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Clustering of Nck by a 12-residue Tir phosphopeptide is sufficient to trigger localized actin assembly

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Enteropathogenic *Escherichia coli* (EPEC) translocates effector proteins into mammalian cells to promote reorganization of the cytoskeleton into filamentous actin pedestals. One effector, Tir, is a transmembrane receptor for the bacterial surface adhesin intimin, and intimin binding by the extracellular domain of Tir is required for actin assembly. The cytoplasmic NH_2 terminus of Tir interacts with focal adhesion proteins, and its tyrosine-phosphorylated COOH terminus binds Nck, a host adaptor protein critical for pedestal formation. To define the minimal requirements for EPEC-mediated actin assembly, Tir derivatives were expressed in mammalian cells in the absence of all other EPEC components. Replacement of the NH_2 terminus of Tir with a viral membrane-targeting sequence promoted efficient surface expression of a COOH-terminal Tir fragment. Artificial clustering of this fusion protein revealed that the COOH terminus of Tir, by itself, is sufficient to initiate a complete signaling cascade leading to pedestal formation. Consistent with this finding, clustering of Nck by a 12-residue Tir phosphopeptide triggered 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 lesions on the intestinal epithelium (Frankel et al., 1998; Nataro and Kaper, 1998; Celli et al., 2000). Attaching and effacing lesions feature a loss of microvilli, intimate adherence of bacteria to host cells, and generation of F-actin pedestal structures beneath attached bacteria. The formation of actin pedestals by EPEC is recapitulated on cultured mammalian cells, and its ability to form pedestals correlates with its ability to cause disease in mammalian hosts (Donnenberg et al., 1993). In addition to its role in pathogenesis, pedestal formation by EPEC serves as a model system for studying the regulation of actin assembly at the plasma membrane.

All of the EPEC genes required for actin pedestal formation are contained within a 35-kb pathogenicity island called the locus of enterocyte effacement (LEE; McDaniel and Kaper, 1997; Elliott et al., 1998). 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. Known substrates of the type III system include the EPEC 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 through which other Esps are delivered to the host cell surface (Ebel et al., 1998; Knutton et al., 1998; Sekiya et al., 2001), whereas EspB and EspD form a pore in the plasma membrane to allow entry of other effectors into the cell (Wolff et al., 1998; Kresse et al., 1999; Wachter et al., 1999; Ide et al., 2001). Among these effectors are EspF, EspG, and Map, which affect several mammalian cell processes but do not play obvious roles in actin pedestal formation (Kenny and Jepson, 2000; Elliott et al., 2001; McNamara et al., 2001), and EspH, which enhances the kinetics of pedestal formation (Tu et al., 2003). Lastly, the

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Abbreviations used in this paper: EPEC, enteropathogenic *Escherichia coli*; LEE, locus of enterocyte effacement; MEF, mouse embryonic fibroblast; TirFL, full-length Tir.

translocated intimin receptor, Tir, is an effector essential for the assembly of F-actin pedestals.

Translocation of Tir into host cells is facilitated by its chaperone, CesT, which binds to the NH_2 terminus of Tir but is not itself secreted (Abe et al., 1999; Elliott et al., 1999). Translocated Tir inserts into the plasma membrane in a hairpin-loop conformation featuring a central extracellular domain that binds to intimin, a bacterial surface adhesin encoded by the *eae* gene of the LEE. Intimin is required for pedestal formation by EPEC (Jerse et al., 1990), and after translocation of Tir and other Esps, the interaction of intimin-coated particles with host cells triggers localized actin assembly into pedestals (Rosenshine et al., 1996; Liu et al., 1999). Intimin binding to endogenous mammalian receptors may also contribute to pedestal formation (Frankel et al., 2001), but the ability of intimin mutants to bind to Tir generally correlates with their ability to promote actin assembly (Liu et al., 2002).

The extracellular region of Tir serves as a bacterial receptor (Kenny et al., 1997; Deibel et al., 1998), and its cytoplasmic domains interact with mammalian components involved in actin assembly. The COOH terminus of Tir contains tyrosine 474, which is phosphorylated after entry into the host cell and is critical for actin pedestal formation (Kenny, 1999). Phosphorylated Y474 and flanking residues bind the host adaptor protein Nck (Gruenheid et al., 2001; Campellone et al., 2002), an activator 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 actin polymerization (Gruenheid et al., 2001), indicating that recruitment of Nck by Tir is an essential step in pedestal formation.

Recombinant derivatives of the cytoplasmic domains of Tir bind additional cytoskeletal components found within pedestals, such as the focal adhesion proteins α-actinin, talin, and vinculin (Freeman et al., 2000; Goosney et al., 2000; Cantarelli et al., 2001; Huang et al., 2002). It has been postulated that binding of α-actinin to the NH_2 terminus of Tir may stabilize the pedestal by anchoring EPEC to the cytoskeleton (Goosney et al., 2000). However, it has not been determined if the ability of Tir to bind focal adhesion proteins is necessary for localized actin assembly.

The roles that type III–secreted proteins other than Tir play during actin polymerization have been difficult to define for several reasons. First, the complete repertoire of Esps is unknown, so other unidentified effectors may be required for pedestal formation. Indeed, it has been proposed that other proteins must be codelivered with Tir to promote its tyrosine phosphorylation (Kenny and Warawa, 2001). Second, Esps with known roles in translocation of other molecules cannot easily be evaluated for additional effector functions. For example, EspB interacts with the actin-binding protein α-catenin (Kodama et al., 2002), EspB expression in mammalian cells induces actin rearrangements (Taylor et al., 1999), and EPEC strains lacking EspB do not generate pedestals (Foubister et al., 1994). However, the analysis of EPEC *espB* mutants is limited by the fact that such mutants are incapable of translocating effectors such as Tir (Kenny et al., 1997). Similarly, it is difficult to assess the roles of EspA and EspD in localized actin assembly because they are also necessary for effector translocation. Hence, the current uncertainty in the set of molecules required for actin pedestal formation has complicated the formulation of models for how EPEC stimulates mammalian signaling cascades.

To determine 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 the tyrosine-phosphorylated COOH terminus of Tir beneath the plasma membrane is sufficient to trigger a Nck-dependent signaling cascade that leads to actin pedestal formation. Consistent with these findings, particles coated with a small Nck-binding phosphopeptide, derived from this domain of Tir, assemble F-actin tails in vitro. Hence, all mammalian components essential for localized actin assembly in this system are recruited as a consequence of Nck binding by the Tir peptide.

Results

Intimin-expressing bacteria attach efficiently to mammalian cells expressing membrane-targeted Tir

To examine the requirements for actin pedestal formation by EPEC, Tir derivatives were ectopically expressed in cultured epithelial cells by transient transfection. First, a full-length Tir (TirFL) containing an NH_2-terminal HA-epitope tag was generated (Fig. 1 A). However, because Tir is normally delivered to the plasma membrane by the bacterial type III translocon, the export of mammalian-expressed TirFL to the cell surface by the eukaryotic secretory machinery might be inefficient. Therefore, a derivative of Tir predicted to be targeted to the plasma membrane was generated by replacing its NH_2-terminal cytoplasmic domain and first transmembrane segment with the NH_2 terminus, transmembrane sequence, and first 18 extracellular residues of the Newcastle Disease Virus HN protein. HN is a type II transmembrane protein, normally inserted into the ER with its NH_2 terminus in the cytoplasm and efficiently transported to the plasma membrane. This HN segment was followed by an HA tag and was predicted to direct surface expression of a fragment of Tir containing the intimin-binding extracellular loop and the COOH-terminal cytoplasmic domain (Fig. 1 A, TirMC).

Microscopic examination of transfected cells using fluorescently labeled anti-HA antibodies indicated that TirFL was enriched in perinuclear regions and was generally present throughout the entire cytoplasm (Fig. 1 B). TirMC, in addition to cytoplasmic staining, localized to the periphery of transfected cells (Fig. 1 B). Ectopic expression of Tir did not appear to promote actin assembly because no gross changes in the distribution of F-actin were observed in Tir-expressing cells (Fig. 1 B).

To determine if Tir was displayed at the cell surface in a functional form, transfected cells were tested for the ability to bind a K-12 laboratory strain of *E. coli* expressing plasmid-encoded intimin independent of other LEE components. Tir-expressing cells were identified by anti-HA fluorescence, and bacterial binding was quantitated microscopically. Approximately 20% of cells expressing TirFL and 90% of cells express-

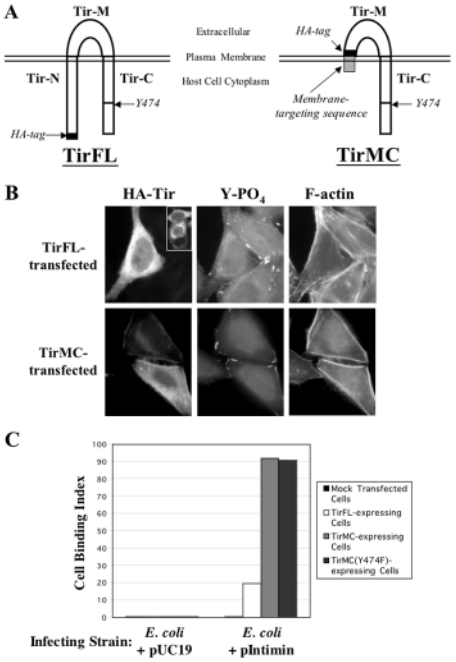

Figure 1. **Intimin-expressing bacteria attach efficiently to mammalian cells expressing membrane-targeted Tir.** (A) Full-length Tir (TirFL) and TirMC, a Tir derivative lacking the NH_2-terminal cytoplasmic domain, are depicted. TirMC was directed to the plasma membrane by an NH_2-terminal targeting sequence derived from a viral HN-protein. Arrows indicate the positions of HA-epitope tags and Y474. (B) HEp-2 cells transfected with plasmids encoding TirFL or TirMC were stained with an mAb to the HA epitope to identify Tir-expressing cells, with an mAb to visualize phosphotyrosine ($Y\text{-}PO_4$), 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 anti-HA fluorescence) were challenged with a laboratory strain of *E. coli* carrying a plasmid-encoding intimin or a pUC19 vector control. Cell binding index, defined as the percentage of cells with at least five bacteria bound, was measured microscopically, and data represent the mean of two experiments.

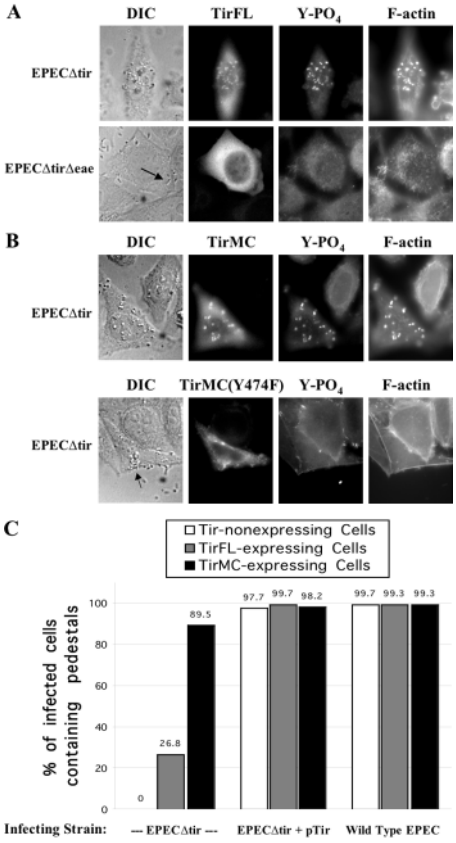

Figure 2. **Tir expressed in mammalian cells allows a *tir*-deficient EPEC strain to form actin pedestals in the absence of the NH_2-terminal cytoplasmic domain.** (A) HEp-2 cells expressing TirFL were infected with either EPEC*Δtir* (top panels) or EPEC*ΔtirΔeae* (bottom panels) and stained as described in the Fig. 1 legend. The arrow indicates positions of several bound bacteria. (B) HEp-2 cells expressing either TirMC (top panels) or a Y474F mutant of TirMC (bottom panels) were infected with EPEC*Δtir* and stained as described in the Fig. 1 legend. The arrow indicates positions of several bound bacteria. (C) HeLa cells transfected with plasmids encoding TirFL or TirMC were infected with wild-type EPEC, EPEC*Δtir* + pTir, or EPEC*Δtir*. Cells with at least five bound bacteria were examined, and the percentage of Tir-expressing cells (identified by anti-HA fluorescence) or Tir-nonexpressing cells generating actin pedestals was quantitated. Data represent the means of triplicate samples of 200 cells each. Similar results were observed in independent experiments.

ing TirMC bound at least five intimin-expressing bacteria, whereas only 0.5% of mock-transfected cells demonstrated this degree of bacterial binding (Fig. 1 C). As expected, TirFL- or TirMC-expressing cells did not bind an isogenic *E. coli* strain that did not express intimin (Fig. 1 C). Thus, TirFL and TirMC both 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, even in the absence of the NH_2-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 EPEC*Δtir*, which expresses intimin but not Tir, and examined for localized actin assembly as indicated by high-intensity F-actin staining beneath bound bacteria. TirFL was enriched at sites of EPEC*Δtir* adherence, and actin pedestals were formed beneath bound bacteria (Fig. 2 A). Intimin was required for actin assembly because interaction of cells with EPEC*ΔtirΔeae* did not alter the distribution of TirFL or F-actin (Fig. 2 A). Thus, Tir does not undergo any essential modifications in the bacterial cytosol, nor does it require codelivery with other effectors through the type III translocon to function in actin pedestal formation.

The NH_2 terminus of Tir, absent in TirMC, is necessary for translocation from EPEC (Abe et al., 1999; Elliott et al., 1999; Crawford and Kaper, 2002) and also binds to focal adhesion proteins (Freeman et al., 2000; Goosney et al., 2000; Huang et al., 2002). The efficient targeting of TirMC to the plasma membrane allowed us to test whether this NH_2-terminal domain is required for actin pedestal formation. When cells expressing TirMC were challenged with EPEC*Δtir*, TirMC was recruited to sites of bacterial attachment and actin pedestals were formed (Fig. 2 B), demonstrating that the NH_2 terminus of Tir is not required for actin assembly.

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 EPEC*Δtir* harboring a plasmid encoding HA-tagged Tir generated pedestals on nearly every cell after 3-h infections, regardless of whether or not these cells expressed TirFL or TirMC (Fig. 2 C). When challenged with EPEC*Δtir*, ~27% of TirFL-expressing cells and nearly 90% of TirMC-expressing cells generated pedestals (Fig. 2 C). These quantities correlate with the surface localization levels of the two Tir derivatives (Fig. 1 C) and indicate that ectopically expressed Tir, even when lacking the entire NH_2-terminal cytoplasmic domain, is capable of efficiently triggering actin pedestal formation.

Clustering of the COOH terminus of Tir beneath the plasma membrane is sufficient to trigger 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 K-12 laboratory strain of *E. coli* that expresses intimin but does not possess a LEE. These bacteria also generated pedestals, whereas bacteria that did not express intimin did not trigger actin assembly (Fig. 3 A). Thus, Tir is the only translocated EPEC effector required for actin pedestal formation. Moreover, latex beads coated with a fusion protein containing the Tir-binding domain of intimin (Liu et al., 1999) also formed pedestals on TirMC-expressing cells (unpublished data), indicating that this domain of intimin is sufficient to trigger localized actin assembly in cells expressing Tir.

It has been suggested that binding of intimin to host cell receptors, in addition to Tir, may contribute to pedestal formation (Frankel et al., 2001). Therefore, we tested the effect of intimin-independent clustering of Tir on pedestal formation in TirMC-expressing cells. After exposure to antibodies that recognize TirM, the extracellular domain of Tir, unbound antibodies were removed and anti-TirM bound to the surface of Tir-expressing cells was clustered by the addition of *Staphylococcus aureus* particles, which bind the Fc portion of antibodies via protein A on their surface. These cell-associated *S. aureus* particles also triggered actin assembly into pedestals in TirMC-expressing cells (Fig. 3 B).

These results suggested that the sole function of the extracellular region of Tir is to promote clustering of its COOH-terminal cytoplasmic domain by virtue of its intimin-binding activity. To cluster this domain without any direct interaction with the intimin-binding region, transfected cells were treated with an anti-HA mAb, which recognizes the extracellular HA-tag of TirMC. Because the murine anti-HA antibody does not bind protein A efficiently, anti-HA antibodies bound to cells were clustered using *S. aureus* coated with anti–mouse IgG. Actin pedestal formation occurred beneath these adherent *S. aureus* particles, whereas TirMC-expressing cells that were not treated with the anti-HA antibody did not form pedestals (Fig. 3 B). Overall, these results indicate that clustering of the cytoplasmic COOH terminus 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.

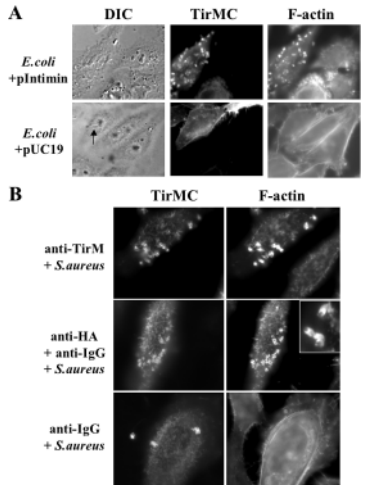

Figure 3. **Clustering of the COOH terminus of EPEC Tir beneath the plasma membrane is sufficient to trigger actin pedestal formation.** (A) HEp-2 cells expressing TirMC were challenged with a laboratory strain of *E. coli* harboring either an intimin-expressing plasmid (top panels) or a vector control (bottom panels). Tir and F-actin were visualized with an anti-HA antibody and phalloidin, respectively. The arrow indicates positions of bound bacteria. (B) 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 (bottom panels). Then, they were challenged with *S. aureus* particles that carry protein A on their surface (top panels) or anti–mouse IgG-coated *S. aureus* (middle and bottom panels). Tir and F-actin were visualized as described in A. *S. aureus* particles are visible when staining for TirMC because they bind the fluorescently labeled secondary antibody. The inset shows a magnification of several pedestals.

Actin pedestal formation resulting from clustering of TirMC is quantitatively, kinetically, and morphologically similar to pedestal formation after EPEC infections

To determine if EPEC effectors other than Tir contribute to actin assembly, the percentage of particles associated with pedestals was quantitated. Approximately 75–85% of wild-type EPEC or EPEC*Δtir* + pTir that were bound to TirMC-expressing cells generated pedestals, and EPEC*Δtir*, *E. coli* K-12 expressing intimin, and anti-HA–coated *S. aureus* each formed pedestals with a similar efficiency (Fig. 4 A). Anti-TirM–coated *S. aureus* was actually slightly more efficient at triggering actin assembly than wild-type EPEC (Fig. 4 A). Hence, neither the NH_2 terminus of Tir nor other EPEC effectors measurably contribute to the efficiency of actin pedestal formation.

Although EPEC effectors other than Tir are not necessary for actin assembly, EspH is reported to enhance the rate of pedestal formation (Tu et al., 2003). To test the effects of EspH and other effectors on the rate of pedestal formation when Tir is already present in the plasma membrane, actin assembly in response to intimin-expressing bacteria was compared in cells expressing TirMC and in cells preinfected ("primed") with an EPEC strain that translocates TirFL and other effectors but lacks intimin. In both cell types, pedestals were observed as early as 5 min after challenge, and the percentage of cells containing pedestals began to plateau after 15 min (Fig. 4 B). In addition, the morphology of pedestals on transfected cells matched pedestals on primed cells; pedestals were long at early time points and more compact at later time

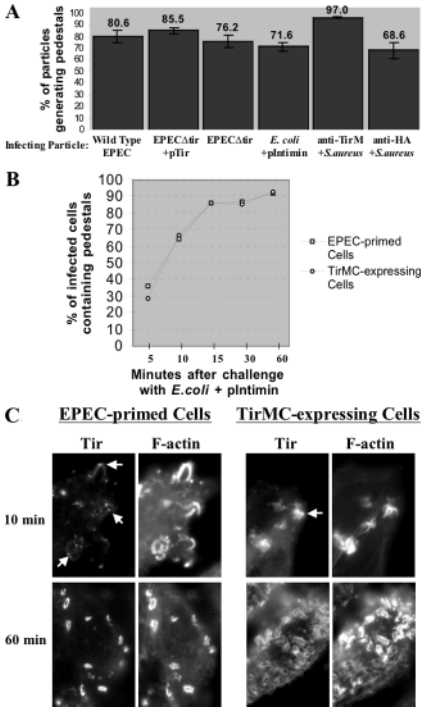

Figure 4. **Actin pedestal formation resulting from clustering of TirMC is quantitatively, kinetically, and morphologically similar to pedestal formation after EPEC infections.** (A) TirMC-expressing HeLa cells were challenged with particles of the indicated type, and the percentage of bound particles generating actin pedestals was quantitated. Data represent the means ± SD of three experiments in which 50 cells were examined for each type of particle in each experiment. A range of 330–635 particles was counted per 50 cells. (B) HeLa cells were either transfected with a plasmid encoding TirMC or were primed with an EPEC strain that delivers TirFL and other Esps to host cells but does not express intimin. Cells were subsequently challenged with *E. coli* expressing intimin, fixed at various time points, and quantitated for pedestal formation as described in the Fig. 2 legend. Each data point represents the mean of two independent experiments in which 200 cells were examined. (C) The transfected or EPEC-primed HeLa cells described in B were examined at 10 or 60 min after 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.

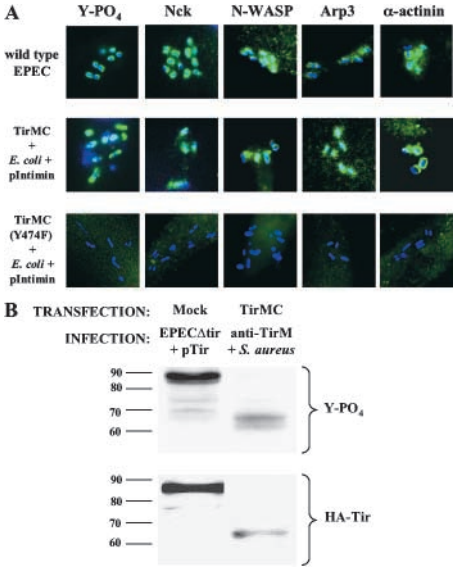

Figure 5. **Clustering results in tyrosine phosphorylation of TirMC and recruitment of Nck, N-WASP, Arp2/3 complex, and α-actinin.** (A) HeLa cells were either mock transfected and infected with wild-type EPEC (top panels) or were transfected with plasmids encoding TirMC or TirMC(Y474F) and challenged with *E. coli* expressing intimin (middle and bottom panels). Cells were stained with antibodies to detect phosphotyrosine (Y-PO_4), Nck, N-WASP, Arp3, or α-actinin (all shown in green). Bacterial DNA was visualized by DAPI staining (blue). (B) HeLa cells were mock transfected and infected with EPEC*Δtir* + pTir (left lane), or were transfected with a plasmid encoding TirMC and treated with particles coated with anti-TirM antibodies (right lane). HA-tagged Tir was immunoprecipitated from cell lysates and Western blotted for HA-Tir and phosphotyrosine.

points (Fig. 4 C). Therefore, once Tir is present in the plasma membrane, the kinetics and patterns of pedestal formation after clustering is independent of other bacterial effectors.

Indistinguishable signaling cascades are initiated by wild-type EPEC and by clustering of the COOH terminus of Tir

After delivery into host cells by the type III translocon, the COOH terminus of Tir is phosphorylated at tyrosine residue 474 to stimulate pedestal formation (Kenny, 1999). To determine whether actin assembly promoted by ectopically expressed Tir also displayed this feature, Tir-transfected cells were examined for phosphotyrosine residues. In the absence of clustering, phosphotyrosine staining of Tir-expressing and nontransfected cells was indistinguishable (Fig. 1 B), which is consistent with the previous observation that Tir expressed in mammalian cells is not efficiently phosphorylated (Kenny and Warawa, 2001). In contrast, clustering of ectopically expressed Tir by intimin presented on the surface of EPEC*Δtir* (Fig. 2, A and B) or *E. coli* K-12 (Fig. 5 A) generated phosphotyrosine-containing pedestals, similar to infection of cells with wild-type EPEC (Fig. 5 A). Immunoblotting confirmed that this staining was due to phosphorylation of Tir (Fig. 5 B). Therefore, Tir, the only EPEC protein present within transfected cells, is phosphorylated by mammalian tyrosine kinases in the absence of other EPEC effectors.

Tyrosine phosphorylation of wild-type Tir delivered by EPEC leads to recruitment of the host adaptor protein Nck and the localized activation of the N-WASP–Arp2/3 actin nucleation machinery (Gruenheid et al., 2001; Lommel et al., 2001; Campellone et al., 2002). To determine if these same factors were recruited to pedestals resulting from clustering of the COOH terminus of Tir, their localization was examined in TirMC-expressing cells. As expected, Nck, N-WASP, and the Arp2/3 complex were recruited to sites of clustered TirMC (Fig. 5 A). In addition, the focal adhesion component α-actinin, an F-actin cross-linking protein normally present in EPEC pedestals (Goosney et al., 2000), also localized to TirMC-derived pedestals (Fig. 5 A).

To test whether binding of Nck to phosphorylated Y474 was required for actin pedestal formation mediated by TirMC, a derivative carrying a Y474F mutation was expressed in mammalian cells. TirMC(Y474F) localizes to the cell surface (Fig. 1 C), but neither EPEC*Δtir* (Fig. 2 B) nor *E. coli* expressing intimin (not depicted) effectively triggered actin assembly in cells expressing this Tir mutant. As pre-

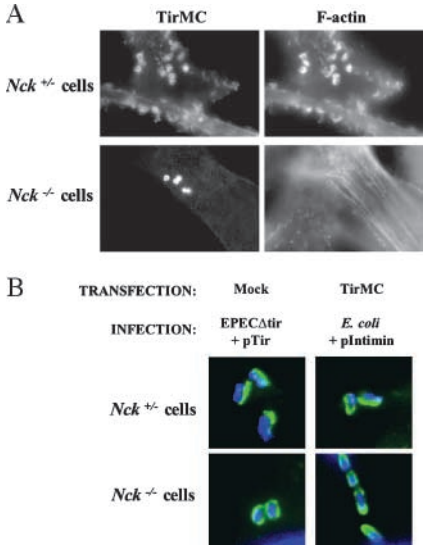

Figure 6. **Clustering of TirMC does not effectively trigger actin pedestal formation in cells that lack Nck.** (A) TirMC was expressed in MEFs that express Nck1 (*Nck* $^{+/-}$ cells; top panels) and MEFs that do not express either Nck1 or Nck2 (*Nck* $^{-/-}$ cells; bottom panels). Cells were treated with anti-TirM antibodies and *S. aureus* particles and visualized as described in the Fig. 3 legend. (B) Nck-proficient MEFs (top panels) and Nck-deficient MEFs (bottom panels) were either mock transfected and infected with EPECΔ*tir* + pTir (left panels) or were transfected with a plasmid encoding TirMC and challenged with *E. coli* expressing intimin (right panels). Cells were stained with antibodies to detect phosphotyrosine (green) and with DAPI to visualize bacterial DNA (blue).

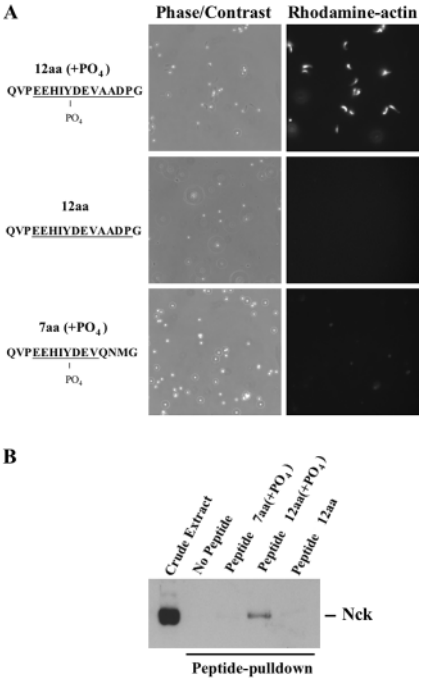

Figure 7. **A clustered Nck-binding peptide from EPEC Tir is sufficient to trigger actin assembly in vitro.** (A) Latex beads were coated with 12aa(+PO_4), a peptide that binds human Nck; 12aa, a nonphosphorylated peptide that lacks Nck-binding activity; or 7aa(+PO_4), a peptide that binds Nck poorly (Campellone et al., 2002). EPEC Tir residues present within the depicted peptides are underlined. Peptide-coated beads were added to *Xenopus* egg extracts supplemented with rhodamine-actin and examined microscopically. (B) *Xenopus* egg extracts were supplemented with the peptides described in A. Biotinylated peptides and associated proteins were collected from extracts using streptavidin-labeled particles and subjected to anti-Nck immunoblot analysis (all lanes except Crude Extract). Crude Extract lane is equivalent to 1/20 of the amount of extract represented in the bead-associated lanes.

dicted, Nck, N-WASP, the Arp2/3 complex, and α-actinin were not efficiently recruited to sites of clustered TirMC(Y474F) (Fig. 5 A). To directly examine a requirement for Nck, TirMC was expressed in Nck-deficient and isogenic Nck-proficient mouse embryonic fibroblasts (MEFs; Gruenheid et al., 2001; Bladt et al., 2003). As expected, actin pedestals were efficiently generated after TirMC was clustered in the plasma membrane of mouse cells that expressed Nck, but not in Nck-deficient MEFs (Fig. 6 A). Moreover, within each cell line, the frequency of pedestal formation after clustering of TirMC was quantitatively indistinguishable from the frequency after infection with EPECΔ*tir* + pTir (unpublished data). The defect in actin assembly in Nck-deficient cells is apparently not due to insufficient tyrosine phosphorylation of Tir because phosphotyrosine staining beneath sites of clustered Tir was similarly evident in the presence or absence of Nck (Fig. 6 B). Overall, these results demonstrate that clustering of the cytoplasmic COOH terminus of Tir likely initiates the same signaling cascade leading to actin assembly that EPEC activates after infection of mammalian cells. Thus, we cannot discern a role in actin assembly for any EPEC-encoded factors other than the COOH terminus of Tir.

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

Because the COOH terminus of EPEC Tir is clearly sufficient to direct localized actin assembly in host cells after clustering and this domain contains the critical Nck-binding site (Gruenheid et al., 2001; Campellone et al., 2002), we sought to determine whether this binding site was sufficient to initiate actin assembly. A small Tir-derived phosphopeptide, termed 12aa(+PO_4), was previously shown to be capable of binding to recombinant Nck (Campellone et al., 2002). Bacterium-sized latex beads coated with this peptide were added to *Xenopus* egg extracts, an experimental system that has been extensively used in the study of actin-based motility. Robust actin tails assembled on these beads but not on beads coated with the nonphosphorylated form of the peptide, 12aa (Fig. 7 A). Beads coated with 7aa(+PO_4), a tyrosine-phosphorylated Tir peptide that binds Nck poorly (Campellone et al., 2002), occasionally generated weak actin clouds (Fig. 7 A). As expected, Nck from *Xenopus* extracts coprecipitated with beads coated with 12aa(+PO_4) (Fig. 7 B).

To test whether Nck is essential for actin assembly in this system, immunodepletion experiments were performed. Actin tail formation was dramatically reduced when beads coated with peptide 12aa(+PO_4) were added to Nck-depleted *Xenopus* extracts (Fig. 8). The addition of recombinant GST-Nck restored actin tail formation to Nck-depleted extracts, and these actin tails appear to be more intense than tails formed in untreated extracts (Fig. 8).

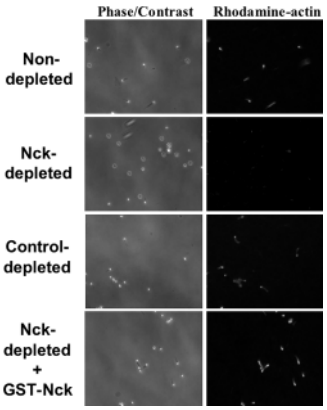

Figure 8. **Nck is critical for actin assembly initiated by Tir peptide 12aa(+PO_4).** *Xenopus* extracts were left untreated or were subjected to two rounds of immunodepletion with beads coated with either an anti-Nck antibody or with a rabbit IgG control antibody. Nck depletion was confirmed by immunoblot (not depicted). Recombinant GST-Nck was added to the Nck-depleted extract in the bottom panels. Extracts were supplemented with rhodamine actin, and beads coated with peptide 12aa(+PO_4) were added.

Overall, these results demonstrate that clustering of Nck by a small Tir phosphopeptide is sufficient to trigger localized actin assembly and suggest that the only critical function that EPEC performs to initiate actin polymerization is to bind and recruit Nck.

Discussion

Formulation of models for how EPEC initiates pedestal formation in mammalian cells has been hampered by difficulties in identifying the entire repertoire of bacterial effectors necessary for actin assembly. Expression of Tir in mammalian cells now demonstrates that no other translocated proteins are required for actin pedestal formation. Cells expressing Tir were fully capable of forming pedestals after challenge with an *E. coli* K-12 strain that expresses intimin, but that does not translocate any bacterial molecules into 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, contained the same principle host factors, 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 pedestal formation.

A previous paper indicated that Tir expressed in mammalian cells was not modified to its mature tyrosine-phosphorylated form, suggesting that other codelivered effectors may be required for Tir to reach its completely modified state (Kenny and Warawa, 2001). In this work, we also found that mammalian expression of Tir did not result in an observable increase in phosphotyrosine staining. However, phosphotyrosine residues colocalized with Tir after clustering, implying that oligomerization induces phosphorylation of Tir by mammalian tyrosine kinases independently of other EPEC effectors. Indeed, immunoprecipitation of Tir and Western blotting with antiphosphotyrosine antibodies confirm that clustering triggers a dramatic increase in Tir tyrosine phosphorylation (unpublished data). Although it remains possible that additional EPEC molecules facilitate serine/threonine phosphorylation of Tir, these 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 participates directly in actin pedestal formation, is to cluster Tir in the plasma membrane, thereby triggering phosphorylation of Y474. This finding is consistent with the previously observed correlation between the ability of intimin to bind to Tir and its ability to initiate actin assembly in EPEC-primed cells (Liu et al., 2002). Here, we demonstrate that intimin can be replaced by entirely unrelated molecules, i.e., antibodies that artificially cluster Tir. The observation that anti-HA antibodies trigger actin assembly in cells that express HA-tagged Tir indicates that Tir signaling does not even require engagement of its intimin-binding domain.

Another domain of Tir, the NH_2-terminal cytoplasmic region, binds the focal adhesion proteins α-actinin, talin, and vinculin in vitro (Freeman et al., 2000; Goosney et al., 2000; Huang et al., 2002). This work reveals that these interactions do not play any observable role in pedestal formation because TirMC, which completely lacks this domain, is fully capable of signaling host cells to generate pedestals. The COOH-terminal cytoplasmic domain of Tir has also been reported to bind to α-actinin (Freeman et al., 2000). However, this interaction is not sufficient to promote recruitment within cells because α-actinin is not detectable beneath sites of clustered TirMC(Y474F), a Tir derivative defective at actin polymerization. Because α-actinin does localize to TirMC-derived pedestals, its recruitment may be a simple consequence of localized actin assembly initiated by the COOH terminus of Tir.

The best-characterized activity of the COOH-terminal domain of Tir is its ability to bind the SH2 domain of Nck, a host adaptor protein required for EPEC pedestal formation (Gruenheid et al., 2001; Campellone et al., 2002). Nck is also critical for actin pedestal formation initiated by ectopically expressed TirMC, and clustering of this Tir derivative triggers recruitment of Nck, N-WASP, and the Arp2/3 actin-nucleating complex, the mammalian components necessary for pedestal formation. Moreover, we found that the minimal Nck-binding phosphopeptide derived from Tir, by itself, is sufficient to stimulate actin assembly in *Xenopus* extracts after immobilization on beads. We cannot rule out the possibility that domains in the COOH terminus of Tir other than the Nck-binding sequence may also contribute to actin assembly, or that the requirements for actin polymerization in vitro may be different than at the membrane of intact cells. Nevertheless, these results support a remarkably simple model for pedestal formation, i.e., the ultimate role of the type III secretion apparatus, intimin, and Tir in actin assembly is to cluster a 12-residue Nck-binding site beneath the plasma membrane. This sequence, after being tyrosine phosphorylated by host kinases, is sufficient to trigger a signaling cascade leading to localized actin polymerization. This model implies that all other components required for actin assembly are recruited subsequent to engagement of Nck by the Tir phosphopeptide.

It is not yet clear if actin assembly is simply a consequence of a high local concentration of Nck, or whether Tir-binding by Nck somehow "activates" the adaptor for downstream signaling. Interestingly, a derivative of TirMC in which the cytoplasmic COOH terminus was replaced with Nck failed to stimulate actin assembly after clustering beneath the plasma membrane (unpublished data), and GST-Nck–coated latex beads did not trigger actin tail formation in *Xenopus* extracts (Ho, H., personal communication; unpublished data). These results suggest that the recruitment of Nck, specifically by Tir, may be critical for triggering localized actin assembly.

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 suggest that recruitment of Nck by A36R, which contains a Nck-binding site nearly identical to that of Tir, may also be sufficient for initiation of actin assembly. After recruitment to the plasma membrane, the SH3 domains of Nck may interact directly with the proline-rich region of N-WASP (Rohatgi et al., 2001), a protein required for actin assembly by both EPEC and vaccinia (Lommel et al., 2001; Snapper et al., 2001). However, this domain of N-WASP is dispensable for the generation of pedestals; rather, the WH1 (WASP–homology-1) domain seems to be critical for recruitment to sites of EPEC adherence (Lommel et al., 2001). This observation suggests that EPEC signaling closely resembles the vaccinia pathway in which the interaction of Nck with N-WASP is apparently mediated by a third protein, WIP (WASP-interacting protein), which binds both to Nck and to the WH1 domain of N-WASP (Moreau et al., 2000).

In addition to binding 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, the 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 Nck signaling.

Materials and methods

Bacterial strains

All EPEC strains were derived from the parental O127:H6 strain JPN15/pMAR7 (Jerse et al., 1990). EPEC*Δtir* (KC14) and EPEC*Δtir* + pTir (pKC17) have been described previously (Campellone et al., 2002). EPEC*ΔtirΔeae* (KC10) was generated from EPEC*ΔtirΔeae::cat-sacB* (KC8) after electroporation of DNA that contains sequence flanking *tir* and *eae* but lacks *cat-sacB* (Campellone et al., 2002). EPEC*ΔtirΔeae* was transformed with pTir (which also encodes CesT) to generate a host cell priming strain. MC1061 is a nonpathogenic laboratory *E. coli* K-12 strain.

Plasmid construction

To create pIntimin, the *eae* gene was PCR amplified from EPEC genomic DNA and cloned into the HindIII and BamHI sites of pUC19. To generate pTirFL (pKC6), a sequence encoding amino acids 1–550 of Tir was inserted as a HindIII–SacII fragment downstream of HA within the transfection vector pHM6 (Roche). To construct a plasma membrane–targeted Tir derivative, a sequence encoding amino acids 1–66 (26 NH_2-terminal cytoplasmic residues, a 22-aa 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, University of Massachusetts Medical School, Worcester, MA) and cloned into the HindIII and XhoI sites of pCDNA3 (Invitrogen) to generate pHN-HA (pKC33). A sequence encoding Tir amino acids 260–550 was cloned into the XhoI and XbaI sites of pHN-HA to create pTirMC (pKC87). pTirMC(Y474F) (pKC187) was generated using PCR primers described previously (Campellone et al., 2002).

Bacterial and mammalian cell culture

For routine passage, all bacteria were cultured in LB at 37°C. To maintain Tir or intimin-expressing plasmids, media were supplemented with kanamycin or ampicillin, respectively. HEp-2 cells were cultured in RPMI-1640 plus 7% FBS, whereas HeLa cells and Nck1/Nck2-deficient (Nck $^{-/-}$) and Nck1-rescued (Nck $^{+/-}$) MEFs (Bladt et al., 2003) were cultured in DME plus 10% FBS.

Transfections and infections

HeLa cells, HEp-2 cells, and MEFs grown in six-well plates were transfected with 500 ng of plasmid per well for 12 h using Lipofectamine with Plus reagent (Invitrogen). Cells were reseeded onto 12-mm glass coverslips to achieve 50–90% confluency after an additional 24 h of growth. Cells were infected with $\sim 2 \times 10^6$ EPEC bacteria or *E. coli* MC1061 harboring pIntimin or pUC19 for 3 h as described previously (Campellone et al., 2002). For antibody clustering experiments, transfected cells were treated with a 1:200 dilution of antibodies raised against the extracellular domain of EHEC Tir (anti-TirM; a gift from A. Donohue-Rolfe, Tufts University School of Veterinary Medicine, North Grafton, MA) or the HA-epitope (HA.11; Covance) for 30 min and then removed. *S. aureus* particles (Pansorbin; Calbiochem) were added to anti-TirM–treated cells and *S. aureus* coated with goat anti–mouse IgG (Zymed Laboratories) was added to anti-HA–treated cells, each for a further 2.5 h. For EPEC-priming experiments, HA-tagged Tir was delivered to the plasma membrane by infecting mock-transfected HeLa cells with 4×10^7 EPEC*ΔtirΔeae* + pTir for 3 h. Bacteria were killed by treatment with 50 μg/ml gentamicin for 30 min and removed. Approximately 2×10^7 *E. coli* + pIntimin were added to transfected or primed monolayers for 5 to 60 min. After infections or particle treatments, cells were fixed and permeabilized (Campellone et al., 2002) and processed for immunofluorescence microscopy. HeLa and HEp-2 cells yielded similar experimental results and were used interchangeably.

Immunofluorescence microscopy

For triple-labeling experiments, transfected cells were treated with mAb HA.11 (1:1,000) and biotinylated antiphosphotyrosine mAb PY66 (1:100; Sigma-Aldrich) for 30 min. They were treated with Alexa488-conjugated streptavidin (1:50; Molecular Probes) for 30 min before the addition of 1 μg/ml TRITC-phalloidin (Sigma-Aldrich) to stain F-actin and Alexa350-conjugated anti–mouse IgG (1:100) for a further 30 min. For double labeling, cells were treated with HA.11 followed by TRITC-phalloidin and Alexa488 anti–mouse IgG (1:200). For single labeling, cells were treated with HA.11 followed by Alexa488 anti–mouse IgG. To examine the composition of actin pedestals, cells were treated with antiphosphotyrosine P-Tyr-100 (1:200; Cell Signaling), anti-Nck (1:200; Upstate Biotechnology), anti–N-WASP (1:1,000; Rohatgi et al., 1999), anti-Arp3 (1:100; a gift from R. Isberg, Tufts University School of Medicine, Boston, MA), or anti–α-actinin BM-75.2 (1:750; Sigma-Aldrich) followed by Alexa488-conjugated anti–mouse or anti–rabbit antibodies and DAPI to stain DNA.

Cell-binding assays

Approximately 2×10^7 *E. coli* MC1061 harboring either pIntimin or pUC19 were added to mock-, pTirFL-, pTirMC-, or pTirMC(Y474F)-transfected HeLa cells for 60 min and processed for DIC microscopy and single-label immunofluorescence. 200 random mock-transfected cells and 200 random cells that were clearly expressing Tir, as determined by intensity of anti-HA staining, were examined in each experiment. Cells containing five or more associated bacteria were scored as positive for cell binding; cells with less than five were scored as negative. Cell binding index equals the percentage of cells harboring at least five bound bacteria.

Pedestal quantitation

To measure the percentage of infected cells generating pedestals, cells containing 5–20 bound particles were examined after double labeling. Tir-expressing cells were identified by their intense anti-HA fluorescence. Cells that exhibited HA fluorescence similar to mock-transfected cells

were considered nonexpressers. A cell was scored as positive if at least 50% of bound particles generated pedestals and negative if <50% of bound particles generated pedestals. 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.

Immunoprecipitation

Monolayers of HeLa cells grown in six-well plates were infected with EPEC*Δtir* + pTir or were treated with anti-TirM and *S. aureus* before lysis with 0.4 mL of lysis buffer (50 mM Hepes, pH 7.4, 50 mM NaCl, 1% Triton X-100, 1 mM Na_3VO_4, 1 mM PMSF, and 10 mg/ml each of aprotinin, leupeptin, and pepstatin [Sigma-Aldrich]) and collection with a cell scraper. Cellular debris and bacteria were removed by centrifugation for 5 min at 10,000 *g* at 4°C. HA-tagged Tir was precipitated by treatment with 1–2 μg of HA.11 plus protein G–agarose beads (Sigma-Aldrich) for 2 h at 4°C. Beads and bound protein were centrifuged, washed thrice with lysis buffer, and resuspended in loading buffer. Boiled samples were resolved by 10% SDS-PAGE and immunoblotted with biotinylated HA.11 or biotinylated PT-66 and developed as described previously (Campellone et al., 2002).

Xenopus extract assays

Xenopus egg extracts (~5 mg/ml) were prepared as described previously (Ma et al., 1998). Biotinylated Tir peptides have also been described previously (Campellone et al., 2002). For peptide pull-downs, streptavidin MagneSphere particles (Promega) were washed and resuspended in wash buffer (20 mM Hepes, pH 7.7, 200 mM KCl, 1 mM $MgCl_2$, 0.1 mM EDTA, 0.5 μM microcystin, and 1 μg/ml ovalbumin). 200 μl of extract was mixed for 45 min at 4°C with 2 μg of each peptide before addition of 50 μl of magnetic particles for a further 15 min. Using a magnetic particle collector (Dynal), beads were washed six times before resuspension in gel-loading buffer. For actin assembly assays, extracts were reconcentrated to 0.2× vol after 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. Tir peptides were bound to streptavidin-labeled 1-μm latex beads (Sigma-Aldrich) in 2× Hepes-buffered saline (50 mM Hepes, pH 7.05, 280 mM NaCl, and 3.0 mM Na_2PO_4) and diluted 10-fold into egg extracts. For immunodepletion studies, Protein A Dynabeads (Dynal) were washed with PBS + 0.1% Triton X-100, and mixed with 7 μg of anti-Nck antibody (NeoMarkers) or rabbit IgG for 1 h. Beads were washed twice with PBS and three times with XB (30 mM Hepes, pH 7.7, 100 mM KCl, 2 mM $MgCl_2$, and 0.1 mM EDTA). Extracts were added to the beads and incubated on ice for 1 h with occasional mixing. Beads were removed using a magnetic particle concentrator, and depletion was repeated with freshly prepared antibody-coated beads. Where indicated, bacterially expressed GST-Nck was added to extracts at a final concentration of 7 nM. Images were collected 20–25 min after addition of beads to extracts.

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Note added in proof. Recently, Rivera et al. (Rivera, G.M., C.A. Briceno, F. Takeshima, S.B. Snapper, and B.J. Mayer. 2004. *Curr. Biol.* 14:11–22) have demonstrated that clustering of a derivative of Nck that contains all three SH3 domains, but not the SH2 domain, induces localized actin assembly at the plasma membrane.

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