen) to generate pHN-HA
(pKC33). Sequence encoding the extracellular domain through the C-terminal cytoplasmic
domain of Tir (amino acids 260-550) was PCR-amplified using primers
GGGCTCGAGGACACACCCGAACCAGATGATC and “ODT401” and cloned into the XhoI and
XbaI sites of pHN-HA to create pTirMC (pKC87). pTirMC(Y474F) (pKC187) was generated
using primers described previously (Campellone et al., 2002).

Bacterial and mammalian cell culture
For routine passage, all E. coli strains were cultured in LB at 37°C. For maintenance of Tir or
intimin-expressing plasmids, media were supplemented with 25μg ml⁻¹ kanamycin or 100μg ml⁻¹
ampicillin, respectively. HEp-2 cells were cultured in RPMI-1640 plus 7% fetal bovine serum,
100U ml⁻¹ penicillin, 100μg ml⁻¹ streptomycin, and 2mM L-glutamine at 37°C in 5% CO₂. HeLa
cells were cultured in DMEM plus 10% fetal bovine serum, 100U ml⁻¹ penicillin, 100µg ml⁻¹ streptomycin, and 2mM L-glutamine at 37°C in 5% CO₂.

**Transfections and infections**

HeLa and HEp-2 epithelial cells grown in 6-well plates were transfected with 500ng of plasmid per well for 12 hours using Lipofectamine with Plus reagent (Invitrogen). Cells were then reseeded onto 12mm glass coverslips in normal growth medium to achieve 50-90% confluency after an additional 24 hours growth. Cells were then infected with approximately 2x10⁶ EPEC bacteria or *E. coli* MC1061 harboring pIntimin or pUC19 for 3 hours as described previously (Campellone et al., 2002). MBP- and MBP-Int277-coated latex beads were generated as described previously (Liu et al., 1999) and similarly added to transfected monolayers. For antibody clustering experiments, Tir-expressing cells were first treated with a 1:200 dilution of antibodies raised against the extracellular domain of EHEC Tir (anti-TirM, a gift from A. Donohue-Rolfe) or the HA-epitope (HA.11, Covance) for 30 minutes. Then a 1:5000 dilution of *S. aureus* "Pansorbin" particles (Calbiochem) was added to anti-TirM-treated cells and *S. aureus* coated with goat anti-mouse IgG (Zymed) was added to anti-HA-treated cells, each for a further 2.5 hours. Subsequent to infections or particle treatments, cells were fixed and permeabilized (Campellone et al., 2002) and processed for immunofluorescence microscopy (see below). HeLa and HEp-2 cells yielded similar results in all experiments and were used interchangeably.

**Immunofluorescence (IF) microscopy**

For triple-labeling experiments, cells were simultaneously treated with mAb HA.11 (1:1000) and biotinylated anti-phosphotyrosine mAb PY66 (1:100) (Sigma) for 30 minutes. They were then washed and treated with Alexa488-conjugated streptavidin (1:50) (Molecular Probes) for 30 minutes before addition of 1 µg ml⁻¹ TRITC-phalloidin (Sigma) to stain F-actin and Alexa350-
conjugated anti-mouse IgG (1:100) for a further 30 minutes. For double-labeling experiments, cells were treated with HA.11, washed, and treated with TRITC-phalloidin and Alexa488-conjugated anti-mouse IgG (1:200). For single-labeling experiments, cells were treated with HA.11 followed by Alexa488-conjugated anti-mouse IgG.

Cell-binding assays

Approximately 2x10⁷ E. coli MC1061 harboring either pIntimin or pUC19 were added to mock-, pTirFL-, pTirMC-, or pTirMC(Y474F)-transfected HeLa cells for 60 minutes and processed for DIC microscopy and single-label IF. 200 random mock transfected cells and 200 random cells that were clearly expressing Tir, as determined by fluorescence intensity of anti-HA-staining, were examined in each experiment. Cells containing 5 or more stably associated bacteria were scored as positive for cell binding; cells with less than 5 were scored as negative. Cell Binding Index equals the percentage of cells harboring at least 5 bound bacteria. Each experiment was performed multiple times and elicited similar results each time.

Pedestal quantitation

Tir-expressing HeLa cells were infected as described above and processed for DIC microscopy and double-label IF. To measure the percentage of infected cells generating pedestals, cells containing 5-20 bound particles were examined. Tir-expressing cells were identified by their intense anti-HA fluorescence. Cells that exhibited Tir fluorescence indistinguishable from mock-transfected cells were considered non-expressers. A cell was scored as positive if at least 50% of bound particles generated pedestals and negative if less than 50% of bound particles generated pedestals. Each experiment was performed multiple times and yielded similar results. Pedestal formation by intimin-coated beads was not quantitated due to variability in coating efficiency and the loss of Tir-binding activity of beads upon storage.
To measure the percentage of particles generating pedestals, TirMC-expressing cells containing 5-20 bound particles were examined. A particle was scored as positive if it was associated with at least one pedestal and as negative if no pedestals were associated with it. Data represent an experiment in which 50 cells were examined in triplicate for each different particle. A range of 330-635 particles was counted per 50 cells.

To measure the kinetics of pedestal formation, HeLa cells expressing TirMC and mock-transfected HeLa cells to which full-length HA-tagged Tir was delivered to the plasma membrane by an EPEC strain that translocates Tir but does not express intimin ("EPEC primed cells") were examined. Priming was performed by preinfecteding HeLa cells with $4 \times 10^7$ EPECΔtirΔcesTΔeae + pTir for 3 hours. Bacteria were then killed by treatment with 50 μg ml$^{-1}$ gentamicin for 30 minutes and removed. TirMC transfected cells were also treated with gentamicin. Approximately $2 \times 10^7$ E.coli + pIntimin were then added to TirMC-expressing or EPEC-primed monolayers for the indicated times prior to fixation and processing for double-label IF. The percentage of infected cells generating pedestals was quantitated as described above.

**Actin assembly assays**

*Xenopus* egg extracts were prepared as described previously (Ma et al., 1998), and were reconcentrated to 0.2X volume following high speed centrifugation. Rhodamine actin was prepared using purified rabbit skeletal muscle actin and tetramethylrhodamine-iodoacetamide rhodamine (Molecular Probes) and added to extracts at a final concentration of 10-20 μg/ml. Biotinylated peptides 16 residues in length (Campellone et al., 2002) were bound to streptavidin-labeled 1 μm latex beads (Sigma) in 2x HEPES buffered saline (HBS) (50mM HEPES pH 7.05, 280mM NaCl, 3.0 mM NaPO$_4$), resuspended in 1x HBS, and diluted 10 fold into egg extracts.
Images were collected 20-25 minutes after addition of beads to extracts, using a Zeiss Axiovert 135 microscope.

**Results**

*Intimin-expressing bacteria attach efficiently to mammalian cells expressing EPEC Tir.*

In order to begin an examination of the requirements for EPEC actin pedestal formation, two derivatives of Tir were ectopically expressed in cultured epithelial cells by transient transfection. First, the entire tir open reading frame was cloned into a mammalian expression vector, downstream of an HA-tag, to direct the cellular expression of a wild type full-length version of Tir (TirFL) as depicted in Fig. 1A. Anticipating that export of TirFL to the cell surface by the mammalian secretory apparatus might be inefficient, a derivative of Tir that should be better targeted to the plasma membrane was also generated by replacing the cytoplasmic N-terminal region and first transmembrane domain of Tir with the N-terminus, transmembrane sequence, and 18 extracellular residues of the Newcastle Disease Virus (NDV) HN protein. NDV-HN is normally efficiently inserted into the plasma membrane with its N-terminus in the cytoplasm. The 66-amino acid HN segment was followed by an HA-epitope tag and was predicted to direct surface expression of a fragment of Tir containing the intimin-binding extracellular loop and the C-terminal cytoplasmic domain (TirMC), as depicted in Figure 1A.

Microscopic examination of transfected cells using fluorescently labeled anti-HA antibodies indicated that TirFL was enriched in perinuclear regions, but generally present throughout the entire cytoplasm (Fig 1B). TirMC, in addition to cytoplasmic staining, appeared to also localize to the plasma membrane, as evidenced by distinct anti-HA staining at the peripheries of transfected cells (Fig. 1B). Tir-expressing and nontransfected cells were
indistinguishable after staining for phosphotyrosine (Fig. 1B), consistent with the previous observation that EPEC Tir expressed in mammalian cells is not efficiently tyrosine phosphorylated (Kenny and Warawa, 2001). The expression of Tir in mammalian cells also did not appear to promote actin assembly, because no gross alterations in the distribution of F-actin were observed in Tir-expressing cells (Fig. 1B).

To determine whether Tir expressed in mammalian cells was displayed at the cell surface in a functional form, transfected cells were challenged with a laboratory strain of E. coli expressing plasmid-encoded intimin, independent of other LEE components. Tir-expressing cells were identified by their intense staining with anti-HA antibodies, and cell binding was quantitated microscopically. Approximately 20% of mammalian cells expressing TirFL bound at least five intimin-expressing bacteria, whereas only 0.5% of mock-transfected cells demonstrated this degree of bacterial binding (Fig. 1C). More than 90% of cells expressing TirMC bound at least five intimin-expressing bacteria (Fig. 1C), with most cells binding more than 20 bacteria (data not shown). As expected, TirFL- or TirMC-expressing cells did not bind an isogenic E. coli strain that did not express intimin (Fig. 1C). Thus, both TirFL and TirMC localize to the plasma membrane and function as intimin receptors, but TirMC does so with considerably greater efficiency.

*Tir expressed in mammalian cells complements a tir-deficient EPEC strain for actin pedestal formation, and complementation does not require the N-terminal cytoplasmic domain.*

To test whether surface-localized TirFL could complement a *tir*-deficient EPEC strain for actin pedestal formation, TirFL-expressing cells were infected with EPECΔ*tir*, which expresses intimin but not Tir, and examined microscopically for localized actin assembly (high intensity F-
actin staining beneath bound bacteria). TirFL was enriched at sites of EPECΔtir adherence and actin pedestals were formed beneath bound bacteria (Fig. 2A). These pedestals also stained with anti-phosphotyrosine antibody (Fig. 2A), similar to pedestals formed on cells by wild type EPEC. Intimin was required for actin assembly, because interaction of cells with EPECΔtirΔcesTΔeae did not produce any apparent changes in the distribution of TirFL, phosphotyrosine residues, or F-actin (Fig. 2A). These results indicate that Tir does not undergo any essential modifications in the bacterial cytosol, nor does it require co-delivery with other effectors through the EPEC type III secretion apparatus to function in actin pedestal formation.

The N-terminus of Tir is necessary for translocation from EPEC (Abe et al., 1999; Crawford and Kaper, 2002; Elliott et al., 1999a) and also binds to focal adhesion proteins *in vitro* (Freeman et al., 2000; Goosney et al., 2000; Huang et al., 2002). The ability to ectopically express membrane-targeted TirMC allowed us to test whether this domain was required for actin pedestal formation. When transfected cells expressing TirMC were challenged with EPECΔtir, TirMC was enriched at sites of bacterial attachment and actin pedestals were formed (Fig. 2B), indicating that the N-terminal cytoplasmic domain of Tir is not required for actin pedestal formation.

To determine the signaling efficiency of ectopically expressed Tir, the percentage of infected cells that gave rise to pedestals was quantitated. Wild-type EPEC or EPECΔtir harboring a plasmid encoding HA-tagged Tir generated pedestals on nearly every cell after 3-hour infections, regardless of whether these cells expressed TirFL or TirMC (Fig. 2C). While cells that did not express Tir did not form pedestals when challenged with EPECΔtir, approximately 27% of TirFL-expressing cells and nearly 90% of TirMC-expressing cells generated pedestals beneath EPECΔtir (Fig. 2C). These quantities correlate with the previously
observed surface localization levels of the two Tir derivatives (Fig. 1C) and indicate that ectopically expressed Tir, even when lacking the entire N-terminal cytoplasmic domain, is capable of efficiently triggering actin pedestal formation.

*Tir expressed in mammalian cells requires an intact Nck binding site to promote actin assembly.*

Tir delivered into host cells by EPEC becomes phosphorylated on tyrosine 474 located in its C-terminus (Kenny, 1999). This phosphotyrosine is required for Tir to bind the host adaptor protein Nck (Campellone et al., 2002; Gruenheid et al., 2001), and a Tir Y474F mutant is defective for Nck binding and actin pedestal formation (Campellone et al., 2002; DeVinney et al., 2001; Goosney et al., 2000; Gruenheid et al., 2001). Phosphotyrosine staining was visualized at sites of F-actin assembly beneath EPECΔ*tir* in TirMC-expressing cells (Fig. 2B), and tyrosine phosphorylated TirMC was also detected by immunoblotting with anti-phosphotyrosine antibody (K.C., manuscript in preparation). To test whether this phosphorylation event was required for actin pedestal formation mediated by TirMC, a derivative carrying a Y474F mutation was expressed in mammalian cells. Intimin-expressing bacteria bound to cells expressing TirMC and TirMC(Y474F) equivalently (Fig. 1C), indicating that TirMC(Y474F) localizes to the cell surface. However, EPECΔ*tir* failed to effectively trigger actin assembly in cells expressing TirMC(Y474F) (Fig. 2B), strongly suggesting that tyrosine phosphorylation of Y474 and Nck-binding are also critical for pedestal formation when Tir is expressed by mammalian cells.

*Tir is the only translocated EPEC effector required for actin pedestal formation.*

To examine if any type III-secreted effector proteins other than Tir are required for actin assembly, TirMC-expressing cells were infected with a laboratory strain of *E. coli* which harbors
an intimin-expressing plasmid, but that does not possess a LEE. Transfected cells that bound these bacteria also generated pedestals efficiently (Fig. 3A). In fact, pedestal formation was quantitatively indistinguishable from pedestal formation by wild type EPEC (compare Fig. 3A to Fig. 2C). Notably, these pedestals contained phosphotyrosine residues (data not shown), indicating that Tir must be phosphorylated by endogenous host cell kinases, since Tir is the only EPEC protein present in these cells. As expected, control bacteria that did not express intimin did not trigger actin assembly (Fig. 3A).

To determine whether any bacterial component other than intimin is required for pedestal formation on transfected cells, bacterium-sized latex beads were coated with an MBP-fusion protein containing the Tir-binding domain of intimin (Liu et al., 1999) and added to TirMC-expressing cells. These beads formed pedestals on TirMC-expressing cells, while control MBP-coated beads did not (Fig. 3B), indicating that the Tir-binding domain of intimin is sufficient to trigger localized actin assembly in cells ectopically expressing Tir.

*Clustering of the C-terminus of EPEC Tir in the plasma membrane is sufficient to trigger actin pedestal formation.*

It has been suggested that binding of intimin to host cell receptors in addition to Tir may contribute to pedestal formation (reviewed in Frankel et al., 2001). Alternatively, the activity of intimin in triggering actin pedestal formation may merely reside in its ability to bind Tir. To distinguish between these two possibilities, we examined whether intimin could be replaced by antibodies that recognize TirM, the extracellular domain of Tir. Therefore, TirMC-transfected cells were treated with antibodies raised against TirM. After removal of unbound antibodies, anti-TirM bound to the surface of TirMC-expressing cells was clustered by the addition of killed
Staphylococcus aureus particles, which bind the Fc portion of antibodies via protein A on their surface. Virtually all of these cell-associated S. aureus particles triggered actin assembly into pedestals in TirMC-expressing cells (Fig. 4).

These results suggested that the sole function of the extracellular region of Tir is to promote clustering of its C-terminal cytoplasmic domain by virtue of its intimin-binding activity. To cluster this domain without any direct interaction with the intimin-binding domain, TirMC-expressing cells were treated with anti-HA monoclonal antibodies, which recognize the extracellular HA-epitope tag of TirMC. Since the isotype of the mouse anti-HA monoclonal antibody, IgG1, is not efficiently recognized by protein A, anti-HA antibody bound to cells was clustered using S. aureus coated with goat anti-mouse IgG. Efficient pedestal formation occurred beneath these adherent S. aureus particles (Fig. 4). In contrast, TirMC-expressing cells that were challenged with S. aureus coated with anti-mouse IgG alone did not form pedestals (Fig. 4).

Overall, these results demonstrate that clustering of the cytoplasmic C-terminal domain of Tir beneath the plasma membrane is sufficient to trigger actin pedestal formation and that in this experimental system the only role for intimin is to cluster Tir.

Actin pedestals derived from clustering of TirMC are generated as efficiently as pedestals formed by wild type EPEC.

As described above, the percentage of TirMC-expressing cells that formed pedestals upon challenge with particles capable of clustering Tir was not significantly affected by the ability of the particle to translocate effectors or by the particular manner in which Tir was clustered. To test whether a somewhat more rigorous method of assessing the efficiency of pedestal formation would lead to the same conclusion, the percentage of bound particles, rather than the percentage
of TirMC-expressing cells that were associated with actin pedestals, was quantitated.
Approximately 75-85% of wild type EPEC or EPECΔtir + pTir that were bound to TirMC-expressing cells generated pedestals, and EPECΔtir, non-pathogenic E. coli expressing intimin, and anti-HA-coated S. aureus each formed pedestals with a similar efficiency (Fig. 5A). Anti-TirM coated S. aureus was actually slightly more efficient at pedestal formation than wild type EPEC (Fig. 5A). These results indicate that the N-terminus of Tir and other EPEC effectors do not measurably contribute to pedestal formation. Furthermore, the C-terminus of Tir efficiently triggers actin assembly irrespective of the manner by which it becomes clustered in the plasma membrane.

*Actin pedestals derived from clustering of TirMC or from clustering of Tir translocated by EPEC are generated at similar rates and exhibit equivalent morphologies.*

While EPEC effectors other than Tir are clearly not necessary for actin assembly, EspH has been shown to facilitate the rate of pedestal formation (Tu et al, 2003). Therefore, we assessed whether EspH, or other effectors, might accelerate the kinetics of pedestal formation when Tir is already present in the membrane. Two sets of cells were challenged with E. coli expressing intimin. One set was transfected with a plasmid encoding TirMC, while the other set was preinfected ("primed") with an EPEC strain that translocates Tir and other effectors but that cannot cluster Tir because it lacks intimin. Each set of cells was fixed at various time points and examined for localized actin assembly. Pedestals were observed on EPEC-primed cells as early as five minutes after challenge, and the percentage of cells containing pedestals began to plateau after 15 minutes (Fig. 5B). The rate of pedestal formation upon challenge of TirMC-expressing cells paralleled that of EPEC-primed cells (Fig. 5B), indicating that once Tir is present in the
plasma membrane, the kinetics of pedestal formation after clustering by intimin is independent of other effectors. Furthermore, at each time point the morphology of pedestals on transfected cells matched that of pedestals on primed cells, i.e., pedestals were long at early time points and shorter and more compact at later time points (Fig. 5C). Therefore, we cannot discern a role in actin pedestal formation for any EPEC-encoded factors other than Tir.

*A clustered Nck-binding peptide from EPEC Tir is sufficient to trigger actin assembly in vitro.*

Since the C-terminus of EPEC Tir is clearly sufficient to direct localized actin assembly in host cells after clustering, and this domain of Tir contains the critical Nck-binding site (Campellone et al., 2002; Gruenheid et al., 2001), we tested whether clustering of this binding site was sufficient to trigger actin assembly. We utilized *Xenopus* egg extracts, which have been extensively employed in the study of actin-based motility (Goldberg, 2001). Latex beads were coated with: “12aa (+PO₄)”, a tyrosine phosphorylated Tir peptide that binds Nck; “12aa”, a non-phosphorylated form of the same peptide which does not bind Nck; or “7aa (+PO₄)” a tyrosine phosphorylated Tir peptide with a low affinity for Nck (Campellone et al., 2002). After these beads were added to *Xenopus* egg extracts, robust actin tails assembled on beads coated with the Nck-binding peptide, but not on beads coated with the non-phosphorylated form of the peptide (Fig. 6). Beads coated with the peptide that binds Nck poorly occasionally generated weak actin clouds (Fig. 6). These results demonstrate that the clustering of Nck by a small phosphopeptide is sufficient to trigger localized actin assembly, and suggest that the only critical function that EPEC performs to initiate actin polymerization is to recruit Nck.
Discussion

Tir is an effector that is critical for actin pedestal formation, but formulation of a complete model of how EPEC activates actin assembly in the mammalian cell has been hampered by difficulties in identifying the entire repertoire of bacterial effectors necessary for this process. In this study, ectopic expression of Tir in mammalian cells revealed that no other translocated proteins are essential for actin pedestal formation. Indeed, F-actin staining of cells expressing Tir revealed that they were fully capable of forming pedestals upon challenge with an *E. coli* K-12 strain that expresses intimin, but that does not translocate any bacterial molecules to the host cell. Actin pedestals formed on transfected cells were morphologically similar to those formed on cells after translocation of the entire repertoire of EPEC effectors, and were generated at an indistinguishable rate and efficiency. Therefore, translocated EPEC molecules other than Tir play at most an auxiliary role in stimulating actin polymerization.

A previous study indicated that Tir expressed in mammalian cells by transfection was not modified to its mature tyrosine-phosphorylated form, suggesting that other co-delivered effector(s) may be required for Tir to reach its completely modified state (Kenny and Warawa, 2001). In the current study, we also found that mammalian expression of Tir did not result in an observable increase in phosphotyrosine staining, as assessed by immunofluorescence. However, phosphotyrosine residues colocalized with Tir after clustering (Fig. 2; data not shown), suggesting that clustering induces tyrosine phosphorylation of Tir by endogenous mammalian tyrosine kinases independently of other EPEC effectors. In fact, immunoprecipitation of Tir and western blotting with anti-phosphotyrosine antibodies confirm that clustering triggers a dramatic increase in Tir tyrosine phosphorylation (K.C., manuscript in preparation). Although it remains possible that additional EPEC molecules facilitate other modifications of Tir, such as
serine/threonine phosphorylation, such modifications are apparently not required for Tir to stimulate localized actin assembly.

These results suggest that the central role for intimin, the only bacterial molecule other than Tir that we have shown participates in actin pedestal formation, is to cluster Tir in the host plasma membrane, consistent with the previously observed correlation between the ability of intimin to bind to Tir and its ability to trigger actin assembly in EPEC-primed cells (Liu et al., 2002). Here we demonstrate that intimin can be functionally replaced by entirely unrelated molecules, i.e., antibodies that artificially cluster Tir. The observation that anti-HA antibodies trigger actin assembly in transfected cells that express HA-tagged Tir indicates that Tir signaling does not even require the specific engagement of its intimin-binding domain.

Another domain of Tir, the N-terminal cytoplasmic region, binds the mammalian focal adhesion proteins α-actinin, talin, and vinculin in vitro (Freeman et al., 2000; Goosney et al., 2000; Huang et al., 2002). However, the current study reveals that these interactions are not necessary for pedestal formation since TirMC, which completely lacks this domain, is fully capable of signaling host cells to generate pedestals. It remains to be determined if focal adhesion proteins are recruited to pedestals independently of this portion of Tir.

This work demonstrates that the critical signaling ability of Tir resides entirely within its C-terminal cytoplasmic region. The best-characterized activity of this domain is its ability to recruit Nck, a host adaptor protein that is required for EPEC pedestal formation (Campellone et al., 2002; Gruenheid et al., 2001). Indeed, preliminary work indicates that two critical components of the actin assembly machinery, Nck and the downstream signaling protein N-WASP, localize to TirMC-derived pedestals in a manner similar to wild type EPEC pedestals (K.C., unpublished observations). Remarkably, the minimal Nck-binding phosphopeptide
derived from Tir is, by itself, sufficient to stimulate actin assembly in *Xenopus* egg extracts after immobilization on beads. Two related peptides that bound Nck poorly or not at all demonstrated a parallel decrease in their ability to induce actin assembly. We cannot rule out the possibility that domains of the C-terminus of Tir other than the Nck-binding sequence may also contribute to actin assembly, or that the requirements for actin assembly in *Xenopus* extracts may be different from that at the membrane of intact mammalian cells. Nevertheless, these results support an incredibly simple model for pedestal formation, i.e., that within the entire LEE pathogenicity island, a mere 12 amino acid sequence of Tir is sufficient to recruit Nck and trigger a complete signaling cascade that leads to localized actin polymerization at the plasma membrane. This model then predicts that all other components required for actin assembly in mammalian cells must be recruited subsequent to engagement of Nck by the Tir phosphopeptide.

Interestingly, the SH2 domain of Nck also binds the tyrosine phosphorylated membrane protein A36R of vaccinia virus, an interaction required for efficient actin tail formation by that pathogen (Frischknecht et al., 1999). Our results imply that Nck recruitment by A36R, which contains a Nck binding site nearly identical to that of Tir, may also be sufficient for initiating actin assembly during infection with vaccinia. After recruitment to the plasma membrane, Nck, which harbors three SH3 domains, may stimulate actin polymerization by interacting directly with the proline-rich domain of N-WASP, an activator of the Arp2/3 actin nucleating complex (Goldberg, 2001; Welch and Mullins, 2002) that is required for pedestal formation by EPEC (Lommel et al., 2001) and for tail formation by vaccinia (Snapper et al., 2001). However, the N-WASP proline-rich domain is dispensable for the generation of pedestals; rather, the WH1 (WASP-homology-1) domain appears to be critical for recruitment to sites of EPEC adherence (Lommel et al., 2001). This observation is consistent with the vaccinia signaling pathway in
which the interaction of Nck with N-WASP is mediated by a third protein, WIP (WASP interacting protein), which binds to Nck and to the WH1 domain of N-WASP (Moreau et al., 2000).

Aside from Tir and A36R, the SH2 domain of Nck binds at least 12 mammalian phosphoproteins, including many receptor tyrosine kinases such as EGFr, PDGFr, and VEGFr, as well as proteins downstream of tyrosine kinases, like Dok and IRS-3 (Buday et al., 2002). The manner in which the signaling cascade exploited by the Tir phosphopeptide resembles pathways stimulated by engagement of these receptors remains to be determined. However, our establishment of easily manipulated experimental systems, such as Tir-expressing cells and peptide-coated beads in Xenopus extracts, will greatly facilitate the further dissection of these pathways, leading to a more complete understanding of the relationship of Nck signaling to disease.
**Figure 1.** Intimin-expressing bacteria efficiently attach to mammalian cells expressing EPEC Tir.

(A.) TirFL, full-length Tir, and TirMC, a Tir derivative lacking its N-terminal cytoplasmic domain, are depicted. TirMC was directed to the plasma membrane by an N-terminal targeting sequence derived from an HN-protein (see Materials and methods). Arrows indicate the positions of HA-epitope tags and Y474.
Intimin-expressing bacteria efficiently attach to mammalian cells expressing EPEC Tir. (B.) HEp-2 cells transfected with plasmids encoding TirFL or TirMC were stained with a monoclonal antibody directed against the HA epitope to identify Tir-expressing cells, with a monoclonal antibody to visualize phosphotyrosine (Y-PO4), and with phalloidin to visualize F-actin. The inset shows several TirFL-expressing cells. (C.) Mock transfected HeLa cells, or cells expressing TirFL, TirMC, or TirMC(Y474F) (identified by high intensity anti-HA fluorescence), were challenged with laboratory strains of E. coli carrying a plasmid encoding intimin or a pUC19 vector control. Cell Binding Index, defined as the percentage of cells containing at least 5 bacteria bound, was measured microscopically, and data represent the mean of duplicate samples of 200 cells each.
Figure 2. Tir expressed in mammalian cells allows a tir-deficient EPEC strain to trigger actin pedestal formation.

(A.) HEp-2 cells transfected with a plasmid encoding TirFL were infected with either EPECΔtir (top panels) or EPECΔtirΔcesTΔeae (lower panels) and stained as described in the legend to Fig. 1. The arrow indicates positions of several bound bacteria.

(B.) HEp-2 cells transfected with a plasmid encoding either TirMC (top panels) or a Y474F mutant of TirMC (lower panels) were infected with EPECΔtir and stained as described in the legend to Fig. 1. The arrow indicates positions of several bound bacteria.
Figure 2. Tir expressed in mammalian cells allows a tir-deficient EPEC strain to trigger actin pedestal formation.

(C.) HeLa cells transfected with plasmids encoding TirFL or TirMC were infected with wild type EPEC, EPECΔtir + pTir, or EPECΔtir. Cells expressing Tir were identified by intense anti-HA fluorescence while Tir-nonexpressing cells were identified by their lack of anti-HA fluorescence. Cells with at least 5 bound bacteria were examined and the percentage of Tir-expressing cells or Tir-nonexpressing cells generating actin pedestals was quantitated microscopically. Wild type EPEC and EPECΔtir + pTir formed pedestals at identical frequencies on TirMC- and TirFL-nonexpressing cells (data not shown). Data represent the means of triplicate samples of 200 cells each.
**Figure 3.** Tir is the only translocated EPEC effector required for actin pedestal formation.

(A.) HEp-2 cells expressing TirMC were challenged with a laboratory strain of *E. coli* harboring either an intimin-expressing plasmid (top panels) or a control plasmid (lower panels). Tir and F-actin were visualized with an anti-HA antibody and phalloidin, respectively. The arrow indicates positions of bound bacteria. The percentage of TirMC-expressing cells containing actin pedestals (+/- standard deviation) was quantitated as described in the legend to Fig. 2.

(B.) HEp-2 cells expressing TirMC were challenged with latex beads coated with either the C-terminal 277 amino acids of intimin fused to MBP (top panels) or with beads coated with MBP alone (lower panels). Cells were stained as described in part A. Arrows indicate positions of bound beads.
Figure 4. Clustering of the C-terminus of EPEC Tir in the plasma membrane is sufficient to trigger actin pedestal formation.

HeLa cells expressing TirMC were treated with antibodies to either TirM, the extracellular domain of Tir (top panels), to the HA epitope (middle panels), or left untreated (lower panels). They were then challenged with *S. aureus* particles which carry protein A on their surface (top panels) or anti-mouse IgG-coated *S. aureus* (middle and lower panels). TirMC and F-actin were visualized as described in the legend to Fig. 3. *S. aureus* particles are visible when staining for TirMC, since they bind the fluorescently labeled secondary antibody. The inset shows a magnification of several pedestals. The percentage of TirMC-expressing cells containing particles generating pedestals was quantitated as described in the legend to Fig. 2.
Figure 5. Actin pedestals resulting from clustering of TirMC are quantitatively, kinetically, and morphologically similar to pedestals derived from EPEC infections.

(A.) TirMC-expressing HeLa cells were challenged with particles of the indicated type, and the percentage of bound particles generating actin pedestals was quantitated microscopically. Data represent the means +/- standard deviations of triplicate samples.

(B.) HeLa cells were either transfected with a plasmid encoding TirMC or were primed with an EPEC strain that delivers full length Tir and other EspS to host cells, but does not generate pedestals because it does not express intimin. Cells were subsequently challenged with an E. coli strain expressing intimin, fixed at the indicated time points, and quantitated for pedestal formation as described in the legend to Fig. 2. Each data point represents the mean of two independent experiments in which 200 cells were examined.
Figure 5. Actin pedestals resulting from clustering of TirMC are quantitatively, kinetically, and morphologically similar to pedestals derived from EPEC infections.

(C.) The transfected or EPEC-primed HeLa cells described above were examined microscopically at 10 or 60 minutes post challenge. Tir and F-actin were visualized with an anti-HA antibody and phalloidin, respectively. Arrows indicate examples of Tir localizations at the tips of pedestals.
Figure 6. A clustered Nck-binding peptide from EPEC Tir is sufficient to trigger actin assembly in vitro.
Latex beads were coated with "12aa(PO₄)", a peptide that binds Nck, "12aa", a non-phosphorylated peptide that lacks Nck-binding activity, or "7aa(PO₄)", a peptide that weakly binds Nck (Campellone et al., 2002). Peptide-coated beads were added to Xenopus egg extracts supplemented with rhodamine-actin and examined microscopically.
CHAPTER IV

CLUSTERING OF EPEC TIR TRIGGERS TWO PHOSPHOTYROSINE-DEPENDENT PATHWAYS OF ACTIN ASSEMBLY

Abstract

Enteropathogenic Escherichia coli (EPEC) triggers the formation of filamentous actin pedestals in epithelial cells beneath extracellularly bound bacteria. To stimulate localized actin assembly into pedestals, EPEC translocates the signaling protein Tir (translocated intimin receptor) into the host plasma membrane where it serves as a receptor for the bacterial surface adhesin intimin. The essential signaling functions of Tir reside within its tyrosine phosphorylated C-terminal cytoplasmic region, and clustering of this domain is sufficient to trigger pedestal formation. A Tir peptide encompassing phosphotyrosine 474 binds to Nck, a host protein required for EPEC to stimulate actin polymerization, and the interaction between clustered Tir and Nck is sufficient to trigger localized actin assembly in Xenopus egg extracts. To determine the role that clustering plays in triggering actin assembly, we examined the tyrosine phosphorylation state of Tir present in the plasma membrane of epithelial cells. We found that clustering stimulates the tyrosine phosphorylation of Tir Y474 and thereby triggers efficient Nck binding. In addition to Y474, Y454 of Tir is also phosphorylated. This tyrosine does not contribute to Nck binding, and is required for a second pathway of actin assembly in host cells.
Introduction

Enteropathogenic *Escherichia coli* (EPEC), a major cause of infantile diarrhea in developing countries, induces the formation of attaching and effacing (AE) lesions on the intestinal epithelium (Campellone and Leong, 2003; Celli et al., 2000; Frankel et al., 1998). AE lesions feature the loss of microvilli, intimate adherence of bacteria to host cells, and the generation of filamentous-actin (F-actin) rich pedestal structures beneath attached bacteria. The formation of actin pedestals by EPEC, as well as by other attaching and effacing organisms, such as the human pathogen enterohemorrhagic *E. coli* (EHEC), rabbit diarrheagenic *E. coli* (RDEC), and the mouse pathogen *Citrobacter rodentium*, is recapitulated on cultured mammalian cells. In addition to a role in pathogenesis, pedestal formation by EPEC serves as a model system for understanding mechanisms by which mammalian cells control actin assembly at the plasma membrane.

All of the gene products required for EPEC to form actin pedestals are contained on a chromosomal pathogenicity island called the locus of enterocyte effacement (LEE), which encodes a type III secretion apparatus for translocation of bacterial effector proteins into host cells (Elliott et al., 1998; McDaniel and Kaper, 1997). Tir, the translocated intimin receptor, is the effector that is critical for triggering actin pedestal formation (Deibel et al., 1998; Kenny et al., 1997b). Upon entry into host cells, Tir integrates into the plasma membrane in a hairpin-loop conformation that features a central extracellular domain that binds to the LEE-encoded surface adhesin intimin (Batchelor et al., 2000; Luo et al., 2000). Both Tir and intimin are required for pedestal formation by EPEC, and the role of intimin in this process is to cluster Tir in the plasma membrane, since intimin can be functionally replaced by antibodies that recognize the extracellular domain of Tir (Campellone et al., submitted; P. Radhakrishnan, in preparation).
The N-and C-terminal domains of translocated Tir reside in the mammalian cell cytoplasm and are available to interact with host cell components. The cytoplasmic C-terminus of Tir is tyrosine phosphorylated after entry into the host cell and is critical for actin assembly (Kenny, 1999). Clustering of this Tir domain beneath the plasma membrane is both necessary and sufficient to trigger pedestal formation (Campellone et al., submitted). Notably, Tir ectopically expressed in mammalian cells by transient transfection localizes to the plasma membrane, but these cells do not contain detectable increases in levels of tyrosine phosphorylated proteins, suggesting that Tir is not efficiently tyrosine phosphorylated (Kenny and Warawa, 2001; Campellone et al., submitted). Consistent with an absence of tyrosine phosphorylation, Tir expressed in mammalian cells does not appear to reorganize the actin cytoskeleton (Campellone et al., submitted). However, when Tir-expressing cells interact with bacterium-sized particles that cluster Tir, actin pedestals are formed and these pedestals accumulate phosphotyrosine residues (Campellone et al., submitted).

Phosphorylation of tyrosine residue 474 in the C-terminus of Tir is critical for stimulating actin assembly in host cells (Goosney et al., 2000; Kenny, 1999). Phosphorylated Y474 and flanking residues bind the host adaptor protein Nck (Campellone et al., 2002; Gruenheid et al., 2001), a key activator of the N-WASP-Arp2/3 pathway of actin assembly in mammalian cells (Rohatgi et al., 2001; Welch and Mullins, 2002). Tir is incapable of activating N-WASP in the absence of Nck (Gruenheid et al., 2001), indicating that Nck is necessary for EPEC pedestal formation. Furthermore, the binding of Nck by Tir is also sufficient to trigger localized actin assembly in vitro, since latex beads coated with a tyrosine phosphorylated Tir peptide that binds Nck form actin tails in Xenopus egg extracts (Campellone et al., submitted). While the recruitment of Nck appears to be the primary function of Tir in triggering actin polymerization, it
is unknown if Nck binding is the only function that the C-terminus of Tir performs to stimulate actin assembly at the plasma membrane of intact mammalian cells.

Since both clustering and tyrosine phosphorylation of the C-terminus of Tir are prerequisites for EPEC-mediated actin assembly, understanding the relationship between these two events is critical for establishing a detailed model for the mechanism of actin pedestal formation. We have delivered Tir to the plasma membranes of cultured mammalian cells by the EPEC type III translocation system and by transient transfection, and examined the tyrosine phosphorylation state of Tir before and after treatment with particles capable of clustering Tir. Our results demonstrate that both the tyrosine phosphorylation of Tir and the ability of Tir to bind to Nck is greatly enhanced after clustering. In addition, we have identified a second phosphotyrosine of Tir, Y454, that contributes to pedestal formation by EPEC by stimulating a second pathway of actin assembly without directly binding Nck.

**Experimental procedures**

*Bacterial strains and plasmids*

All EPEC strains used in this study were derived from the O127:H6 wild type strain E2348/69 (JPN15/pMAR7) and have been described previously (Campellone et al., 2002; Jerse, 1990; Campellone et al., submitted). Plasmids used for transfection of mammalian cells and for expression of intimin in *E. coli* have also been described previously (Campellone et al., submitted).

*Bacterial and mammalian cell culture*

For routine passage, all *E. coli* strains were cultured in LB media at 37°C. Prior to infections, EPEC was cultured in DMEM + 100mM HEPES pH 7.4 in 5% CO₂, growth conditions known
to enhance type III secretion. HeLa and COS1 cells were cultured in DMEM plus 10% fetal bovine serum, 100U ml\(^{-1}\) penicillin, 100\(\mu\)g ml\(^{-1}\) streptomycin, and 2mM L-glutamine at 37°C in 5% CO\(_2\). Mammalian cell transfections were performed as described previously (Campellone, submitted).

_Infections_

Ninety percent confluent monolayers of HeLa or COS1 cells grown in 6-well plates were infected with approximately 5 x 10\(^7\) EPEC bacteria for 3.5 hours (Campellone et al., 2002). Bacteria were then killed by treatment with 50\(\mu\)g ml\(^{-1}\) gentamicin for 30 minutes. In some experiments, gentamicin treatment was followed by addition of 10\(^7\) _E. coli_ (MC1061, a K-12 laboratory strain) harboring either an intimin-expressing plasmid or a control plasmid, for 2 additional hours. Alternatively, monolayers were treated with anti-TirM antibodies (diluted 1:200) (Campellone et al., submitted) for 30 minutes followed by addition of approximately 10\(^7\) fixed _S. aureus_ particles (Pansorbin; Calbiochem) for 1.5h. Following challenge with _E. coli_ or _S. aureus_, cells were again treated with gentamicin.

_Preparation of whole-cell lysates_

HeLa and COS1 cell monolayers, which were infected as described above, were collected from 6-well plates after the addition of PBS + 2mM EDTA. Cells were centrifuged, washed once with PBS, and lysed in 30 \(\mu\)l of lysis buffer (50 mM HEPES, pH 7.4, 50 mM NaCl, 1% Triton X-100, 1 mM Na\(_3\)VO\(_4\), 1 mM phenylmethylsulphonyl fluoride (PMSF) and 10 \(\mu\)g ml\(^{-1}\) each of aprotinin, leupeptin and pepstatin (Sigma)). Loading buffer was added to the samples and they were resolved by SDS-PAGE as described below. To prepare EPEC lysates, bacteria cultured in DMEM + 100mM HEPES were centrifuged, washed once with PBS, and resuspended in SDS-PAGE loading buffer.
**Immunoprecipitation**

Cell monolayers, which were infected as described above, were treated with 0.4 mL lysis buffer and collected from 6-well plates with a cell scraper. Lysates were added to microfuge tubes on ice and homogenized with 26\(\times\)8 syringes. Cellular debris was pelleted by centrifugation for 5 minutes at 10000G at 4°C. Supernatents were then added to 1.5 μL of anti-HA mAb HA.11 (Covance) and incubated at 4°C for 2 hours with mixing. Protein G-agarose beads (Sigma), previously washed with lysis buffer, were then added to this mixture and the suspensions were incubated for an additional 2 hours at 4°C with mixing. Beads and bound protein were centrifuged and washed twice with 0.5X lysis buffer. Beads were then resuspended in SDS-PAGE loading buffer and immunoprecipitates were resolved as described below.

**Western blotting**

Protein samples prepared as described above were boiled, separated by 10% SDS-PAGE, and transferred to PVDF membranes. For whole cell lysates, membranes were blocked for 45 minutes in PBS + 5% milk (PBSM) before treatment with HA.11 (1:1000 in PBSM), biotinylated anti-phosphotyrosine mAb PT-66 (1:300) (Sigma), goat anti-Arp3 (1:250) (Santa Cruz), or rabbit anti-TirM (1:1000) (Campellone et al., submitted) for 2.5 hours. For immunoprecipitates, membranes were probed with biotinylated mAb HA.11 (1:1000), and biotinylated mAb PT-66. Following washes, membranes were treated with alkaline phosphatase conjugates of anti-mouse, anti-biotin, anti-goat, or anti-rabbit antibodies, and developed as described previously (Campellone et al., 2002). Treatment with two other anti-phosphotyrosine antibodies, mAb PY99 (1:250) (Santa Cruz) and mAb P-Tyr-100 (1:250) (Cell Signaling Technology), resulted in similar staining patterns as PT-66, but PT-66 was the most sensitive
among the three antibodies (K.C., unpublished). Each experiment was performed at least three times and yielded similar results each time.

Far-western analysis

Purified GST and GST-Nck (1μg per lane) were separated by 10% SDS-PAGE and blotted to PVDF membranes. HeLa cells were either infected with EPEC strains that co-express intimin and Tir, or were preinfected with EPEC and challenged with *E. coli* or anti-TirM + *S. aureus* as described above. Membranes were then probed with these HeLa cell lysates and bound Tir was detected with an anti-HA antibody as described previously (Campellone et al., 2002).

Experiments were performed at least three times and yielded similar results each time.

Immunofluorescence microscopy

HeLa cells grown on 12mm glass coverslips to 70-90% confluency were infected with approximately 5x10⁵ EPEC bacteria for 3.5 hours as described previously (Campellone et al., 2002). Cells were fixed and permeabilized, treated with mAb P-Tyr-100 (1:100) for 30 minutes to stain phosphotyrosine, washed, and treated with TRITC-phalloidin (Sigma) to visualize F-actin and Alexa488-conjugated anti-mouse IgG (1:200) (Molecular Probes) for 30 minutes. To determine the efficiency of EPEC pedestal formation, cells containing 5-20 bound bacteria were scored for pedestal formation (Campellone et al., submitted). Triplicate samples of 50 cells each were examined for each different strain. More than 350 bound bacteria were counted per 50 cells.

Peptide binding assays

*E. coli* lysates containing GST, GST-Nck and GST-Grb2 were resolved by 10% SDS-PAGE and blotted to PVDF membranes. Membranes were probed with tyrosine phosphorylated peptides “Tir-Y474-PO₄” (Biotin-QVPEEHIpYDEVAADPG), “Tir-Y454-PO₄” (Biotin-
SSEVVNPpYAEVGGARN), or “A36R-Y132-PO₄” (Biotin-GGEQTIpYQNTT) as described previously (Campellone et al., 2002). Experiments were performed at least three times and yielded similar results each time.

Results

*Intimin is required for efficient tyrosine phosphorylation of EPEC Tir.*

Clustering of EPEC Tir in the plasma membrane and tyrosine phosphorylation of Tir are two crucial events that lead to actin pedestal formation (Campellone et al., submitted; Kenny, 1999). To begin an analysis of the relationship between these two events, we examined the tyrosine phosphorylation state of Tir translocated into host cells from JPN15/pMAR7, a wild type EPEC strain that co-express Tir and intimin, and from JPN15.96/pMAR7, a strain that has a transposon insertion in *eae*, the gene that encodes intimin (Jerse, 1990). Cultured mammalian epithelial (HeLa) cells were infected with each of these EPEC strains, non-intimately adherent bacteria were killed with gentamicin, and the remaining HeLa cells were collected, lysed, separated by SDS-PAGE, and analyzed by western blot. Translocated Tir, which is modified by serine/threonine phosphorylation and migrates at nearly 90kDa in SDS-PAGE gels, can be easily differentiated from non-translocated (bacterial) Tir, which migrates at less than 80kDa (Kenny, 1999; Kenny et al., 1997b). Staining with antibodies to Tir demonstrated that wild type EPEC and JPN15.96 translocated Tir into mammalian cells equivalently, as evidenced by the presence of the 90kDa form of Tir (Fig. 1A, lanes 5-6). Cells that were infected with wild type EPEC contained a robust phosphotyrosine band migrating at approximately 90kDa (Fig. 1A, lane 6), consistent with tyrosine phosphorylation of Tir. In contrast, Tir appeared to be tyrosine
phosphorylated to a significantly lesser extent after translocation into host cells by the strain that did not express intimin (Fig. 1A, lane 5).

To confirm that the reduction in tyrosine phosphorylation after delivery by JPN15.96 was not due to polar effects resulting from the transposon insertion in eae, a separate pair of strains was examined in these assays. HeLa cells were infected with EPECΔtir + pTir, an EPEC strain that lacks a chromosomal copy of Tir but harbors a low-copy number plasmid encoding an N-terminally HA-tagged Tir (plus the Tir chaperone, CesT), and with EPECΔtirΔcesTΔeae + pTir, a strain which contains a complete deletion of tir, cesT, and eae and harbors plasmid-encoded HA-Tir and CesT [hereafter referred to as EPECΔtirΔeae + pTir]. Western blots of HeLa cell lysates from these infections yielded similar results, i.e., phosphotyrosine staining was much greater after Tir translocation into host cells by a strain that also expressed intimin (Fig. 1A, lanes 3-4).

To test whether a similar pattern of tyrosine phosphorylation could be observed in a non-epithelial cell type, experiments with these strains were repeated in COS1 fibroblasts. EPEC translocated Tir into COS cells, as evidenced by a change in Tir mobility from 78kDa to nearly 90kDa (Fig. 1B, compare lanes 2-3, 6-7). As in experiments with HeLa cells, the presence of a 90kDa tyrosine phosphorylated protein was much greater when Tir was delivered to host cells by an EPEC strain that co-expressed intimin (Fig. 1B, lanes 6-7).

To confirm that the 90kDa phosphoprotein was indeed Tir, we took advantage of the presence of the N-terminal HA-tag to immunoprecipitate Tir from cell lysates and examine it by western blot. While Tir was robustly tyrosine-phosphorylated when translocated into host cells during infection with EPEC that expresses both intimin and Tir (Fig. 1C, lane 1), Tir was poorly tyrosine phosphorylated when translocated by a strain that lacks intimin (lane 3). It should be
noted that levels of tyrosine phosphorylated Tir after immunoprecipitation procedures were usually somewhat reduced compared to Tir processed for immunoblotting immediately after cell lysis. Nevertheless, these results indicate that intimin is required for efficient tyrosine phosphorylation of Tir.

*Clustering in the plasma membrane triggers efficient phosphorylation of Tir.*

Tir that is delivered into host cells by strains that lack intimin is capable of triggering actin pedestal formation following interactions with intimin-expressing bacteria (Liu et al., 1999; Rosenshine et al., 1996). Intimin may initiate this actin assembly in host cells merely by creating a high local concentration of previously phosphorylated Tir molecules beneath bound bacteria or, alternatively, intimin-binding may stimulate the tyrosine phosphorylation of Tir. To differentiate between these two possibilities, HeLa cells were preinfected with EPECΔtirΔaeae + pTir to deliver Tir into the plasma membrane and then challenged with an *E. coli* strain harboring an intimin-expressing plasmid (Campellone et al., submitted). Immunoprecipitation of HA-tagged Tir and immunoblotting with an anti-phosphotyrosine antibody indicated that Tir was robustly phosphorylated after challenge with intimin-expressing bacteria but not following treatment with an isogenic strain that harbors a vector control (Fig. 2, lanes 1-2). This level of Tir tyrosine phosphorylation was similar to phospho-Tir levels in cells infected with an EPEC strain that simultaneously co-expresses Tir and intimin (compare Fig. 1C-lane 1, Fig. 2-lane 2).

The role of intimin in triggering actin assembly is to cluster Tir, since intimin-expressing bacteria can be replaced functionally by particles coated with antibodies to TirM, the extracellular domain of Tir (Campellone et al., submitted). To determine whether phosphorylation of Tir in EPEC-preinfected HeLa cells was similarly enhanced after this
artificial method of clustering, these cells were challenged either with anti-TirM antibodies, with
*S. aureus* particles, or with anti-TirM antibodies followed by *S. aureus* particles which bind to
and cluster antibodies due to the presence of Protein A on their surface. Treatment of EPEC-
preinfected cells with *S. aureus* did not alter the tyrosine phosphorylation state of Tir (data not
shown). However, treatment with anti-TirM antibodies resulted in a slight increase in the
tyrosine phosphorylation of Tir (Fig. 2, compare lanes 1, 3). In contrast, treatment with anti-
TirM and *S. aureus* particles yielded a great increase in Tir phosphorylation (Fig. 2, lane 4).
These results demonstrate that clustering is sufficient to trigger the efficient tyrosine
phosphorylation of EPEC Tir.

*Tyrosine phosphorylation of the C-terminal cytoplasmic domain of Tir occurs independently of
other translocated EPEC effectors.*

An HA-tagged derivative of Tir that lacks its N-terminal cytoplasmic domain, TirMC, has been
efficiently expressed at the surface of mammalian cells, and clustering of this version of Tir with
anti-TirM-coated *S. aureus* particles triggers the formation of actin pedestals which contain
phosphotyrosine residues (Campellone et al., submitted). To test whether the phosphorylation
state of Tir ectopically expressed in mammalian cells was also enhanced by clustering, TirMC-
expressing cells were challenged with anti-TirM and *S. aureus*. As a control, TirMC-expressing
cells were also challenged with EPECΔ*tirΔeae*, an EPEC strain that lacks Tir and intimin but
translocates other effectors into host cells. Immunoprecipitation of TirMC and immunoblotting
indicated that TirMC was poorly tyrosine phosphorylated after cells were treated with
EPECΔ*tirΔeae* (Fig. 3, lane 2). This level of phosphorylation was indistinguishable from
uninfected TirMC-expressing cells (data not shown). In contrast, tyrosine phosphorylation of
TirMC was greatly enhanced after treatment with anti-TirM and S. aureus (Fig. 3, lane 3).

Overall, these results demonstrate that tyrosine phosphorylation of the C-terminal cytoplasmic domain of EPEC Tir occurs independently of other translocated EPEC effectors and is not related to the manner in which Tir is delivered to the plasma membrane.

_CLUSTERING OF TIR IN THE PLASMA MEMBRANE PROMOTES PHOSPHORYLATION OF Y474 AND EFFICIENT NCK BINDING._

Tir is phosphorylated on tyrosine 474 located in its C-terminal cytoplasmic domain, an event critical for EPEC-mediated actin assembly (Kenny, 1999). To confirm that Y474 was the residue being phosphorylated after Tir clustering, we generated a Tir derivative containing a Y474F mutation, expressed it in EPECΔtirΔeae, and preinfected HeLa cells with this strain. Treatment of these cells with intimin-expressing bacteria resulted in only a small increase in Tir tyrosine phosphorylation (Fig. 2, lanes 5-6). This amount of phosphorylation was substantially lower than phosphorylation of wild type Tir (compare lanes 2 and 6), and demonstrates that Y474 is the residue that is predominantly phosphorylated following clustering.

Tyrosine 474 and flanking residues of Tir bind to the host adaptor protein Nck in a phosphotyrosine-dependent manner (Campellone et al., 2002; Gruenheid et al., 2001). Since Y474 is efficiently phosphorylated only after clustering, we predicted that Tir, following clustering, would similarly interact efficiently with Nck. To test this, we performed far western blots in which membranes containing purified GST-Nck were probed with lysates of HeLa cells that were preinfected with EPECΔtirΔeae + pTir and challenged with particles either capable or incapable of clustering Tir. Bound Tir was then detected using an anti-HA antibody. As predicted, Tir clustered by either intimin-expressing E. coli or anti-TirM and S. aureus bound...
Nck as efficiently as Tir clustered by a strain that co-expressed intimin (Fig. 4, compare lanes 7 and 9 to lane 1). Furthermore, Tir which was not clustered in the membrane of HeLa cells bound Nck inefficiently (Fig. 4, lane 5), consistent with the previously observed low level of Tir tyrosine phosphorylation in the absence of clustering (Fig. 1). These results clearly demonstrate that Tir is able to efficiently bind Nck only after it has been clustered in the plasma membrane. As expected, Y474 was required for Nck binding, since a clustered Y474F mutant of Tir did not interact with Nck (Fig. 4, lanes 3, 11).

Tyrosine 454 is a second phosphorylated tyrosine within EPEC Tir.

It has been previously demonstrated that clustering of the C-terminal cytoplasmic domain of EPEC Tir is sufficient to trigger the formation of actin pedestals that are quantitatively and morphologically indistinguishable from pedestals generated by wild type EPEC (Campellone et al., submitted). It has also been reported that merely clustering the Nck binding site of Tir on beads triggers actin tail formation in Xenopus egg extracts (Campellone et al., submitted). However, it has not been determined if other domains of Tir, aside from the Nck binding site, contribute to actin assembly at the plasma membrane of intact mammalian cells. When analyzing the phosphorylation state of Tir after clustering, we noticed that EPEC Tir was still tyrosine phosphorylated, albeit inefficiently, in the absence of Y474 (Fig. 1C-lane 2, Fig. 2, lane 6). These results suggested that a second phosphotyrosine residue is present within Tir.

The vaccinia virus A36R membrane protein contains a tyrosine-phosphorylated Nck-binding site nearly identical to that of Tir, and the tyrosine within this sequence, Y112, is critical for the efficient formation of actin tails by vaccinia (Frischknecht et al., 1999). However, A36R contains an additional phosphotyrosine residue, twenty amino acids downstream of Y112, that
also contributes to actin tail formation (Scaplehorn et al., 2002). A survey of the C-terminal cytoplasmic domain of EPEC Tir indicates that it contains three tyrosines in addition to Y474: Y454, Y483, and Y511 (Fig. 5A, data not shown). Among these tyrosines, Y454 lies 20 residues away from the Nck-binding tyrosine of Tir. Notably, Y454 is conserved in all published EPEC, RDEC, and Citrobacter strains, pathogens which also possess consensus Nck-binding sites (Fig. 5A, data not shown).

To test whether Y454 of Tir was tyrosine phosphorylated, a Y454F mutant and a Y454F/Y474F double mutant were generated and expressed in EPEC. After infection of HeLa cells, Tir-Y454F exhibited only a slight reduction in tyrosine phosphorylation compared to wild type Tir (Fig. 5B, lanes 2-3). In comparison, tyrosine phosphorylation of Tir-Y474F was drastically reduced, although not completely abolished (Fig. 5B, lane 4). This remaining phosphotyrosine signal was due to the presence of Y454, since no tyrosine phosphorylation was detected in the Tir Y454F/Y474F double mutant (Fig. 5B, lane 5). These results suggest that Tir contains two phosphorylated tyrosines, Y454 and Y474, and demonstrate that Y474 is the more efficiently phosphorylated residue.

**Tyrosine 454 contributes to actin pedestal formation by EPEC.**

To test whether Tir Y454 contributes to actin pedestal formation by EPEC, HeLa cells were infected with strains expressing wild type Tir or Tir derivatives with point mutations in Y454 and/or Y474, and the percentage of bound bacteria generating actin pedestals was quantitated. Approximately 85% of EPEC expressing wild type Tir formed pedestals and, as observed previously, a Y474F mutation resulted in a dramatic reduction in the efficiency of pedestal formation (Fig. 6; Campellone et al., 2002; DeVinney et al., 2001; Goosney et al., 2000;
Gruenheid et al., 2001). Interestingly, pedestal formation was not completely abolished, since approximately 3% of bound bacteria still generated pedestals (Fig. 6). Regions beneath bacteria expressing Tir-Y474F rarely contained phosphotyrosine residues, but when phosphotyrosine staining was clearly evident beneath a bound bacterium, a pedestal was usually formed (Fig. 6). Tyrosine 454 is required for EPEC to form these pedestals since a Tir-Y454F/Y474F double mutant did not generate any pedestals, similar to an EPEC strain that expresses no Tir at all (Fig. 6). Mutation of tyrosine 454 to phenylalanine by itself had no effect on the efficiency of pedestal formation (Fig. 6), indicating that when Tir possesses a functional Nck binding site, the efficiency of pedestal formation is maximal.

A peptide encompassing phosphotyrosine 454 does not bind Nck or Grb2.

Two SH2-domain-containing adaptor proteins that localize to actin pedestals are Nck, whose role in pedestal formation is well-defined (Campellone et al., 2002; Gruenheid et al., 2001; Campellone et al., submitted) and Grb2, whose function during pedestal formation has not been investigated. While Nck is the best-characterized adaptor protein involved in activating N-WASP-mediated actin assembly (Campellone et al., 2002; Frischknecht et al., 1999; Gruenheid et al., 2001; Rohatgi et al., 2001; Campellone et al., submitted), Grb2 has also been shown to stimulate actin assembly, albeit at a lower efficiency (Rohatgi et al., 2001). In addition, Grb2 is recruited by phosphotyrosine 132 of vaccinia virus A36R to initiate a low level of actin tail formation in infected cells (Scaplehorn et al., 2002). Therefore, we generated a synthetic peptide containing phosphorylated Y454 and tested whether it could bind to Nck, or to Grb2, in peptide overlay assays. While a control peptide encompassing phosphorylated Y474 of Tir bound GST-Nck very efficiently and a peptide containing phosphorylated Y132 of A36R bound to GST-
Grb2, the phosphorylated Y454 peptide of Tir did not bind either molecule in these assays (data not shown). Thus, Y454 apparently does not directly utilize Nck or Grb2 to generate the pedestals observed in the Tir-Y474F mutant. These results imply that Y454 and surrounding residues recruit a different SH2-domain-containing molecule that is capable of stimulating actin assembly in mammalian cells.

**Discussion**

The observation that EPEC Tir ectopically expressed in mammalian cells was not detectable as a tyrosine phosphorylated protein in the absence of clustering (Campellone et al., submitted; Kenny and Warawa, 2001) led us to investigate the relationship between Tir oligomerization and tyrosine phosphorylation. In this study we have determined that efficient phosphorylation of Tir tyrosine 474 is triggered in response to clustering and occurs independently of all other bacterial effectors. Moreover, since Tir clustering essentially serves to generate a binding site for the adaptor protein Nck, we suggest that EPEC possesses the ability to regulate actin assembly not only by controlling the delivery of Tir into host cells, but by modulating the tyrosine phosphorylation state of Tir by altering quantity of intimin displayed on its surface.

An earlier study indicates that Tir translocated into mammalian cells by strains that lack intimin is phosphorylated as efficiently as Tir delivered by a strain that expresses intimin (Rosenshine et al., 1992). Other than differences in experimental procedures, we do not have a clear explanation for the apparent inconsistency between that report and this study. We examined the phosphorylation state of Tir delivered from two strains that lack intimin. One strain was generated in another laboratory and encodes Tir on its chromosome (Jerse, 1990), while a second strain was constructed in our laboratory and expresses Tir from a low copy
number plasmid (Campellone et al., submitted). In each case, tyrosine phosphorylation of Tir translocated by these strains was at least 10-fold lower than Tir delivered by strains that co-expressed intimin. These results are consistent with two other studies in which Tir was not tyrosine phosphorylated when introduced into host cells independently of intimin, either by transient transfection (Campellone et al., submitted; Kenny and Warawa, 2001) or by delivery through the Yersinia type III secretion apparatus (Kenny and Warawa, 2001). In addition, the regulation of Tir phosphorylation that we observe clearly resembles the oligomerization-dependent mechanism of tyrosine kinase activation that typically occurs within mammalian cells (Hubbard and Till, 2000; Schlessinger, 2000).

The tyrosine kinases that phosphorylate Tir have not yet been identified, but sequence upstream of Y474 (EEHIY_{474}) is homologous to a consensus substrate for Src-family kinases (EEEIY) (Songyang et al., 1995). Moreover, the Helicobacter pylori CagA effector protein contains several EEPIY repeats that are tyrosine phosphorylated by Src-family members (Selbach et al., 2002; Stein et al., 2002), and the Nck binding tyrosine within the vaccinia virus A36R protein (TEHIY_{112}) is also phosphorylated by Src-family kinases (Frischknecht et al., 1999). Therefore, it seems reasonable to propose that EPEC Tir similarly recruits Src kinases to phosphorylate Y474 with its Nck binding site.

It is becoming increasingly clear that the signaling cascades that EPEC Tir activates to form actin pedestals are remarkably similar to pathways that vaccinia virus A36R stimulates to promote actin tail formations. Interestingly, in this report we have determined that Tir, like A36R, harbors a second phosphotyrosine located twenty amino acids away from its Nck binding tyrosine. The presence of this residue, Y454, is required for a second pathway of pedestal formation that occurs in the absence of Y474 and direct Nck binding. This observation closely
resembles work in the vaccinia system, where a Y112F mutation in the Nck binding site of A36R yields a dramatic but incomplete reduction of actin tail formation within host cells and a Y112F/Y132F double mutation is required to totally abolish actin assembly (Frischknecht et al., 1999).

Despite the obvious similarities in the phenotypes that mutations of these additional phosphotyrosines exhibit, they are different in several important ways. First, while Y112 and Y132 of A36R are phosphorylated at equivalent efficiencies (Frischknecht et al., 1999), Y474 within Tir is much more efficiently phosphorylated than Y454. Second, residues upstream of Tir-Y454 and A36R-Y132 do not contain significant identity (VQNPYY_{454} versus EQTIY_{132}), suggesting that they may be substrates for different kinases. Third, while Y132 of A36R binds the adaptor protein Grb2 to initiate actin assembly (Scaplehorn et al., 2002), Y454 of Tir does not appear to bind Grb2.

These results imply that Y454 binds some other SH2-domain containing protein that functions to stimulate actin polymerization within host cells. While consensus binding sites for many SH2-domain-containing proteins have been identified (Songyang et al., 1993), none of these clearly resemble residues neighboring Y454. Notable, however is the fact that sequence downstream of EPEC Tir Y454 (Y_{454}AEVG) is somewhat homologous to residues C-terminal to a phosphorylated tyrosine in the Helicobacter pylori CagA phosphoprotein (YAQVA) (Stein et al., 2002), a protein involved in cytoskeletal rearrangements by that pathogen (Censini et al., 2001). No cellular function has yet been attributed this particular tyrosine of CagA, and it is tempting to speculate that these phosphotyrosines within Tir and CagA activate the same signaling cascades after entry into host cells.
We have shown that Y454 does not bind the Nck adaptor directly, but signaling downstream of Y454 may still require Nck for activation of actin assembly, as suggested by the observation that cells lacking Nck are resistant to pedestal formation by EPEC (Gruenheid et al., 2001). Furthermore, we do not yet know if a single Tir molecule can exist in a doubly phosphorylated form, or whether two distinct populations of phosphorylated Tir molecules exist within host cells. Thus, the signaling pathway downstream of phosphotyrosine Y454 and its relationship to Nck remain to be determined.

While the EPEC Y454 pathway to actin pedestal formation is inefficient in vitro, it may be quite effective in vivo. Our laboratory has observed that EHEC, an AE pathogen similar to EPEC, expresses roughly 5-fold higher levels of intimin and Tir when cultivated within the intestines of gnotobiotic piglets than when grown in vitro (P. Radhakrishnan, in preparation). In addition, in vitro grown E. coli engineered to overexpress intimin generate pedestals on transfected cells overexpressing Tir-Y474F with significantly greater efficiency than EPEC strains that express normal levels of intimin and Tir-Y474F (K.C., unpublished). Interestingly, Deng and coworkers have recently reported that a Citrobacter rodentium strain expressing a Tir derivative with a Y471F mutation in its Nck binding site (equivalent to Y474F in EPEC Tir) fails to form pedestals on cultured cells, but still forms AE lesions and causes disease in vivo (Deng et al., 2003); this contradicts all previous reports indicating that the ability of AE organisms to form pedestals on cultured cells correlates with their ability to cause disease in mammalian hosts (Donnenberg et al., 1993a; Marches et al., 2000; Schauer and Falkow, 1993; Tzipori et al., 1995). However, actin pedestals generated by Citrobacter are very difficult to detect in cell culture systems (Deng et al., 2003). Our results with EPEC in an optimized in vitro system suggest that the ability of this Citrobacter Tir mutant to form AE lesions is probably due to
signaling downstream of Y454. Just as earlier studies of Y474 highlighted a role for Nck in actin polymerization, the characterization of factors downstream of Y454 will likely provide further insight into mammalian pathways of actin assembly and their relationship to the pathogenesis of attaching and effacing organisms.
Figure 1. Intimin is required for efficient tyrosine phosphorylation of EPEC Tir.
(A.) HeLa epithelial cells were left uninfected (lane 1) or were infected with EPEC strains that express various combinations of intimin and Tir (lanes 2-6). Non-intimately associated bacteria were killed with gentamicin and removed. The remaining HeLa cells were lysed, separated by SDS-PAGE, and immunoblotted with a monoclonal anti-phosphotyrosine (Y-PO₄) antibody or polyclonal antibodies against Tir (see Experimental procedures). Anti-Ar3 staining indicates similar loading in each lane.
Figure 1. Intimin is required for efficient tyrosine phosphorylation of EPEC Tir.

(B.) EPEC lysates (lanes 1-3) or lysates of COS1 fibroblasts which were infected as described in part A (lanes 4-7) were immunoblotted. HA-tagged Tir was visualized using a monoclonal antibody against the HA-epitope and tyrosine phosphorylated Tir (arrow) was visualized using an anti-phosphotyrosine antibody. Anti-Arp3 staining indicates similar loading in each lane containing COS cell lysates.
### HeLa Cell HA Immunoprecipitates

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>Y474F</th>
<th>WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intimin:</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Y-PO&lt;sub&gt;4&lt;/sub&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<tr>
<td>1</td>
<td>2</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

1. EPEC<sub>Δ</sub>ir + pTir
2. EPEC<sub>Δ</sub>ir + pTir(Y474F)
3. EPEC<sub>Δ</sub>ir <sub>Δ<i>eae</i></sub> + pTir

**Figure 1.** Intimin is required for efficient tyrosine phosphorylation of EPEC Tir.

(C.) HeLa cells were infected as described in part A. HA-tagged Tir was immunoprecipitated from cell lysates using an anti-HA antibody and immunoprecipitates were resolved by SDS-PAGE and immunoblotted as described in part B. Identical results were obtained in experiments using COS1 cells (data not shown).
**HeLa Cell HA Immunoprecipitates**

<table>
<thead>
<tr>
<th>EPECΔtirΔeae + pTir Preinfection</th>
<th>EPECΔtirΔeae + pTir(Y474F) Preinfection</th>
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</thead>
<tbody>
<tr>
<td><strong>E. coli + pUC19:</strong></td>
<td>+</td>
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<tr>
<td><strong>E. coli + pIntimin:</strong></td>
<td>-</td>
</tr>
<tr>
<td>anti-TirM Ab:</td>
<td>-</td>
</tr>
<tr>
<td><strong>S. aureus:</strong></td>
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</table>

*Figure 2.* Clustering in the plasma membrane triggers efficient phosphorylation of Tir. HeLa cells were preinfected with EPEC strains that deliver Tir to the plasma membrane but that do not express intimin. Preinfecting bacteria were then killed with gentamicin and removed prior to challenge with particles of the indicated type. HA-tagged Tir was immunoprecipitated from HeLa cell lysates and immunoblotted as described in the legend to Fig. 1.
**Figure 3.** Tyrosine phosphorylation of the C-terminal cytoplasmic domain of Tir occurs independently of other translocated EPEC effectors. HeLa cells were mock transfected and infected with EPECΔtir + pTir (lane 1), or were transfected with a plasmid encoding TirMC, and infected with EPECΔtirΔeae (lane 2) or with particles coated with antibodies to the extracellular domain of Tir (lane 3). HA-tagged TirMC was immunoprecipitated from HeLa cell lysates and immunoblotted as described in the legend to Fig. 1. Arrows indicate the positions of tyrosine phosphorylated TirMC.
Figure 4. Clustering of EPEC Tir in the plasma membrane promotes efficient Nck binding. Equal amounts of purified GST ('GST') and GST–Nck ('Nck') were separated by SDS–PAGE, transferred to PVDF membranes and probed with lysates of HeLa cells which were previously infected with EPEC strains alone (lanes 1-4), or which were preinfected with EPEC strains and challenged with particles of the indicated type (lanes 5-12). Bound Tir was detected using an anti-HA monoclonal antibody.
Figure 5. Tyrosine 454 is a second phosphorylated tyrosine within EPEC Tir.

(A) Amino acid alignment of amino acids 446-488 within the C-terminal cytoplasmic domains of Tir molecules derived from EPEC strains E2348/69, 87A, and 413/89-1 (Deibel et al., 1998; Elliott et al., 1998; Paton et al., 1998), rabbit diarrheagenic E. coli (RDEC) strains RDEC-1, 83/39, and B10 (Marches et al., 2000; Tauschek et al., 2002; Zhu et al., 2001), and Citrobacter rodentium (Citro) strains CDC 1843-73T and DBS100 (Luperchio et al., 2000). Tyrosine 454 and tyrosine 474 are specified by arrows, and the Nck binding site is depicted beneath a black bar. Numbering is based upon the EPEC E2348/69 prototype.
### HeLa Cell

**HA Immunoprecipitates**

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<tr>
<th>Tir:</th>
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<th>Y454F</th>
<th>Y474F</th>
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<tr>
<td></td>
<td></td>
<td>Y-PO₄</td>
<td>HA-Tir</td>
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</tbody>
</table>

1. EPEC Δtir
2. EPEC Δtir + pTir
3. EPEC Δtir + pTir(Y454F)
4. EPEC Δtir + pTir(Y474F)
5. EPEC Δtir + pTir(Y454F/Y474F)

**Figure 5.** Tyrosine 454 is a second phosphorylated tyrosine within EPEC Tir.
(B) HeLa cells were infected with the indicated EPEC strains and HA-tagged Tir was immunoprecipitated from the HeLa cell lysates and immunoblotted as described in the legend to Fig. 1.
Tyrosine 454 contributes to actin pedestal formation by EPEC. HeLa cells were either infected with EPECΔtir or with EPECΔtir harboring plasmids for the expression of wild type Tir or Tir point mutants. Cell associated bacteria were detected by DIC microscopy, phosphotyrosine (Y-PO₄) was visualized with an anti-phosphotyrosine monoclonal antibody, and F-actin was visualized with phalloidin. Staining of infected cells with an anti-HA antibody to detect the N-terminus of Tir indicated that each Tir derivative was properly translocated (not shown). The percentage of bound bacteria generating pedestals (+/- standard deviation) was quantitated microscopically (see Experimental procedures). Arrows indicate positions of several bound bacteria.

<table>
<thead>
<tr>
<th></th>
<th>DIC</th>
<th>Y-PO₄</th>
<th>F-actin</th>
<th>% of Bound Bacteria Generating Pedestals</th>
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<td>EPECΔtir + pTir (Y474F)</td>
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<td>3.3 +/- 0.8</td>
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<tr>
<td>EPECΔtir + pTir (Y454/474F)</td>
<td></td>
<td></td>
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<td>0.0 +/- 0.0</td>
</tr>
</tbody>
</table>

**Figure 6.** Tyrosine 454 contributes to actin pedestal formation by EPEC. HeLa cells were either infected with EPECΔtir or with EPECΔtir harboring plasmids for the expression of wild type Tir or Tir point mutants. Cell associated bacteria were detected by DIC microscopy, phosphotyrosine (Y-PO₄) was visualized with an anti-phosphotyrosine monoclonal antibody, and F-actin was visualized with phalloidin. Staining of infected cells with an anti-HA antibody to detect the N-terminus of Tir indicated that each Tir derivative was properly translocated (not shown). The percentage of bound bacteria generating pedestals (+/- standard deviation) was quantitated microscopically (see Experimental procedures). Arrows indicate positions of several bound bacteria.
CHAPTER V

DISCUSSION

A protein that interacts with EPEC Tir but not EHEC Tir: the Nck adaptor

The inability of EHEC Tir to promote pedestal function in EPEC provided an opportunity to identify sequences of EPEC Tir critical for actin signaling. An EPEC/EHEC Tir hybrid that contains only a 12-amino-acid sequence of EPEC Tir encompassing the critical Tyr474 can promote pedestal formation by EPEC (Campellone et al., 2002). Interestingly, simple tyrosine phosphorylation of Tyr474 is not sufficient to activate the host actin signaling machinery, because a hybrid containing a seven-amino-acid sequence of EPEC Tir encompassing Tyr474 becomes tyrosine-phosphorylated but does not signal (Campellone et al., 2002). Thus, it seemed likely that sequences flanking phosphorylated EPEC Tir Tyr474, together with the phosphorylated tyrosine itself, were being recognized by a host actin signaling protein.

A breakthrough in deciphering the function of this segment of EPEC Tir occurred when two laboratories recognized that it was highly homologous to Nck-binding sequences, particularly the region surrounding Tyr112 of the vaccinia virus A36R protein (Campellone et al., 2002; Gruenheid et al., 2001). Phosphotyrosine 112 has previously been implicated in Nck recruitment and actin-tail formation by vaccinia (Frischknecht et al., 1999), suggesting that EPEC Tir similarly recruits Nck during pedestal formation.

In fact, two recent studies demonstrate that Tir peptides encompassing Tyr474 directly bind to Nck in vitro in a phosphotyrosine-dependent manner (Campellone et al., 2002; Gruenheid et al., 2001), and that Tir molecules containing intact Nck-binding sites recruit Nck to
sites of actin assembly (see introduction, Figure 1c; Table 1). Gruenheid et al. (2001) further demonstrate that Nck is essential for actin focusing triggered by EPEC, because cell lines that do not express Nck proteins are not capable of supporting pedestal formation by infecting EPEC.

**Signaling downstream of Nck**

The identification of Nck binding as a proximal and essential interaction in actin pedestal formation by EPEC was a seminal finding, leading to straightforward models for EPEC actin signaling (Figure 1a). Nck can directly stimulate N-WASP to activate the Arp2/3 complex *in vitro* (Rohatgi et al., 2001), suggesting that after recruitment by tyrosine-phosphorylated Tir, Nck might simply bind and activate N-WASP to trigger actin assembly. Alternatively, Nck binding by EPEC Tir might indirectly recruit N-WASP during actin pedestal formation. This parallels the model in vaccinia, where Nck bound to the A36R protein is thought to interact with N-WASP via an intermediate called WIP (WASP-interacting protein), and overexpression of the WIP WASP-binding domain inhibits tail formation (Moreau et al., 2000). Consistent with the suggestion that Nck indirectly recruits N-WASP during pedestal formation is the finding that the localization of N-WASP to sites of EPEC adherence requires its WIP-binding (WH1) domain, but not its proline-rich Nck-binding or GTPase-binding domains (Lommel et al., 2001). However, EPEC can generate pedestals on cells that lack WIP (S. Snapper, personal communication), suggesting that other proteins with WIP-like functions might exist in mammalian cells. Given that Nck also binds to other proteins that regulate the actin cytoskeleton, such as p21-activated kinases (Buday et al., 2002), a more complete picture of EPEC pedestal formation will require that the signaling components that function directly downstream of Nck be definitively identified.
Management of Nck signaling

Molecules that might modulate activation of N-WASP by Nck also deserve consideration, as these simple models are developed further. For example, phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂) and Nck synergistically activate N-WASP in vitro (Rohatgi et al., 2001). PI(4,5)P₂ can accumulate in lipid rafts, which serve as platforms for the activation of signaling components at the plasma membrane (Caroni, 2001; Simons and Toomre, 2000). Interestingly, several raft-associated proteins, such as CD44 and annexin-2, as well as glycosylphosphatidylinositol (GPI)-anchored proteins, localize to sites of EPEC adherence (see introduction, Table 1). Thus, by its Tir binding activity, Nck might be brought into the proximity of membranous PI(4,5)P₂, resulting in even more efficient activation of N-WASP. The raft-associated proteins are recruited to EPEC binding sites in a manner that is independent of Tir, but dependent upon an otherwise functional type III secretion system (Zobiack et al., 2002). Thus, it is intriguing to speculate that other type III secreted proteins are involved in targeting pedestal-forming bacteria to lipid rafts and enhancing Tir–Nck triggered actin condensation.

Potential roles of other actin-associated molecules in pedestal formation

The composition of the actin pedestal is extremely complex (see introduction, Table 1), and many of its constituents besides Nck, N-WASP and Arp2/3 might play important roles. For example, cortactin, which has the ability to stimulate Arp2/3 in a manner similar to N-WASP (Olazabal and Machesky, 2001), localizes to sites of bacterial adherence in a Tir-independent manner (see introduction, Table 1). Moreover, overexpression of a fragment of cortactin
incapable of stimulating the Arp2/3 complex impairs the generation of EPEC pedestals (Cantarelli et al., 2002). The actin depolymerizing factor cofilin, the actin-filament-severing protein gelsolin, and the polymerization rate regulator VASP (vasodilator-stimulated phosphoprotein) also accumulate within actin pedestals (see introduction, Table 1). Although no evidence directly implicates these molecules in actin pedestal formation, cofilin and gelsolin could help provide free actin subunits for incorporation into the growing end (tip) of the pedestal. Furthermore, cofilin and VASP have been shown to play important roles in the actin-based motility of *Shigella* and *Listeria*, respectively, when assayed in a system of purified components (Loisel et al., 1999). Finally, several mammalian focal adhesion proteins, such as α-actinin, talin and vinculin, are recruited to actin pedestals (see introduction, Table 1), and have been shown to bind cytoplasmic domains of EPEC Tir *in vitro* (Cantarelli et al., 2001; Freeman et al., 2000; Goosney et al., 2000; Huang et al., 2002). The interactions of these proteins with Tir might serve to anchor EPEC to the host-cell cytoskeleton; however, it is not known whether these molecules play a direct role in pedestal formation.

**The enteropathogenic *E. coli* Tir–Nck interaction is sufficient to initiate actin assembly**

Although the identification of Nck as a signaling molecule acting directly downstream of Tir clearly narrowed the universe of plausible models of actin-pedestal formation by EPEC, several aspects of this process have complicated the identification of the minimal elements critical for actin assembly. For example, it was not clear if other translocated EPEC molecules were essential for actin condensation. Eight EPEC-secreted proteins have been identified so far, and it has been difficult to ascertain whether some of these, such as EspB and EspD, are required for pedestal formation owing to their direct roles in actin signaling or solely because of their roles in...
the translocation of Tir. Recently, this limitation was circumvented by the expression of functional EPEC Tir within transfected mammalian cells (Campellone et al., submitted). These studies demonstrate definitively that clustering of EPEC Tir, in the absence of any other secreted EPEC proteins, is sufficient to trigger actin pedestal formation. Therefore, other translocated EPEC molecules play at most an auxiliary role in promoting actin assembly.

EPEC Tir binds *in vitro* to several actin-associated host molecules in addition to Nck, such as α-actinin, vinculin and talin, but determining whether the interaction of Tir with these proteins is required for actin assembly has been difficult. Our ability to test for Tir function in transfected cells allowed for the evaluation of the actin signaling activities of Tir derivatives that would fail to be translocated by the type III system. These studies demonstrated that artificially clustering the carboxyl terminus of EPEC Tir within the host plasma membrane is sufficient to trigger actin assembly into pedestals (Campellone et al., submitted). Consequently, the amino-terminal cytoplasmic domain of Tir, the domain that best binds focal adhesion proteins (Goosney et al., 2000; Huang et al., 2002), is apparently dispensable for pedestal formation. Surprisingly, particles coated with a minimized Nck-binding peptide of EPEC Tir assemble actin tails in *Xenopus* egg extracts (Campellone et al., submitted), indicating that Nck binding by Tir is sufficient to trigger actin polymerization *in vitro*.

Collectively, the observations discussed above suggest that, when stripped down to bare essentials, the initiation of actin assembly by EPEC Tir is remarkably simple, comprising recruitment of Nck via a 12-amino-acid sequence encompassing phosphorylated Tyr474.
**Nck-independent pedestal formation by enterohemorrhagic *E. coli* O157:H7**

Compared with the relative simplicity of actin assembly by EPEC, pedestal formation by EHEC is more complex and less well characterized. EHEC generates pedestals independently of Nck, because infecting EHEC do not recruit Nck to sites of actin polymerization (see introduction, Figure 1d; Table 1) and can form pedestals on cell lines that do not express Nck (Gruenheid et al., 2001). DeVinney et al. (DeVinney et al., 2001) demonstrated that Nck-independent actin signaling by EHEC requires translocation of one or more bacterial factors in addition to Tir (Figure 1b). Given the critical role of the adaptor protein Nck in actin assembly mediated by EPEC Tir, it is tempting to speculate that the putative EHEC factor(s) might also function as an adaptor that directly or indirectly links EHEC Tir to host actin-signaling molecules such as N-WASP. Such a factor(s) might be encoded within the LEE, a suggestion supported by the observation that the most divergent sequences within the EPEC and EHEC LEE elements are those comprising the open reading frames of putative secreted proteins (i.e. those presumed to interact with the host) rather than the bacterial type III secretion machinery itself (Perna et al., 1998). Alternatively, these additional factors may be encoded outside the LEE, a possibility supported by the observation that the cloned EPEC LEE can confer the pedestal-forming phenotype to nonpathogenic *E. coli* (McDaniel and Kaper, 1997), whereas a cloned EHEC LEE cannot (Elliott et al., 1999b). Clearly, identification of these putative effectors is the next important step in elucidating the mechanism of actin assembly by EHEC, and is likely to be facilitated by the recent publications of the genome sequences of EHEC O157:H7 strains (Hayashi et al., 2001; Perna et al., 2001).
Conclusions and future work

Through the years, the vast majority of studies designed at elucidating the mechanism of actin-pedestal formation have focused on EPEC and its effectors. Until recently, the manner by which EHEC and EPEC formed pedestals was assumed to be virtually identical, because the LEE pathogenicity islands were highly conserved and the pedestals appeared morphologically similar. However, recent studies clearly dictate that these two pathogens have evolved different Tir-based schemes to usurp host-cell signaling networks. The ability of EPEC Tir to bind Nck is apparently sufficient to initiate actin assembly. In contrast, EHEC Tir does not require Nck to form actin pedestals, but probably acts in concert with other translocated bacterial factors to promote N-WASP activation. Just as the identification of Nck binding as the essential activity of EPEC Tir has highlighted the role of this adaptor protein in actin assembly, the characterization of essential activities of EHEC Tir and other EHEC effectors is certain to provide new insights into mammalian pathways of actin signaling.
Figure 1. Models of actin signaling cascades activated by EPEC and EHEC during pedestal formation. (a) EPEC Tir becomes tyrosine-phosphorylated in the host-cell plasma membrane and binds the adaptor protein Nck. Nck recruits N-WASP or a WIP—N-WASP complex to trigger activation of the Arp2/3 complex, which leads to actin assembly. (b) EHEC Tir localizes to the plasma membrane, but is not tyrosine phosphorylated. Other EHEC proteins (‘X’) in addition to Tir are translocated into host cells. This combination of Tir and other factors promotes recruitment and activation of N-WASP by an unidentified mechanism (designated with a question mark). N-WASP then stimulates Arp2/3-based actin nucleation.
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