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Interaction of the Enteropathogenic *Escherichia coli* Protein, Translocated Intimin Receptor (Tir), With Focal Adhesion Proteins

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When enteropathogenic *Escherichia coli* (EPEC) attach and infect host cells, they induce a cytoskeletal rearrangement and the formation of cytoplasmic columns of actin filaments called pedestals. The attached EPEC and pedestals move over the surface of the host cell in an actin-dependent reaction [Sanger et al., 1996: *Cell Motil Cytoskeleton* 34:279–287]. The discovery that EPEC inserts the protein, translocated intimin receptor (Tir), into the membrane of host cells, where it binds the EPEC outer membrane protein, intimin [Kenny et al., 1997: *Cell* 91:511–520], suggests Tir serves two functions: tethering the bacteria to the host cell and providing a direct connection to the host's cytoskeleton. The sequence of Tir predicts a protein of 56.8 kD with three domains separated by two predicted trans-membrane spanning regions. A GST-fusion protein of the N-terminal 233 amino acids of Tir (Tir1) binds to alpha-actinin, talin, and vinculin from cell extracts. GST-Tir1 also coprecipitates purified forms of alpha-actinin, talin, and vinculin while GST alone does not bind these three focal adhesion proteins. Biotinylated probes of these three proteins also bound Tir1 cleaved from GST. Similar associations of alpha-actinin, talin, and vinculin were also detected with the C-terminus of Tir, i.e., Tir3, the last 217 amino acids. Antibody staining of EPEC-infected cultured cells reveals the presence of focal adhesion proteins beneath the attached bacteria. Our experiments support a model in which the cytoplasmic domains of Tir recruit a number of focal adhesion proteins that can bind actin filaments to form pedestals. Since pedestals also contain villin, tropomyosin and myosin II [Sanger et al., 1996: *Cell Motil. Cytoskeleton* 34:279–287], the pedestals appear to be a novel structure sharing properties of both focal adhesions and microvilli. Cell Motil. Cytoskeleton 47:307–318, 2000.

Key words: enteropathogenic *Escherichia coli*; attachments; translocated intimin receptor; fusion proteins; alpha-actinin; talin; vinculin

INTRODUCTION

Actin, and the proteins that interact with it, form an indispensable network in cells. This dynamic filament system is crucial for a number of cellular functions including locomotion, cell division, shape changes in cells, and various forms of intracellular motility. Control of the site-specific assembly and disassembly of actin filaments is critical for directing these activities to the appropriate position in the cell. Microorganisms have also been shown to trigger such rearrangements of the actin cytoskeleton. For example, *Listeria monocytogenes* [Tilney and Portnoy, 1989] enters a living cell and uses the actin and actin-related molecules to locomote in the

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Abbreviations used: EPEC = enteropathogenic *Escherichia coli*; EHEC = enterohemorrhagic *Escherichia coli*; Tir = translocated intimin receptor.

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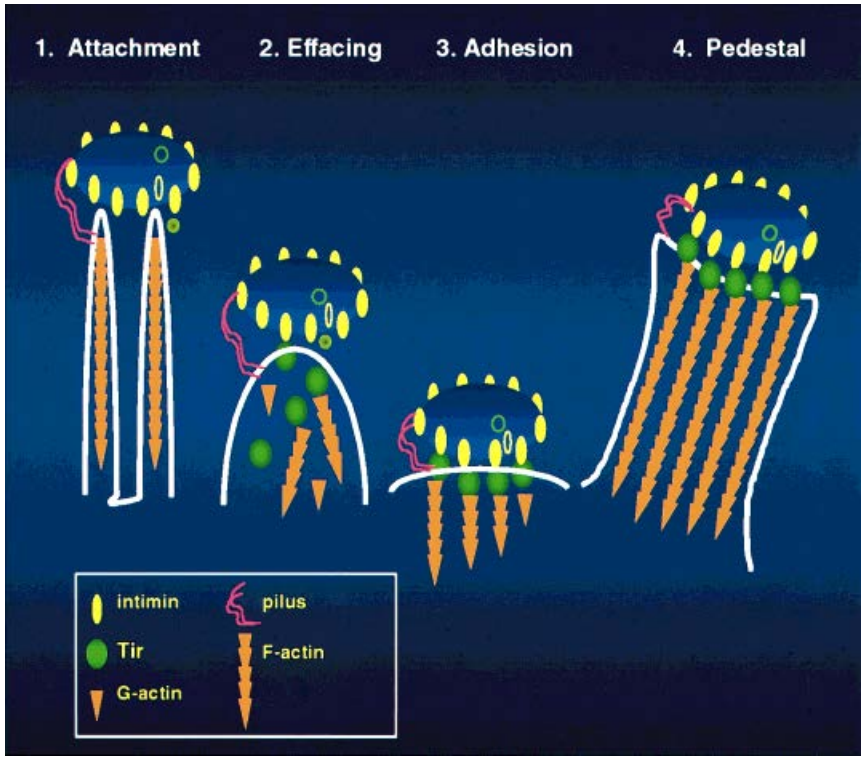

Fig. 1. Diagram summarizing the four stages of EPEC attachment. **1:** Attachment of EPEC to the host intestinal epithelium. **2:** Effacement of the intestinal microvilli. **3:** Adhesion of the infectious bacterium to the surface of the host cell and (**4**) formation of a complete actin bundle that emanates from the externally attached EPEC, i.e., the pedestal. **Inset** (bottom): Nomenclature key of proteins depicted.

host cytoplasm [Dabiri et al., 1990; Sanger et al. 1992], exit and infect neighboring cells. *Shigella, Rickettsia* and vaccinia viruses also share *Listeria*'s *modus operandi* [Higley and Way, 1997].

The gram-negative enteric *Escherichia coli* has two subtypes that can induce remodeling of the actin cytoskeleton of eukaryotic cells: enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC). This organism attaches to the surface of intestinal cells and induces the loss or effacement of the microvilli, leading to diarrhea. Additionally, EHEC also produces toxins, which can lead to death following consumption of food contaminated with only a small number of these bacteria. The basic manifestation of EPEC and EHEC colonization of intestinal cells is an attachment of the bacteria to the extracellular surfaces of the cells accompanied by the formation of a column of cytoplasmic actin filaments under the attachment sites [Knutton et al., 1989; Donnenberg et al., 1997]. The attachment sites resemble membrane-covered mounds and are called pedestals. Actin-binding proteins, alpha-actinin, Arp2/3, ezrin, talin, villin, and WASP have been localized directly beneath these EPEC attachments [Finlay et al., 1992; Kalman et al., 1999; Sanger et al., 1996].

EPEC move, not inside the host cell cytoplasm like *Listeria*, but while remaining attached to the outside of the host cell membrane with the pedestal of actin filaments trailing behind (Fig. 1) [Sanger et al., 1996]. The bundles extend from 1 to 10 μm above the cell, and the bacteria and attached membrane move together with the pedestal over the cell surface. Actin polymerization is required for the movement [Sanger et al., 1996], but it is not known how actin polymerization participates in the reactions that produce the movement, nor which of the other cytoskeletal proteins known to be present in pedestals are required for pedestal formation and/or motility. Furthermore, mutants of EPEC that attach to the host but do not induce the formation of pedestals are not as virulent as those that do [Donnenberg et al., 1997].

It is not clear how actin filaments form pedestals after infection of cells with EPEC. Evidence to date indicates that the bacterial protein, Tir (previously called Hp90), must be inserted into the host membrane, and then bound to the EPEC protein intimin, before pedestals form underneath the EPEC attachment site at approximately 2–3 h post-infection (Fig. 1) [Rosenshine et al., 1992]. During infection of host cells, the EPEC Tir is tyrosine phosphorylated and EHEC Tir is not; however, inhibition of tyrosine phosphorylation in EPEC-infected cells inhibits pedestal formation [Kenny, 1999]. The complete sequence of the Tir protein is available from Genbank and shows little homology to known pathogenic bacterial proteins [Kenny et al., 1997]. From its sequence, Tir has a predicted size of 56.8 kD but has an SDS/PAGE gel mobility of 78 kD. There are two predicted alpha-helical transmembrane domains that divide

the protein into three discrete domains: the N-terminal domain of 233 amino acids (Tir1), the second domain of 106 amino acids including one tyrosine (Tir2), and the third domain of 165 amino acids containing three tyrosines (Tir3) [Kenny et al., 1997]. The intracellular and extracellular membrane positions of the three protein domains with respect to the infected cell are well established. The first and third domains are cytoplasmic exposed domains while the second domain remains extracellular to the infected cell [Kenny, 1999; Hartland et al., 1999]. Since actin filaments usually do not appear to interact with transmembrane proteins directly [Stossel, 1993], we assumed that Tir may be interacting with some cytoskeletal protein(s) that can then interact with actin filaments. In this report, we demonstrate that the two cytoplasmic domains of Tir interact with three proteins—alpha-actinin, talin, and vinculin—that are components of focal adhesions and that can bind actin filaments. A preliminary report of this work was presented at the annual meeting of the Amrerican Society for Cell Biology [Freeman et al., 1999].

MATERIALS AND METHODS

Bacteria and Imaging

Enteropathogenic *E. coli* (EPEC), the wild type strain, E23348/69, and the JPN-15 strain lacking the pilus gene, *bfpA* [Jerse et al., 1990] (gifts of Dr. J. Kaper, Department of Medicine, University of Maryland, Baltimore, MD) were used to infect cultured cells: Caco-2, HeLa, PtK2, and REF-52 [Sanger et al., 1996]. Cells were infected for 4 h with bacteria from overnight cultures grown in BHI (Beef Heart Infusion) or LB (Luria broth) as described previously [Sanger et al., 1996]. Infected cells were prepared for immunofluorescence as previously described [Sanger et al., 1996]. Briefly, cells were fixed in 4% paraformaldehyde, permeabilized and incubated with either vinculin, alpha-actinin, or talin antibodies (Sigma, St. Louis, MO), followed by incubation with secondary fluorescein labeled anti-mouse IgG antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). The cells were washed and counterstained with rhodamine labeled phalloidin (Fluka Chemie AG, Ronkokoma, NY) and mounted in Mowiol [Ayoob et al., 2000]. Images of double-labeled cells were obtained as previously described [Ayoob et al., 2000]. Fluorescent images were obtained using a Nikon Diaphot 200 inverted fluorescence microscope and a Zeiss Confocal microscope. Photographic images were assembled using Metamorph (Universal Imaging Corp., West Chester, PA) and Adobe Photoshop (San Jose, CA).

GST Fusion Proteins.

Tir1 and Tir3 domains were expressed as glutathione *S*-transferase (GST) fusion proteins (Amersham Pharmacia Biotech, Piscataway, NJ). All expressed constructs resulted from cloning polymerase chain reaction (PCR) amplified products using E. coli (EPEC strain E2348/69) genomic DNA into the plasmid vector pGEX 4T-1. pGST-Tir1 expresses Tir amino acids 1 to 233 and results from cloning a 706 base pair amplified *Bam*HI/*Hind*III PCR fragment using the 5′ PCR primer TTGGGGATCC ATG CCT ATT GGT AAC CTT GG and the 3′ PCR primer CCCCAAGCTT TTA TTT AGG ATC TGA GCG AAC G. pGST-Tir3 expresses Tir amino acids 333 to 550 and results from cloning a 517 bp PCR amplified *Bam*HI/*Hind*III fragment using the 5′ PCR primer AATTGGATCC ACG ACT GCG CTC CAT AGA CG and the 3′ PCR primer GGGGAAGCTT TTA AAC GAA ACG TAC TGG. Amplified products were gel purified and cloned into vector pET28a (Novagen, Madison, WI) and the inserts verified by restriction digest analysis. Tir1 and Tir3 fragments were subcloned via *Bam*HI/*Xho*I restriction sites into pGEX 4T-1 and pGEX6p-1, transformed into DH5α (Life Technologies, Inc., Grand Island, NY) and the plasmid DNA's purified and sequenced (CHOP, Philadelphia, PA). Following sequence verification, both GST-Tir1 and GST-Tir3 fusion constructs were transformed into *Escherichia coli* BL-21(DE3)pLysS and induced with IPTG for protein expression. GST fusion proteins were purified according to the manufacturer's specifications (Amersham Pharmacia Biotech, Piscateway, NJ). Additionally, Tir1 was cloned into the pGEX6p-1 plasmid and the purified GST-Tir1 proteolysed using Prescission protease (Amersham Pharmacia Biotech, Piscateway, NJ) to prepare free Tir1.

Protein Purifications, Biotinylation, and Protein Overlays

Purified alpha-actinin, talin, and vinculin were prepared using standard techniques [Feramisco and Burridge, 1980; Hock et al., 1989; Danowski et al., 1992; Sanger et al., 2000]. These three proteins were used in standard coprecipitation assays and, additionally, as biotinylated probes in overlay assays. Biotinylation of the proteins was accomplished following the manufacturer's recommendations (BiotinTAG; Sigma, St. Louis, MO). Purified proteins were run on gels and transferred to nitrocellulose membranes. Membranes were incubated with purified biotinylated proteins, washed, and binding detected using Extravidin® peroxidase conjugate (Sigma, St. Louis, MO) and visualized using chemiluminescence.

Coprecipitations

Coprecipitation experiments were performed using either purified GST-Tir1, GST-Tir3, or GST proteins

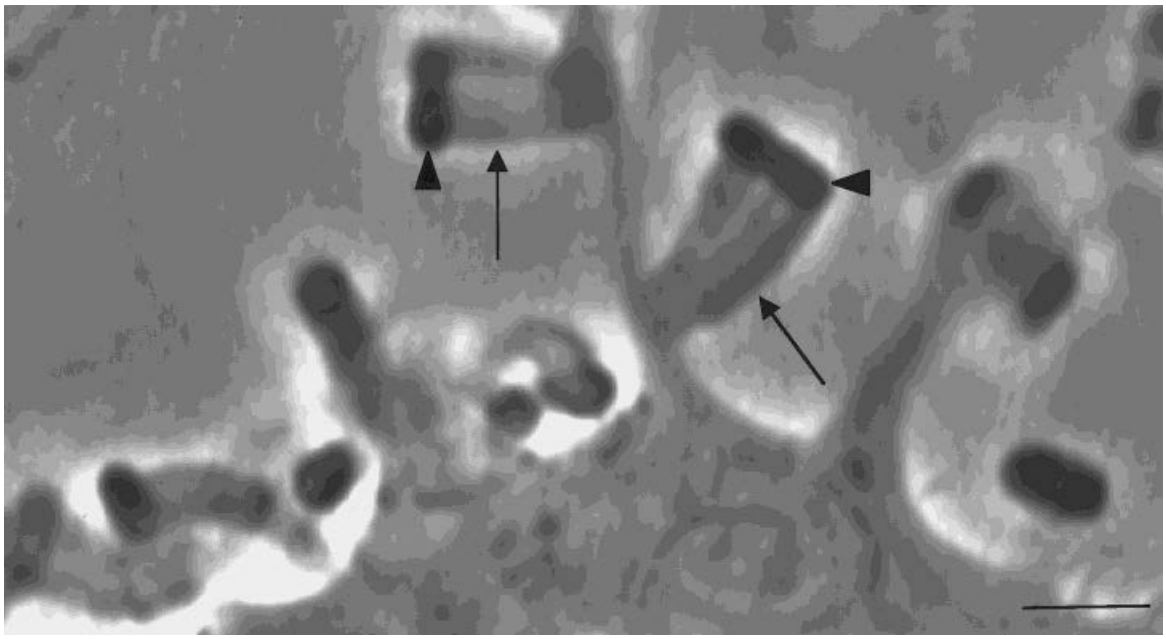

Fig. 2. A single time-point from a sequence showing a live PtK2 cell infected with EPEC. The phase dense bacteria (*arrowheads*) are at the top of cellular extensions called pedestals (*arrows*). Bar = 1 μm.

combined with chicken gizzard extracts in a 1.5-ml microfuge tube. Similar precipitation experiments were performed with the three purified focal adhesion proteins using either GST-Tir1, GST-Tir3, or GST alone. The mixtures were incubated for 1 h on ice and then centrifuged 14,000*g* in an Eppendorf microfuge. Supernatants were carefully removed and transferred to a clean microfuge tube with 35 μl of a 50% glutathione bead slurry (Pharmacia, Sweden) and incubated for 30 min on ice. The beads binding the protein samples were washed twice in phosphate buffered saline (PBS), 1% triton-X (Fisher Biochemicals) followed by a single wash in PBS alone. These beads were suspended and boiled in 30 μl of SDS-sample buffer (Bio-Rad, Richmond, CA) and run on 10% SDS gels. The proteins were transferred to nitrocellulose membrane and treated as described above.

Other Methods

Genomic E2348/69 bacterial DNA was purified using the CTAB (cetyltrimethylammonium bromide) protocol [Ausbel et al., 1998]. Briefly, overnight cultures were grown in 1.5 ml Luria Broth (LB). The culture was pelleted and suspended in STET buffer with lysozyme (50 μg/ml) and the mixture incubated briefly at room temperature and then heated to 95°C. The suspension was centrifuged for 10 min at 14,000 rpm in an Eppendorf microfuge and the viscous pellet removed with a toothpick. To the remaining solution, CTAB was added and the mixture was centrifuged. The pellet was suspended in 1.2 M NaCl and EtOH to precipitate the DNA. Genomic DNA was then suspended in TE (10 mM Tris-HCL, pH 8.0, 1 mM EDTA) and used for subsequent PCR reactions. Plasmid DNA was routinely purified using the Wizard® Plus Miniprep DNA purification system (Promega, Madison, WI).

Polyclonal antisera was generated by subcutaneous injection of New Zealand white rabbits with either GST-Tir1 or GST-Tir3 (Cocalico Biologicals, Reamstown, PA). GST-Tir1/3 purified protein was separated on SDS/PAGE and stained with Coomassie blue. Representative protein bands were removed and used for rabbit inoculation. Animals were periodically boosted and the antisera tested for immunfluorescence reactivity. Pre-immune sera was assayed and used as a negative control.

Preparation of gizzard extracts have been described elsewhere [Sanger et al., 2000]. Briefly, frozen gizzards (Pel-Freeze Biologicals, Rogers, AR) were thawed, homogenized, and extracted with a 2 mM Tris, pH 9, yielding a protein extract enriched for alpha-actinin, vinculin, actin, and talin. After titration to pH 7.2 and centrifugation, the supernatant was aliquoted and stored at −80°C in 1.0-ml aliquots.

RESULTS

Subcellular Localization of Actin, Alpha-Actinin, Talin, and Vinculin With EPEC

When EPEC attach to the surface of host cells, actin filaments form beneath them in bundles that vary in

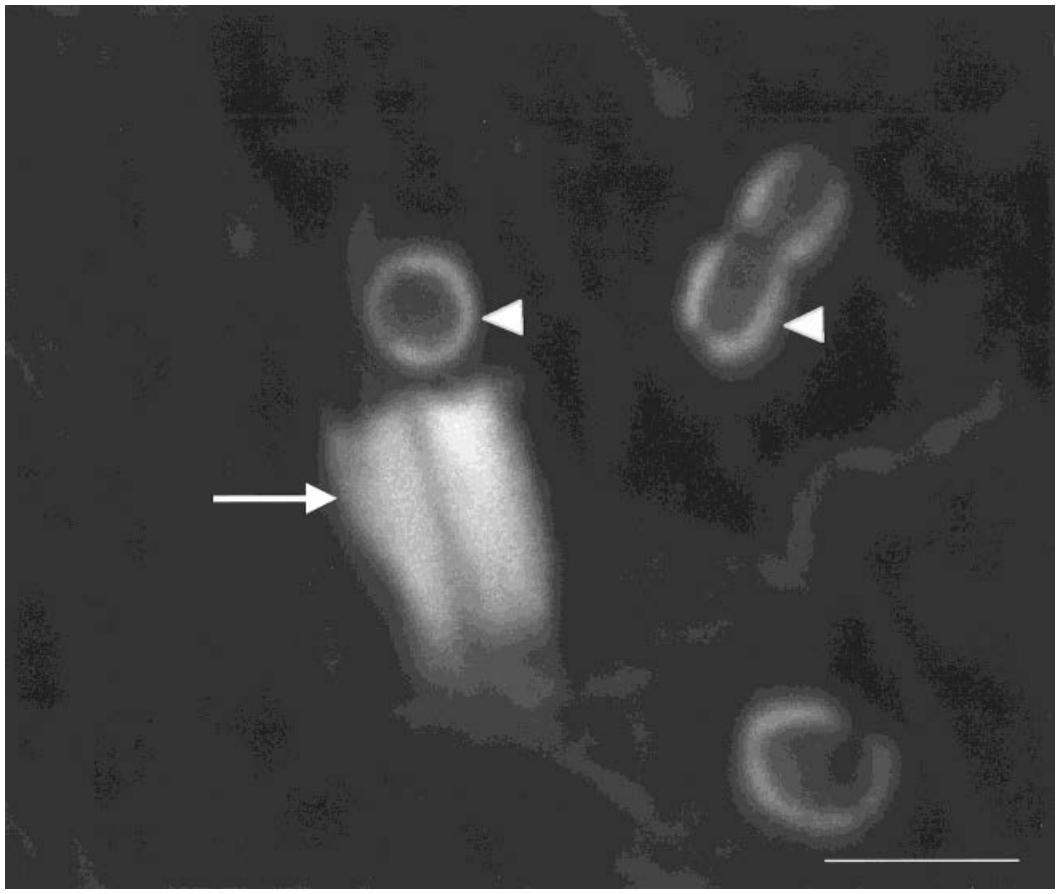

Fig. 3. Summed Z-series image of an EPEC-infected cell obtained with confocal microscopy. Phalloidin stained bundles of actin are distributed along the shaft of the pedestal (*arrow*). The surface attached bacterium can be detected in the negative image at the top of the pedestal. Longer pedestals (*arrow*) usually are positioned parallel to the surface of the cell. Actin filaments also form in shorter pedestals (*arrowheads*) that extend only 1 to 2 μm into the host cytoplasm beneath the perimeter of attached EPEC. The dark images of the EPEC can be observed in the middle of the actin filaments. These short actin filaments are seen from a top view looking into the host cell. Bar = 1 μm.

length. Long bundles project from the host cell forming pedestals with EPEC at the tips (Fig. 2). Measurements of phalloidin-stained cells with confocal microscopy indicate that the actin bundles are about 1 to 10 μm in length (Fig. 3). An individual bacterium can induce a single pedestal or two pedestals (Fig. 4). Whereas phalloidin and anti-alpha-actinin antibodies react along the length of the pedestals, antibodies directed against the two different cytoplasmic domains of Tir (Tir1 and Tir3) detect the antigen only at the cell membrane directly beneath the externally attached EPEC (Fig. 5).

A number of actin-associated proteins have been localized to the EPEC pedestals by immunofluorescence microscopy, including actin, alpha-actinin, Arp2/3, ERM, non-muscle myosin II, talin, tropomyosin, villin, and VASP [Finlay et al., 1992; Kalman et al., 1999; Sanger et al., 1996]. Two of these proteins, alpha-actinin and talin, are also localized in attachment plaques, and we have found that another attachment plaque component, vinculin, can be found in pedestals associated with EPEC (Fig. 6). In contrast to alpha-actinin and talin localization, however, the vinculin localization was not always seen beneath all the attached EPEC on an infected cell. The same localization results were seen when PtK2 cells were transfected with either GFP-alpha-actinin or GFP-vinculin and then infected with EPEC. In cells transfected with GFP-vinculin, not all attached bacteria had fluorescence concentrated beneath them, whereas in cells transfected with GFP-alpha-actinin, all attached EPEC were associated with concentrations of alpha-actinin (data not shown).

Tir1 and Tir3 Associate With the Focal Adhesion Proteins, Talin, Alpha-Actinin, and Vinculin

As an approach to biochemically identify Tir-associated host proteins, we generated GST-fusion proteins to

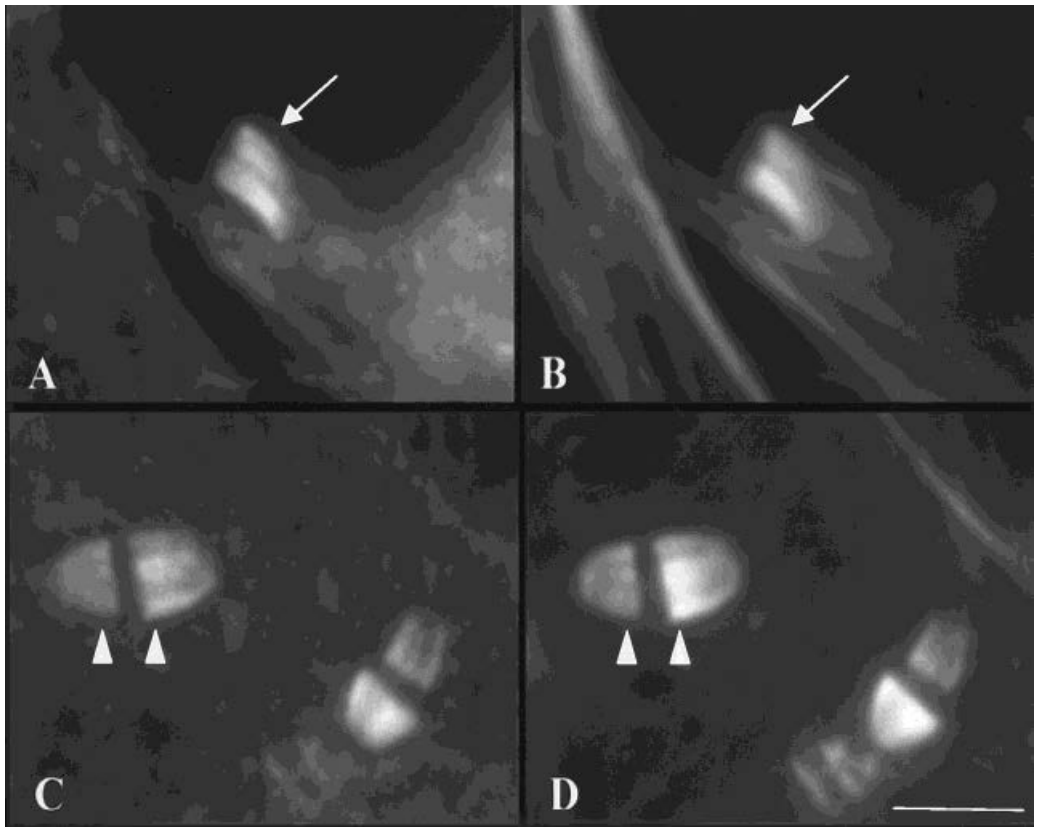

Fig. 4. Double immunofluorescence of (**A,B**) single and (**C,D**) double pedestals stained with rhodamine phalloidin (B,D) and (A,C) an alpha-actinin antibody. *Arrows* indicate co-localization of actin and alpha-actinin in single pedestals, and *arrowheads* indicate co-localization in double pedestals. Bar = 1 μm.

the Tir intracellular domains, Tir1 and Tir3 (Fig. 7A). Purified GST-Tir fusion proteins were used in coprecipitation assays with protein extracts prepared from chicken gizzards, and analyzed by Western blot using antibodies recognizing a variety of cytoskeletal proteins. Three proteins, talin, alpha-actinin, and vinculin, all major constituents of focal adhesions were found to be associated with the intracellular domain of Tir1 (Fig. 7B–D). The predicted molecular weight of each focal adhesion protein observed in the whole protein gizzard extract was identical to the proteins coprecipitated with GST-Tir1 (Fig. 7B–D, lanes 1 and 2, respectively). There was minimal antibody signal detected with GST alone (Fig. 7B–D, lanes 3). Coprecipitations with Tir1 with gizzard extracts also showed the presence of actin when incubated with an actin antibody (data not shown). These same Tir1 protein associations with talin, alpha-actinin, and vinculin, detected using chicken gizzards, were also observed using protein extracts made from either whole spleen or human platelets (data not shown). To further assess host cytoskeletal protein interactions, similar coprecipitation experiments were done with gizzard extracts and the Tir3 domain. As with Tir1, talin, alpha-actinin, and vinculin were also found to interact with Tir3 (Fig. 8A–C, lanes 1, 2, respectively), and, again, no significant coprecipitation was observed with GST alone (Fig. 8A–C, lanes 3).

To address the question of whether Tir directly interacts with talin, alpha-actinin, or vinculin, co-precipitation experiments were done using purified preparations of the individual proteins. Analysis of each of the preparations with Coomassie blue stained SDS/PAGE showed each protein representing >95% of visible bands (data not shown). Western blot analysis indicated a small amount of alpha-actinin in the vinculin preparation (Fig. 9A, lane 4), and a minor amount of vinculin in the talin preparation (Fig. 9C, lane 7), whereas the alpha-actinin preparation showed no indication of talin or vinculin (Fig. 9B, lane 5; Fig. 9C, lane 6). None of the three protein preparations showed evidence of actin. All three protein preparations, talin, alpha-actinin, and vinculin, coprecipitated with GST-Tir1 and GST-Tir3, but not GST alone, suggesting direct interaction of the proteins with the Tir domains (Fig. 9A, lanes 1 and 2; B, lanes 2 and 3; C, lanes 2 and 3). The small amount of other proteins evidenced by Western blot analyses in the purified preparations were all below detectable levels in

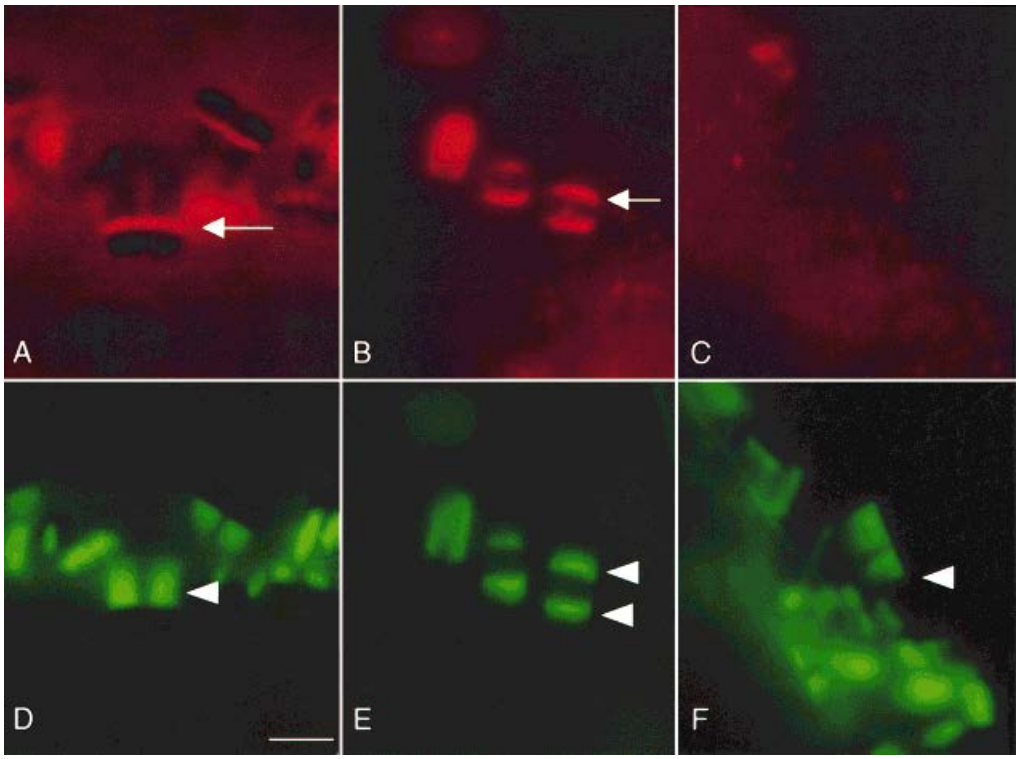

Fig. 5. Localization of Tir1 (**A**) and Tir3 (**B**) beneath externally attached EPEC. (A) Tir1 and (B) Tir3 (*arrows*) are only localized in the surface attachment of the bacteria. Actin filaments (*arrowheads*) are distributed all along the shafts of the pedestals (D,E, and F). **C:** Control infected cell(s) stained with the preimmune sera and secondary fluorescent antibody and (**F**) rhodamine phalloidin. Note that the preimmune sera (C) does not stain any region of the pedestal (F). Bar = 1 μm.

coprecipitation experiments. These results provide the first biochemical association of Tir1 and Tir3 with host cytoskeletal associated proteins. Supporting evidence for this conclusion was provided by protein overlays of Tir domains with biotinylated alpha-actinin, talin, and vinculin. Each of the three proteins was biotinylated and reacted with blots of GST-Tir1, Tir1 from which GST had been cleaved, GST-Tir3, and free GST. The biotinylated proteins bound to the Tir peptides and not to the free GST, indicating direct interaction of the Tir cytoplasmic domains and host cytoskeletal proteins (data not shown).

DISCUSSION

The attachment of EPEC to a host cell is achieved by the binding of the bacterial surface protein, intimin, to Tir, a protein that is secreted into the host cell during EPEC infection [Kenny et al., 1997]. Tir becomes inserted into the host membrane with both ends in the host cytoplasm and the intimin-binding domain extended outside the host cell [Kenny, 1999]. Immunolocalization of Tir shows it to be positioned at the site of attachment of EPEC to the host membrane; it does not extend along the pedestal [Kenny et al., 1997]. Tir has 233 amino acids from the amino terminus to the first predicted transmembrane domain and 217 amino acids from the second transmembrane domain to the C-terminus. The biochemical work in this report demonstrates that several proteins found in focal adhesions, i.e., alpha-actinin, talin, and vinculin, bind to the two cytoplasmic domains of Tir. Figure 10 summarizes the current status of the cytoskeletal proteins associated with the pedestals beneath EPEC.

When EPEC attach to the cell membrane of infected host cells and induce a recruitment of host cell actin to the attachment sites, a number of actin-associated proteins are also recruited to the sites [Finlay et al., 1992; Kalman et al., 1999; Sanger et al., 1996]. The presence of villin along the lengths of the pedestals, and non-muscle myosin myosin II and tropomyosin at the base of the pedestals, suggested that the pedestals resembled large microvilli [Sanger et al., 1996]. However, the detection of alpha-actinin, talin, and vinculin along the lengths of the pedestals is inconsistent with this proposal since microvilli do not have these proteins distributed along their actin shafts. These three proteins are typical of focal adhesions [Longhurst and Jennings, 1999], which do not

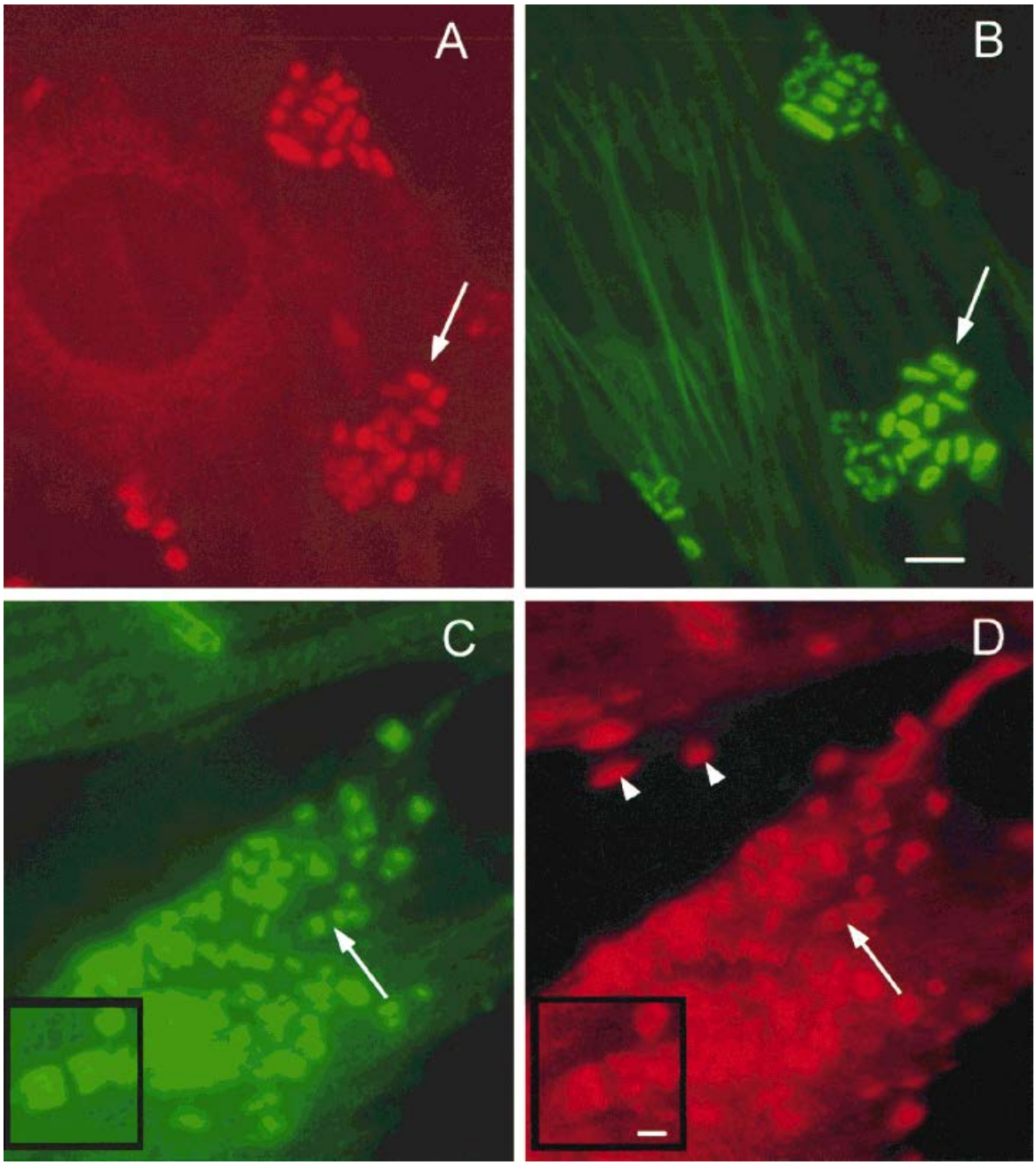

Fig. 6. Immunofluorescent patterns of EPEC on PtK2 cells. **A:** Talin staining of EPEC. **B:** Same cell stained with phalloidin-FITC. Staining of the same infected PtK2 cells with antibodies directed against (**C**) alpha-actinin and (**D**) vinculin. **Insets:** Double pedestal; *arrow* indicates the non-staining position of the bacterium. Note that alpha-actinin, talin, and vinculin are co-localized in the cytoplasm beneath the externally attached bacteria. D: The *arrowheads* indicate the localization of vinculin in focal adhesions. Scale bar in B = 5 μm for A–D, in inset (D) = 2.5 μm.

contain villin, tropomyosin, and myosin II. Thus, the pedestals appear to be a novel structure sharing properties of both focal adhesions and microvilli (Fig. 10). The proteins detected in the pedestals may localize there due to their ability to bind directly and indirectly the actin filaments so abundant in the pedestals. We have observed that as infection proceeds, and the number of EPEC on the cells increases, the number of stress fibers decreases. We suggest that these Tir molecules and pedestal actin bundles are recruiting the focal adhesion proteins and actin from the existing stress fibers, resulting in stress fiber disassembly.

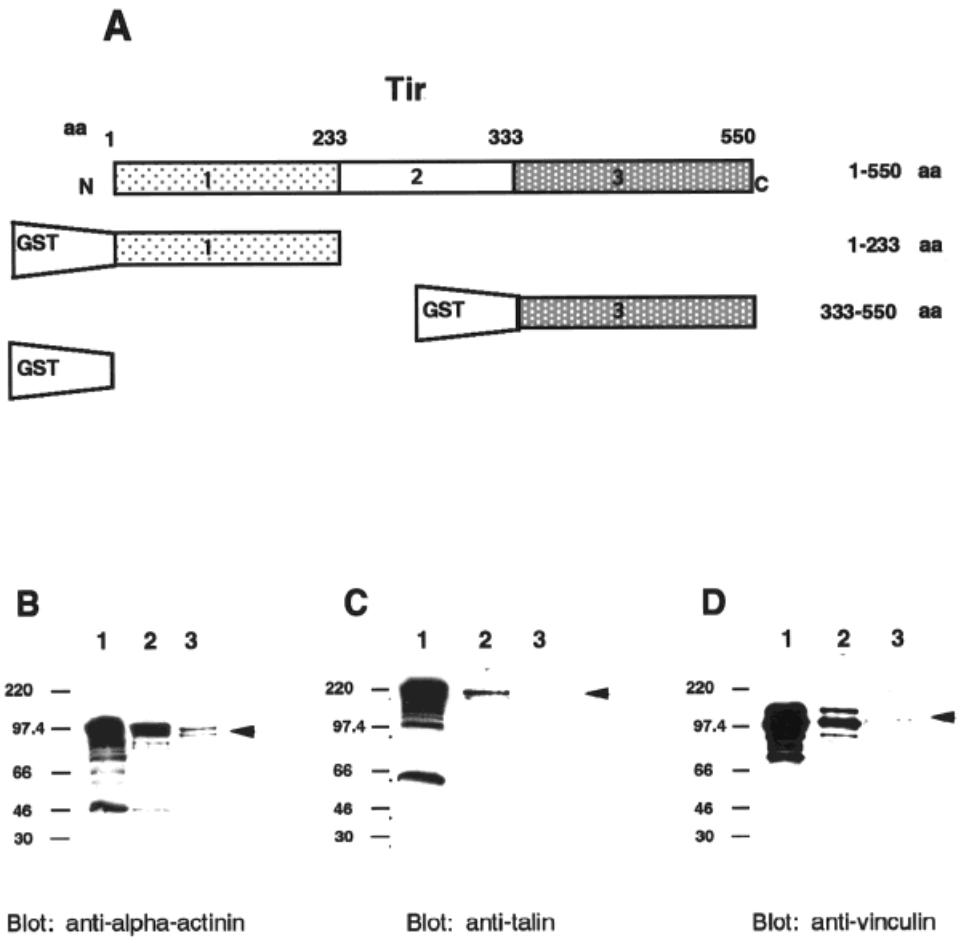

Fig. 7. **A:** Illustration of GST-Tir fusion proteins. The 550 amino acid full-length Tir is depicted (**top**). The three domains of Tir, Tir1 (amino acids 1-233), Tir2 (amino acids 234–332), and Tir3 (amino acids 333–550) are indicated as the *light spotted box*, *white box*, and *dark spotted box*, respectively. Diagrams of the various GST-fusion proteins of the Tir1 and Tir3 cytoplasmic amino acid domains are indicated beneath. **B–D:** ~20 μg of total protein from chicken gizzard extracts were combined with 5–10 μg of either GST-Tir1 or GST, glutathione sepharose beads, washed and proteins separated on 10% SDS/PAGE. Proteins were transferred to nitrocellulose and probed with either (A) polyclonal alpha-actinin antibody or monoclonal (B) talin or (C) vinculin antibody. Detection of alpha-actinin, vinculin or talin was done using a goat anti-rabbit or anti-mouse horseradish peroxidase (HRP) conjugate and visualized by chemiluminescence. **Lanes 1:** ~1.5 μg of total chicken gizzard extract, alone. **Lanes 2:** Coprecipitation of GST-Tir1 and chicken gizzard extract. **Lanes 3:** Coprecipitation of GST and chicken gizzard extract. *Arrowheads* indicate appropriate gel mobilities of alpha-actinin, talin, or vinculin.

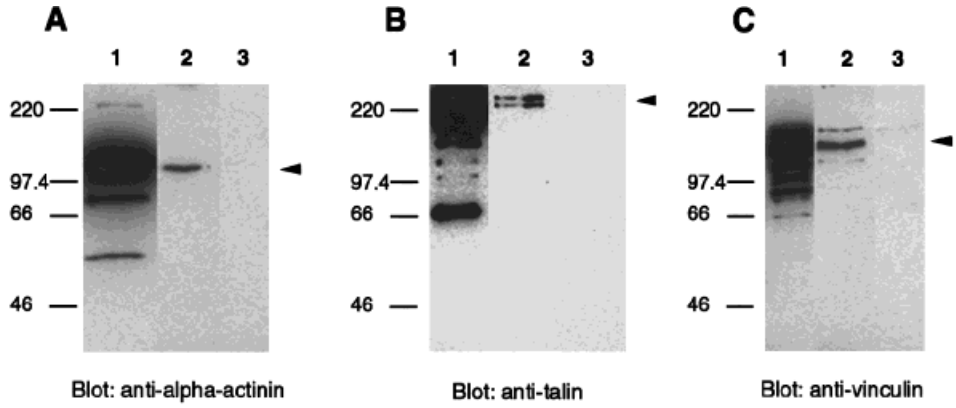

Fig. 8. **A–C:** Co-precipitation of focal adhesion proteins alpha-actinin, talin, and vinculin with Tir3. Co-precipitations using ~20 μg of total chicken gizzard protein extracts were combined with 5–10 μg of either GST-Tir3 or GST. Samples were prepared as described in Figure 7 and Material and Methods. Precipitated proteins were probed with either (A) polyclonal alpha-actinin antibody or monoclonal (B) talin or (C) vinculin antibody and detection visualized by chemiluminescence. **Lanes 1:** ~1.5 μg of total chicken gizzard extract, alone. **Lanes 2:** Coprecipitation of GST-Tir3 and chicken gizzard extract. **Lanes 3:** Coprecipitation of GST and chicken gizzard extract. *Arrowheads* indicate appropriate gel mobilities of alpha-actinin, talin, or vinculin.

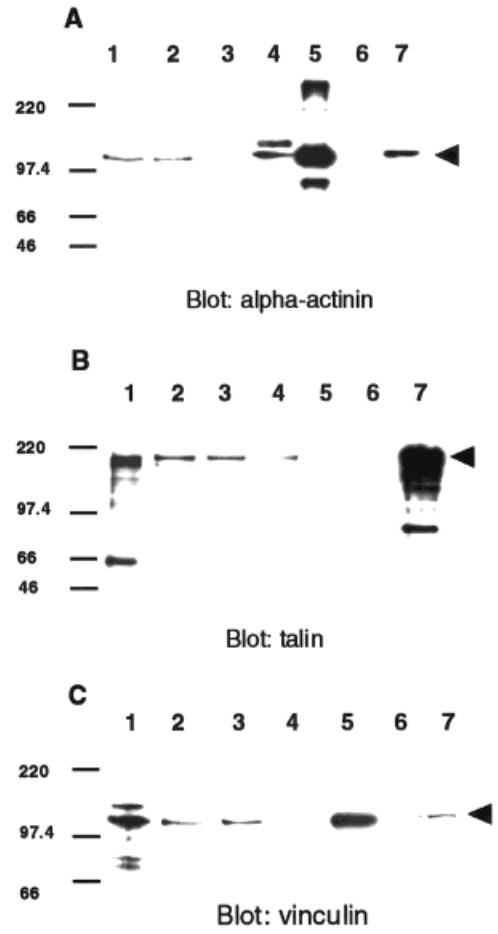

Fig. 9. In vitro interaction of purified Tir1 and purified alpha-actinin, talin, and vinculin. Purified GST-Tir1, Tir3, or GST were combined with purified (A) alpha-actinin, (B) talin, or (C) vinculin. Samples were prepared, detected, and visualized as previously described. *Arrows* indicate correct gel mobilities of alpha-actinin, talin, or vinculin. **A: Lane 1:** Coprecipitation of GST-Tir1 and alpha-actinin; **lane 2:** coprecipitation of GST-Tir3 and alpha-actinin; **lane 3:** coprecipitation of GST and alpha-actinin; **lane 4:** vinculin; **lane 5:** alpha-actinin; **lane 6:** talin; **lane 7:** gizzard extract. **B: Lane 1:** gizzard extract; **lane 2:** coprecipitation of GST-Tir1 and talin; **lane 3:** coprecipitation of GST-Tir3 and talin; **lane 4:** coprecipitation of GST and talin; **lane 5:** alpha-actinin; **lane 6:** vinculin; **lane 7:** talin. **C: Lane 1:** gizzard extract; **lane 2:** coprecipitation of GST-Tir1 and vinculin; **lane 3:** coprecipitation of GST-Tir3 and vinculin; **lane 4:** GST alone and vinculin; **lane 5:** vinculin; **lane 6:** alpha-actinin; **lane 7:** talin. *Arrowheads* indicate appropriate gel mobilities of alpha-actinin, talin, or vinculin.

The localization of Tir in the cell membrane with an extracellular binding domain and two cytoplasmic domains that may bind to the actin cytoskeleton suggests a similarity with integrins. The integrins are concentrated at the focal adhesions and are composed of two different polypeptide subunits (alpha and beta subunits) that form heterodimers. Interactions with the cytoskeleton occur through the beta subunits [Critchley et al., 1999; Longhurst and Jennings, 1999]. The beta-1 cytoplasmic domain of integrins binds the focal adhesion proteins alpha-actinin, talin, tensin, and FAK [Longhurst and Jennings, 1999]. Vinculin does not bind directly to the cytoplasmic domains of integrin, but its binding to alpha-actinin, talin, and tensin positions it close to integrin in the focal adhesions. Comparison of the cytoplasmic domains of Tir with the cytoplasmic domains of integrins revealed no homologies.

Although vinculin co-precipitated with GST-Tir1 and GST-Tir3, and biotinylated vinculin bound with Tir1, GST-Tir1, and GST-Tir3 in gel overlays, the microscopic localization of vinculin with EPEC attachment sites was not consistently seen. This was observed in studies using both immunolocalization and GFP-vinculin-expressing cells infected with EPEC. The variability in detection of vinculin underneath the attached EPEC may be related to the episodic movements of the EPEC attachments [Sanger et al., 1996]. Galbraith et al. [1999] have recently demonstrated that the localization of vinculin beneath external fibronectin-coated beads was a function of movement. When these beads were stationary, there was no vinculin localization beneath the beads, and when the beads were pulled with laser tweezers, vinculin localized beneath the beads. The ability of EPEC Tir to bind vinculin is also shared by the *Shigella* surface protein Ipa [Bourdet-Sicard et al., 1999].

Sequence comparisons of the Tir1 domain to other known proteins identified espin, a microvillus protein, and talin, the focal adhesion protein currently discussed in this manuscript, as two proteins sharing amino acid sequence homologies (Fig. 11). Similarly, sequence comparison with the Tir3 domain did not reveal any shared homologies with other known proteins. Tir1 amino acids 161 to 210 had a 34% identity and 54% similarity with a 60 amino acid region of talin that binds the head domain of vinculin (Fig. 11). When vinculin is in a folded configuration, this head domain binds to the vinculin C-terminal tail domain and talin binding is reduced [Johnson and Craig, 1994]. Thus, the physiological conformation of vinculin could dictate its interactions with Tir; the vinculin tail bound by the head could prevent the binding to Tir1 whereas an extended motif would permit binding of Tir1 to the head, and actin binding by the extended tail. Furthermore, talin itself may compete with Tir for binding interactions with vinculin. It has recently been suggested that vinculin heads can form dimers and trimers stabilized by VASP with the vinculin tail domains extended to interact with actin filaments [Huettelmaier et al., 1998]. It is of interest to note that VASP has also been immunolocalized to the pedestals (Fig. 10) [Kalman et al., 1999].

The other region of Tir1, amino acids 17–52, has significant homology with another actin-binding protein, espin. Espin, composed of 837 amino acids, binds and bundles actin, and is a major cytoskeletal protein (1

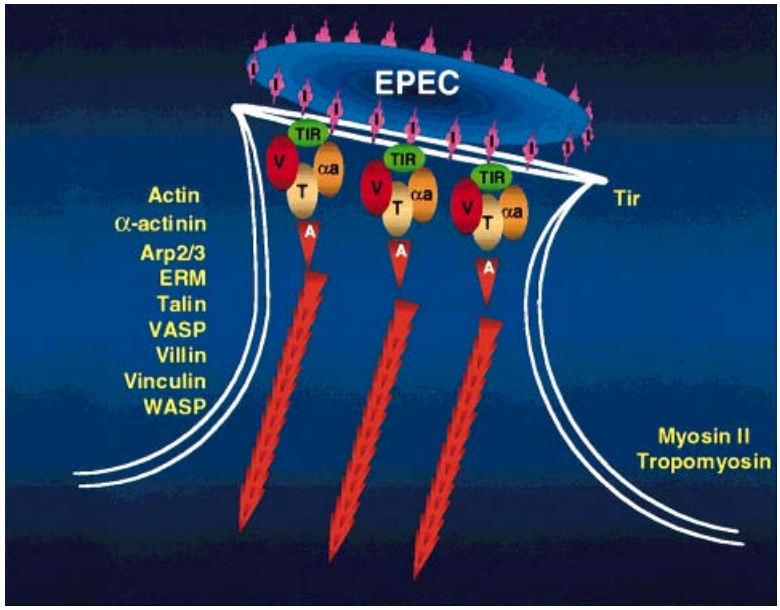

Fig. 10. Diagram showing the putative position of the Tir binding proteins in the pedestal and their position with respect to the F-actin core. The proteins listed on the left side of the pedestal have been reported to be distributed all along the length of the pedestal while those on the right have been detected at its base. Illustrated proteins: αA, alpha-actinin; A, actin; I, intimin; T, talin; Tir, translocated intimin receptor; V, vinculin.

```
Tir1:   17  PPAPPLPSQTDGAARGGTGHLISSTGALGSRSLFSP 52
            PPAPPLP + GAA G SSTG+ S ++ SP
Espin: 598  PPAPPLPIEGAGAA-CGQRRSSSTGSTKSFNMMSP 632

Tir1:  161  GGSGHPMVTVASDIAEARTKILAKLDPDNHGGRQPKDVDTRSVGVGSASG 210
            GGSG P++ A +A A +++L P + RQ ++ + VG ASG
Talin: 602  GGSGRPLLQAAKGLAGAVSELLRSAQPASAEPRQ--NLLQAAGNVGQASG 649
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Fig. 11. Sequence comparisons of the Tir1 domain with regions of espin and talin. The Tir amino acid sequence was analyzed using the standard the National Center for Biotechnology Information (NIH) QBLAST program. Two proteins sharing amino acid sequence homology to the Tir1 domain were talin, amino acids 602–649 and espin, amino acids 598–632. Amino acid identity is indicated in shaded areas and similarity as +.

espin/20 actin monomers) in the Sertoli cells of the testes [Chen et al., 1999]. A smaller espin, lacking the first 484 amino acids of the N-terminus of the larger form, is present in brush borders (1 small espin/130 actin monomers) [Chen et al., 1999]. Both espins contain a region of 34 amino acids that has 50% identity and 61% similarity with amino acids 17–52 of Tir1 (Fig. 7). This espin region has not been implicated in any known functions, including binding or bundling actin filaments [Chen et al., 1999]. But given the shared sequence homologies with Tir1, these sequences may prove important for microvillar association.

Whether the association of actin and actin-associated proteins beneath the attached EPEC plays a role in the pathology of EPEC infection is an open question. Mutants lacking Tir are able to associate with the surfaces of host cells, but phalloidin staining of these infected cells reveals unorganized F-actin in the regions below the extracellularly attached bacteria [Kenny, 1999]. Intimin mutants also associate with host cells, but do not induce pedestal formation [Kenny, 1999] and are not as virulent as with wildtype EPEC [Donnenberg et al., 1993]. Hecht [1999] reviews how the ". . .flushing action of secretory diarrhea is a host defense mechanism against enteric pathogens." Thus, the Tir molecules in the pedestals with their ability to bind alpha-actinin, vinculin, and talin, may confer an evolutionary advantage in enabling the EPEC to be held on the surfaces of infected cells and to resist the flushing action of the infected host animal.

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NOTE ADDED IN PROOF

Goosney et al., [2000, Current Biology 10:735–738] report that the Tir1 domain interacts directly with alpha-actinin.

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