Insulin receptor tyrosine kinase substrate links the *E. coli* O157:H7 actin assembly effectors Tir and $EspF_U$ during pedestal formation

Didier Vingadassalom[a], Arunas Kazlauskas[b], Brian Skehan[a], Hui-Chun Cheng[c], Loranne Magoun[a], Douglas Robbins[a], Michael K. Rosen[c], Kalle Saksela[b], and John M. Leong[a,1]

[a]Department of Molecular Genetics and Microbiology, University of Massachusetts Medical School, Worcester, MA 01655; [b]Department of Virology, Haartman Institute, University of Helsinki and HUSLAB, Helsinki University Central Hospital, FIN-00014, Helsinki, Finland; and [c]Department of Biochemistry and Howard Hughes Medical Institute, University of Texas Southwestern Medical Center, Dallas, TX 75390

Edited by R. John Collier, Harvard Medical School, Boston, MA, and approved March 2, 2009 (received for review September 12, 2008)

Enterohemorrhagic *Escherichia coli* O157:H7 translocates 2 effectors to trigger localized actin assembly in mammalian cells, resulting in filamentous actin ''pedestals.'' One effector, the translocated intimin receptor (Tir), is localized in the plasma membrane and clustered upon binding the bacterial outer membrane protein intimin. The second, the proline-rich effector $EspF_U$ (aka TccP) activates the actin nucleation-promoting factor WASP/N-WASP, and is recruited to sites of bacterial attachment by a mechanism dependent on an Asn-Pro-Tyr (NPY_{458}) sequence in the Tir C-terminal cytoplasmic domain. Tir, $EspF_U$, and N-WASP form a complex, but neither $EspF_U$ nor N-WASP bind Tir directly, suggesting involvement of another protein in complex formation. Screening of the mammalian SH3 proteome for the ability to bind $EspF_U$ identified the SH3 domain of insulin receptor tyrosine kinase substrate (IRTKS), a factor known to regulate the cytoskeleton. Derivatives of WASP, $EspF_U$, and the IRTKS SH3 domain were capable of forming a ternary complex in vitro, and replacement of the C terminus of Tir with the IRTKS SH3 domain resulted in a fusion protein competent for actin assembly in vivo. A second domain of IRTKS, the IRSp53/MIM homology domain (IMD), bound to Tir in a manner dependent on the C-terminal NPY_{458} sequence, thereby recruiting IRTKS to sites of bacterial attachment. Ectopic expression of either the IRTKS SH3 domain or the IMD, or genetic depletion of IRTKS, blocked pedestal formation. Thus, enterohemorrhagic *E. coli* translocates 2 effectors that bind to distinct domains of a common host factor to promote the formation of a complex that triggers robust actin assembly at the plasma membrane.

enterohemorrhagic *Escherichia coli* | IRSp53/MIM homology domain | IRTKS | N-WASP | SH3 domain

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 is a food-borne pathogen that is an important agent of both diarrheal and systemic disease (1). Along with the closely related pathogen, enteropathogenic *E. coli* (EPEC), it is a member of the attaching and effacing (AE) family of Gram-negative enteric pathogens, so named because they generate striking histopathological lesions on intestinal epithelia, characterized by a loss of microvilli, intimate attachment of the bacteria to the host cell, and the formation of filamentous (F)-actin-rich pedestal structures beneath the host cell membrane at sites of bacterial attachment (1). The ability to form AE lesions correlates with the ability to colonize the intestine and cause disease in animal models (2, 3). In addition, the ability to stimulate the localized assembly of F-actin in the host cell has been a model for understanding the control and modification of the mammalian cytoskeleton.

Actin pedestal formation by EHEC and EPEC depends on the delivery of bacterial effector proteins into host cells via a type III secretion system (4, 5). One effector required for pedestal formation is the translocated intimin receptor (Tir) (6, 7). After translocation into host cell, Tir adopts a hairpin loop conformation in the host cell plasma membrane with N- and C-terminal intracellular domains and a central extracellular domain that binds to the bacterial outer membrane protein intimin. Clustering of Tir in the host cell membrane upon intimin binding initiates a signaling cascade, ultimately leading to actin pedestal formation.

For the canonical EPEC strain, serotype O127:H6, Tir is the only translocated effector required for pedestal formation, and after becoming phosphorylated on tyrosine residue 474 (Y474) by mammalian kinases, recruits the SH2 domain-containing mammalian adapter protein Nck (8, 9). Nck promotes recruitment of the neuronal Wiskott-Aldrich syndrome protein (N-WASP), which in turn activates actin assembly by stimulating the actin nucleating complex Arp2/3 (10).

In contrast, EHEC O157:H7 Tir generates pedestals independent of Nck (11). The C-terminal cytoplasmic domain of EHEC Tir harbors an Asn-Pro-Tyr_{458} (NPY_{458}) sequence that is essential for actin signaling (12–14). In addition, EHEC translocates into host cells a second effector, $EspF_U$ (aka TccP) that acts in concert with Tir to promote pedestal formation (15, 16). An EHEC$\Delta espF_U$ mutant generates pedestals at approximately one tenth the efficiency of WT on cultured monolayers (15) and is impaired at the expansion of an initial infectious niche during infection of infant rabbits (17). $EspF_U$ contains multiple 47-aa proline-rich repeats, and a 20-residue sequence of the repeat is capable of binding and activating WASP/N-WASP (15, 18–20). $EspF_U$ is recruited to sites of bacterial attachment in a manner dependent on the Tir NPY_{458} sequence (13), and Tir and $EspF_U$ form a co-immunoprecipitable complex with N-WASP in infected cells (15).

Although N-WASP and $EspF_U$ are in complex with Tir, neither protein appears to directly bind this protein (15, 16), suggesting that another factor (or factors) binds Tir and promotes complex formation. No other bacterial effectors besides Tir and $EspF_U$ are required for pedestal formation (21), so this putative factor is likely of host origin. In addition, given that actin pedestal formation occurs, albeit at low levels, in the absence of $EspF_U$, the putative host factor may itself stimulate actin assembly. In the current study, we report that the insulin receptor tyrosine kinase substrate (IRTKS), a homologue of insulin receptor substrate protein of 53 kDa (IRSp53) and thus a

Author contributions: D.V., A.K., B.S., H.-C.C., M.K.R., K.S., and J.M.L. designed research; D.V., A.K., B.S., H.-C.C., L.M., and D.R. performed research; M.K.R., K.S., and J.M.L. contributed new reagents/analytic tools; D.V., A.K., B.S., H.-C.C., M.K.R., K.S., and J.M.L. analyzed data; and D.V. and J.M.L. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

See Commentary on page 6431.

[1]To whom correspondence should be addressed. E-mail: john.leong@umassmed.edu.

This article contains supporting information online at www.pnas.org/cgi/content/full/0809131106/DCSupplemental.

member of a protein family that is capable of transducing actin assembly signals in mammalian cells, is targeted by both Tir and $EspF_U$ and is thus essential to the formation of a potent actin assembly complex during EHEC pedestal formation.

Results

The SH3 Domains of IRTKS and IRSp53 Bind the C-Terminal Proline-Rich Region of EHEC $EspF_U$, Localize to Actin Pedestals, and Trigger Pedestal Formation When Artificially Clustered as Tir Fusion Proteins. The C-terminal 47-residue repeats of $EspF_U$ each contain an amphipathic helix that interacts with WASP/N-WASP (18, 20), as well as a region that harbors up to 3 copies of the sequence PxxP, a motif associated with recognition by SH3 domain-containing proteins (22). To identify possible SH3 domain-containing host proteins that could link $EspF_U$ and Tir, we screened an essentially complete collection of human SH3 domains expressed on phage surface (23) for the ability to bind to GST-$EspF_U$C, a GST fusion protein containing 6 C-terminal proline-rich repeats of $EspF_U$ [supporting information (SI) Fig. S1]. Affinity panning of the phage display library revealed that GST-$EspF_U$C bound avidly to SH3 clones, as indicated by more than 100-fold higher recruitment of phages than observed with a GST protein that was used as a negative control (not shown). Sequencing of resultant phagemids revealed that the SH3 domains of IRTKS (24) or its close homologue IRSp53 were the only clones consistently enriched, and constituted 70% of the selected phages isolated from these enrichments. IRSp53, via its SH3 domain, interacts with known regulators of actin assembly, such as Scar2/WAVE2 and N-WASP (25). In addition to the SH3, IRSp53 and IRTKS contain an N-terminal IRSp53/MIM-homology domain (IMD) that may bundle actin, bind and deform membranes, and interact with small G proteins (26).

To better define the region of $EspF_U$ recognized by the SH3 domains of IRSp53 and IRTKS, derivatives of $EspF_U$C were tested in yeast 2-hybrid assays for their ability to interact with the IRSp53 or IRTKS SH3 domains. A single 47-residue repeat ("R47," Fig. S2) of $EspF_U$ was capable of SH3 binding because co-expression of $SH3_{IRTKS}$ or $SH3_{IRSp53}$ fusions with an R47 fusion activated the β–galactosidase reporter between 35- and 120-fold (Fig. S2). This signal was specific to SH3 domains, as neither IMD_{IRTKS} nor IMD_{IRSp53} interacted with R47, and required the proline-rich sequence of an $EspF_U$ repeat, because R33, a 33-residue fragment of $EspF_U$ that lacks most of the proline-rich sequence, did not bind either SH3 domains.

To determine if the interaction of IRSp53 and IRTKS with $EspF_U$ detected in vitro is reflected by recruitment to actin pedestals, we examined the distribution of IRSp53 and IRTKS in infected cells by immunofluorescence microscopy. Upon EHEC infection of HeLa cells, both IRSp53 and IRTKS were recruited to the tip of phalloidin-stained actin pedestals (Fig. 1*A*), similar to the localization of $EspF_U$ (15, 16).

As pedestal formation involves direct interaction of $EspF_U$ with the GTPase binding domain (GBD) of WASP/N-WASP (15, 16, 18–21), we tested whether binding of the IRTKS SH3 domain to $EspF_U$ was compatible with simultaneous binding to GBD_{WASP}. GBD_{WASP}, fluorescently labeled with FITC, was added to $EspF_U$-5R, a 5-repeat derivative of $EspF_U$ (26), or to both $EspF_U$-5R and GST-$SH3_{IRTKS}$ (Fig. S1), all at equivalent molar concentrations (taking into account the 5 repeats of $EspF_U$-5R). The relative size of GBD_{WASP}-containing complexes, detected by absorbance at 494 nm, was determined by gel filtration chromatography. As expected, GBD_{WASP} bound to $EspF_U$-5R, as indicated by an increase in the apparent size (i.e., earlier elution) of FITC-GBD_{WASP} (Fig. 2, blue vs. purple traces). The addition of GST-$SH3_{IRTKS}$ caused a further shift of the GBD to a more rapidly eluting peak (Fig. 2, green trace).

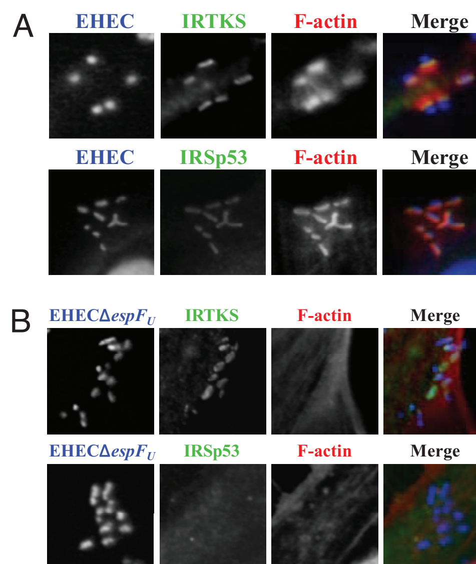

Fig. 1. IRTKS and IRSp53 are recruited to actin pedestals but only IRTKS localizes to sites of bacterial attachment independently of $EspF_U$. (*A*) HeLa cells were infected with EHECΔ*dam*, which generates actin pedestals more efficiently on cultured mammalian cells than does WT EHEC (thereby facilitating evaluation of recruitment; ref. 38), and examined after staining with anti-IRSp53 or anti-IRTKS antibody (green), DAPI to localize attached bacteria (blue), and Alexa568-phalloidin (red). (*B*) HeLa cells were infected with EHECΔ*dam*Δ*espF_U* and examined after staining as in *A*.

The GBD did not shift upon addition of GST-$SH3_{IRTKS}$ alone (Fig. 2, orange trace). Thus, the earliest eluting peak represents a ternary complex of GBD, $SH3_{IRTKS}$, and $EspF_U$-5R (confirmed by SDS/PAGE; not shown). These data indicate that $SH3_{IRTKS}$ and the WASP GBD domain can simultaneously bind $EspF_U$.

The localization of IRSp53 and IRTKS at the tips of pedestals and the ability of the IRTKS SH3 domain to bind an $EspF_U$-5R/GBD complex in vitro raised the possibility that IRSp53 and/or IRTKS might promote pedestal formation by recruiting $EspF_U$/N-WASP. To test whether the requirement for the C-terminal domain of Tir, which is normally essential for $EspF_U$ recruitment and pedestal formation, can be bypassed by direct fusion of Tir to the IRSp53 and IRTKS SH3 domains, we replaced the Tir C terminus with $SH3_{IRTKS}$ or

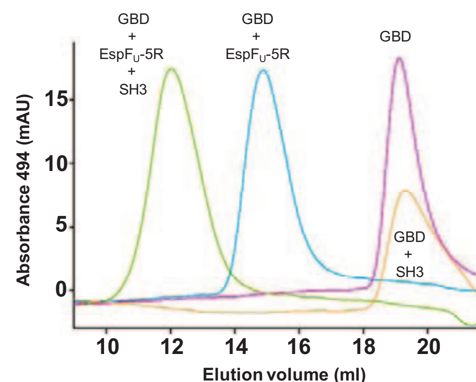

Fig. 2. The IRTKS SH3 domain, $EspF_U$ proline-rich domain, and WASP GBD form a tripartite complex in vitro. Interactions between GST-$SH3_{IRTKS}$ (50 μM) and $EspF_U$-5R (10 μM) in complex with FITC-labeled GBD (50 μM) were examined by gel filtration chromatography. The A_{494} profile, which detects FITC-GBD, is shown.

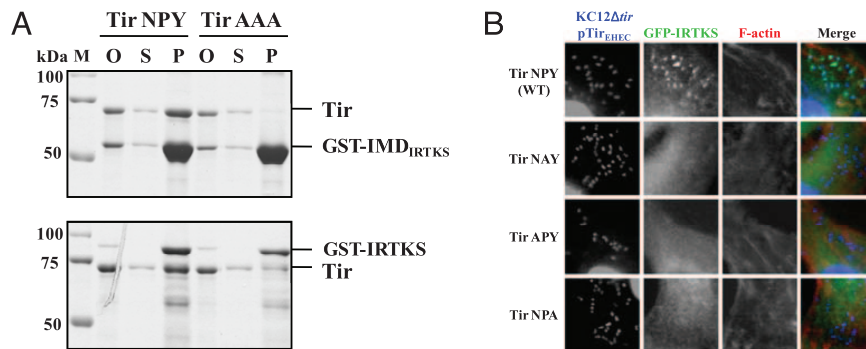

Fig. 3. The EHEC Tir NPY_{458} sequence is required for binding of IRTKS to Tir and its recruitment to sites of bacterial attachment. (*A*) GST-IRTKS derivatives were incubated with $TirNPY_{458}$ or $TirAAA_{458}$ and pulled down using glutathione magnetic beads. Proteins present in the original incubation (O), the supernatant (S) or the pull-down (P) were visualized by Coomassie staining after 10% SDS/PAGE. (*B*) HeLa cells transfected with GFP-IRTKS were infected with KC12Δ*tir* (8) harboring plasmids encoding EHEC Tir carrying the WT NPY_{458} sequence (Tir NPY) or alanine substitutions of this sequence, as indicated (*Left*). Monolayers were examined after staining with DAPI to localize bacteria (blue), Alexa568-phalloidin (red), and anti-myc antibody to detect GFP-IRTKS-myc (green).

$SH3_{IRSp53}$, and infected HeLa cells ectopically expressing these fusions with KC14/pEspF_U, an EPEC strain engineered to translocate EspF_U but that does not normally generate pedestals because it lacks Tir (8). In fact, infection of transfected HeLa cells expressing either TirΔC-$SH3_{IRTKS}$ or TirΔC-$SH3_{IRSp53}$ resulted in the formation of phalloidin-stained actin pedestals beneath bound bacteria, and in a manner dependent on EspF_U (Fig. S3). Thus, the C terminus of Tir can be functionally replaced by the IRSp53 or IRTKS SH3 domains, indicating that the interactions of these domains with EspF_U are sufficient to trigger EspF_U-mediated pedestal formation in mammalian cells.

IRTKS Binds to Tir and Localizes at Sites of Bacterial Attachment Independently of EspF_U. Given ability of IRSp53 and IRTKS to bind EspF_U, their localization at the pedestal tip could simply reflect the interaction of these proteins with EspF_U. To test this hypothesis, we assayed recruitment of IRSp53 and IRTKS upon infection of HeLa cells with an *espF_U* mutant of EHEC. As expected given the absence of EspF_U, actin pedestals were not readily observed under adherent bacteria (Fig. 1*B*). IRSp53 was not associated with bound bacteria, indicating that this protein requires EspF_U for localization to these sites. In contrast, IRTKS was readily recruited to sites of bacterial attachment in the absence of EspF_U (Fig. 1*B*). Thus, whereas localization of IRSp53 at the pedestal tip is likely secondary to binding to EspF_U, IRTKS might be actively involved in recruiting EspF_U to these sites.

The EspF_U-independent localization of IRTKS at the sites of bacterial attachment raised the possibility that IRTKS could bind to the Tir C-terminal cytoplasmic domain. To determine whether IRTKS or IRSp53 interacts with TirC, we used the yeast 2-hybrid assay and analyzed the IMD and SH3 domains separately. Neither the SH3 nor the IMD of IRSp53 bound to TirC (Fig. S4), an observation consistent with the lack of recruitment of IRSp53 to sites of bacterial attachment in the absence of EspF_U. In contrast, co-expression of IMD_{IRTKS} and TirC derivatives indicated an interaction, resulting in an 8-fold induction of β–galactosidase reporter activity (Fig. S4). These data suggest that the IMD of IRTKS mediates its recruitment to sites of bacterial attachment by binding to the C-terminal cytoplasmic domain of translocated Tir.

The EHEC Tir NPY_{458} Sequence Is Required for Binding of IRTKS to Tir and Its Recruitment to Sites of Bacterial Attachment. The Tir tripeptide NPY_{458} within the C-terminal cytoplasmic domain of EHEC Tir is critical for Tir function and alanine substitution of any of these residues resulted in severe defects in both EspF_U recruitment and pedestal formation (13). To test whether the ability of IRTKS to bind Tir requires the NPY_{458} sequence, we assessed IRTKS-Tir interaction in GST pull-down assays using purified derivatives of these proteins (Fig. S1). GST-IMD_{IRTKS} bound to WT Tir ("Tir NPY") in this assay, but was not capable of binding to a mutant Tir harboring substitutions of the NPY_{458} motif to alanine residues ("Tir AAA"; Fig. 3*A Upper*). Full-length GST-IRTKS also interacted with WT Tir, and the efficiency of binding was significantly diminished by mutation of the NPY_{458} sequence (Fig. 3*A Lower*).

Single alanine substitutions of the Tir NPY_{458} sequence abrogate both EspF_U recruitment and pedestal formation (13). To determine if these mutants are also incapable of recruiting IRTKS, HeLa cells that ectopically express GFP-IRTKS were infected with KC12Δ*tir*/$pTir_{EHEC}$, an EPEC strain engineered to express EHEC Tir (8), or isogenic strains that express alanine-substituted Tir NPY_{458} mutants. When HeLa cells ectopically expressing GFP-IRTKS (Fig. S5) were infected with KC12Δ*tir* expressing WT Tir, IRTKS was recruited to sites of bacterial attachment (Fig. 3*B*), consistent with our previous finding (Fig. 2). As expected because of the lack of EspF_U, no actin pedestals were formed. In contrast, no recruitment of GFP-IRTKS to sites of bacterial attachment was detected when the transfected HeLa cells were infected with bacteria expressing Tir derivatives that carry alanine substitutions in N456, P457, or Y458 (Fig. 3*B*). Thus, IRTKS directly binds to Tir via the IMD and is recruited to sites of bacterial attachment in an NPY_{458}-dependent manner.

Ectopic Expression of the IRTKS SH3 or IMD Domain Inhibits EspF_U-Dependent Pedestal Formation. To examine the functional role of IRTKS in actin signaling by EHEC, we assessed pedestal formation after ectopic expression of its IMD or SH3 domain in HeLa cells. HeLa cells were transfected with plasmids producing a variety of GFP derivatives, including GFP-$SH3_{IRTKS}$ and GFP-IMD_{IRTKS}. Immunoblotting confirmed that all GFP derivatives were efficiently produced (Fig. S5). Expression of the GFP control had no effect on pedestal formation, as virtually all transfected cells displayed actin pedestals (Fig. 4 *Top*). In contrast, expression of GFP-$SH3_{IRTKS}$ strongly inhibited pedestal formation: only 15% of cells expressing high levels of GFP-$SH3_{IRTKS}$ exhibited pedestals (Fig. 4, row 3). Consistent with the hypothesis that this inhibition was caused specifically by the ability of GFP-$SH3_{IRTKS}$ to bind EspF_U, expression of a GFP fusion contain-

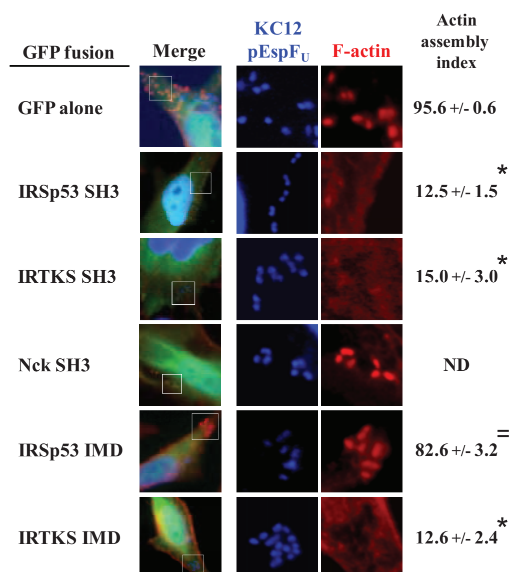

Fig. 4. Ectopic expression of the IRTKS SH3 or IMD domain inhibits $EspF_U$-dependent pedestal formation. Transfected HeLa cells expressing GFP or GFP fusion proteins were infected with KC12/p$EspF_U$ (15). Transfected cells were identified by GFP fluorescence (*Merge*), and monolayers were stained with DAPI (blue) and Alexa568-phalloidin (red). (The lack of localization of GFP-IMD to sites of bacterial attachment may be related to the unexplained paucity of Tir foci.) The percentage of cells competent for actin pedestal formation after infection is shown (*Right*). Shown is the mean ± SD of at least 3 experiments; **P* < 0.0001; $^{=}P < 0.01$.

ing the SH3 domain of IRSp53, which also binds $EspF_U$, blocked pedestal formation (Fig. 4, row 2), whereas expression of a fusion containing an SH3 domain of Nck, which was not enriched from the SH3 phage display library by affinity panning on $EspF_U$, had no discernible effect (Fig. 4, row 4). Importantly, inhibition by GFP-$SH3_{IRTKS}$ and GFP-$SH3_{IRSp53}$ was specific to $EspF_U$-mediated pedestals and not caused by non-specific inhibition of translocation or the actin assembly machinery, because expression of these fusions did not inhibit pedestal formation by EPEC (Fig. S6), which generates pedestals independent of $EspF_U$ (8, 9, 15, 16).

As shown in Fig. 4, expression of GFP-IMD_{IRTKS} also efficiently inhibited actin pedestal formation, because only 12.6% of cells that expressed GFP-IMD_{IRTKS} and bound bacteria demonstrated pedestals (Fig. 4, row 6). This inhibition was specific because expression of GFP-IMD_{IRTKS} had no effect on actin pedestal formation by EPEC (Fig. S6). In contrast to the strong inhibitory activity of GFP-IMD_{IRTKS}, the pedestal index for cells expressing GFP-IMD_{IRSp53} was 82.6% (Fig. 4, row 5), which, although somewhat lower than for cells expressing GFP alone (i.e., 95.6%), is consistent with our inability to discern recruitment of IRSp53 to sites of bacterial attachment in the absence of $EspF_U$.

Genetic Depletion of IRTKS Inhibits $EspF_U$-Dependent Pedestal Formation. To further examine whether IRTKS function is required for EHEC actin assembly, we used an RNAi approach based on previously published siRNA sequences that efficiently and specifically silence expression of IRTKS or IRSp53 (27). RT-PCR analysis of cells transfected with a combination of 2 IRTKS siRNAs showed an approximately 90% depletion of IRTKS mRNA compared with control siRNA (Fig. S7*A*). Similarly, a combination of 2 IRSp53 siRNAs knocked down IRSp53 mRNA more than 90% (Fig. S7*A*).

To assess the role of IRTKS in pedestal formation, IRTKS-depleted and control cells were infected with KC12/p$EspF_U$. As expected, pedestals formed with high efficiency on control siRNA-treated cells—visual quantitation revealed that virtually all infected cells displayed pedestals. In contrast, pedestal formation was diminished more than 5-fold on cells treated with a combination of the 2 IRTKS siRNAs (Fig. 5 and Fig. S7*B*). IRTKS depletion with only 1 siRNA resulted in partial (≈50%) but significant inhibition (Fig. S7*B*). The decrease in pedestal formation was specific for IRTKS, as cells depleted for IRSp53 generated pedestals with undiminished efficiency (Fig. 5 and Fig. S7*B*), and the cells depleted for both IRTKS and IRSp53 generated pedestals at a frequency indistinguishable from cells depleted only for IRTKS (Fig. 5 and Fig. S7*B*). Importantly, EPEC formed pedestals with high efficiency on IRTKS-depleted cells (Fig. S7*C*). Thus, in agreement with the data on ectopic expression of IRTKS SH3 or IMD domains, these RNAi studies indicate that IRTKS is specifically required for $EspF_U$-mediated actin assembly.

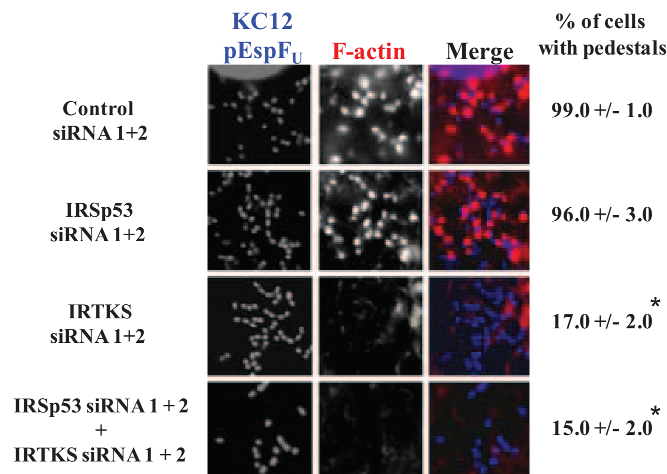

Fig. 5. Genetic depletion of IRTKS inhibits $EspF_U$-dependent pedestal formation. HeLa cells transfected with pairs of control, IRTKS, or IRSp53 siRNAs, or a pool of the pair of IRTKS and IRSp53 siRNAs, were infected with KC12/p$EspF_U$ (15). Monolayers were examined after staining with DAPI (blue) and Alexa568-phalloidin (red). The percentage of cells competent for actin pedestal formation after infection is shown (*Right*). Shown is the mean ± SD of at least 3 experiments; **P* < 0.0001.

Discussion

$EspF_U$ binds and activates WASP-family actin nucleation-promoting factors (15, 16) and artificial fusion of $EspF_U$ to Tir clustered at the plasma membrane is sufficient to trigger actin assembly (18, 20, 21). However, although Tir, $EspF_U$, and N-WASP are associated in host cells, neither $EspF_U$ nor N-WASP directly interact with Tir (15, 16), indicating that a (host-encoded) factor is required for formation of this actin assembly complex. Because $EspF_U$ contains multiple PxxP sequences, we screened an essentially complete collection of human SH3 domains (23) and identified IRTKS as an avid binding partner of $EspF_U$. Detection of a ternary complex of $SH3_{IRTKS}$, $EspF_U$, and GBD_{WASP} in vitro supports the model that IRTKS is part of an $EspF_U$/N-WASP-containing complex that potently stimulates Arp2/3. Consistent with this hypothesis, a Tir-$SH3_{IRTKS}$ fusion lacking the Tir C terminus, which is normally required for function, generated robust $EspF_U$-mediated pedestals upon clustering by intimin.

IRTKS was localized to the tip of actin pedestals, raising the possibility that it mediated Tir-$EspF_U$ interaction. Indeed, whereas the SH3 domain of IRTKS bound to $EspF_U$, its IMD

bound to Tir in a manner dependent on the NPY_{458} sequence, which has previously been shown to be critical for $EspF_U$ recruitment. Furthermore, IRTKS was recruited to sites of bacterial attachment, dependent on Tir NPY_{458} but independent of $EspF_U$ and actin assembly. Finally, ectopic expression of either the IMD or SH3 of IRTKS, or RNAi silencing of IRTKS, inhibited $EspF_U$-dependent pedestal formation without affecting $EspF_U$-independent pedestal formation by EPEC. These results provide compelling evidence that IRTKS, by interacting with Tir and $EspF_U$, promotes the formation of a complex of bacterial and host factors that trigger robust actin assembly beneath bound bacteria. The SH3 domain of the IRTKS homologue IRSp53 appears to be functionally equivalent to that of IRTKS because it promotes $EspF_U$-mediated actin assembly when clustered at the plasma membrane. However, in contrast to IRTKS, IRSp53 was not detectably recruited to sites of bacterial attachment in the absence of $EspF_U$, and ectopic expression of the IRSp53 IMD, or siRNA-mediated depletion of IRSp53, had no marked effect on pedestal formation, correlating with our inability to detect interaction of the IRSp53 IMD with the Tir C terminus in a yeast 2-hybrid assay. It should be noted, however, that we have been able to detect binding of recombinant IRSp53 to recombinant Tir in vitro (D.V., unpublished data), and this activity might be reflected in the mild ($\approx$15%) inhibition of pedestal formation by ectopic expression of GFP-IMD_{IRSp53} (see Fig. 4). In addition, Stradal and coworkers have implicated IRSp53 in pedestal formation using murine cell lines (39), and the relative roles of members of this family during pedestal formation in different cell types remains to be fully determined.

IRTKS, as a member of the IRSp53 family, is involved in signal transduction pathways that link deformation of the plasma membrane and remodeling of the actin cytoskeleton (26). IRTKS promotes actin assembly and membrane protrusions when overexpressed in mammalian cells (24), so it is possible that its role in pedestal formation may extend beyond simply recruiting $EspF_U$ to sites of clustered Tir at the plasma membrane. In fact, an EHECΔ*espF$_U$* mutant retains the ability to generate low-level Tir-mediated localized actin assembly in vitro (15) and to trigger some AE lesions during infection of the mammalian host (17). The IRSp53 C-terminal SH3 domain has been shown to interact with cytoskeletal factors such as the Ena/VASP protein Mena, Eps8, and the formin mDia, as well as the Arp2/3 activators WAVE2 (see ref. 26 for review) and N-WASP (25). The IMD binds F-actin, the GTPase Rac, and lipids, and additionally is structurally reminiscent of a Bin-amphiphysin-Rvs167 (BAR) domain, which binds and deforms membranes, generating invaginations during endocytosis. IMDs, also known as I-BAR (inverse BAR) domains because of their opposite curvature, triggers protrusive membrane deformation (26, 28), and it is tempting to speculate that this activity of the IRTKS IMD might contribute to the morphology of AE lesions.

Recent work has shown that, within a 47-residue C-terminal $EspF_U$ repeat, a segment consisting of approximately 20 residues forms an amphipathic helix and an extended arm that binds and activates WASP/N-WASP (18, 20). We show here that a different segment of the repeat, one that is rich in prolines, is required for binding to the IRTKS/IRSp53 SH3 domains, and that IRTKS and N-WASP can bind $EspF_U$ simultaneously. The division of a repeat unit into 2 functional recognition elements parallels that of the $EspF_U$-related *E. coli* effector EspF. Like $EspF_U$, EspF consists of an N-terminal translocation domain and several 47-residue C-terminal repeats, each of which contains an N-WASP binding segment and a proline-rich sequence that is recognized by an SH3 domain-containing protein that binds and deforms membranes. In the case of EspF, the SH3-containing protein is SNX9 (29, 30), which contains a BAR domain and participates in membrane remodeling during endocytosis (31). Although $EspF_U$ can complement some functions of EspF (32), EspF plays no apparent role in pedestal formation (15), presumably because its proline-rich sequences target a different SH3 domain. This difference notwithstanding, both EspF and $EspF_U$ alter membrane and actin dynamics by acting as modular and repetitive adaptor proteins that link N-WASP to a membrane-deforming protein.

With the identification of IRTKS as an essential link between Tir and $EspF_U$, a striking feature of many components of the actin pedestal signaling cascade is the ability to multimerize. The membrane anchoring domain of intimin and the extracellular domain of Tir each encode elements that promote homotypic dimerization (33, 34), leading to the hypothesis that intimin-Tir interactions result in a reticular array-like superstructure of Tir cytoplasmic domains beneath the clustered receptor. This putative array of Tir cytoplasmic domains is recognized by the IRTKS IMD, which, upon dimerization, would be predicted to present physically linked pairs of IRTKS SH3 domains to recruit $EspF_U$. In this regard, it is notable that the presence of at least 2 $EspF_U$ repeats is required for recruitment to sites of bacterial attachment (19). Finally, the repetitive nature of $EspF_U$ is also critical for downstream signaling, because the tandem N-WASP-binding elements synergistically activate the N-WASP/Arp2/3 pathway for actin assembly (20, 21, 35). Thus, by targeting distinct domains of IRTKS, Tir and $EspF_U$ promote the formation of a multimeric complex containing N-WASP-binding and activation elements that triggers the robust actin assembly.

Materials and Methods

Strains, Plasmids, and DNA Manipulations. The bacterial strains and plasmids used in this study are listed in Table S1 (40). Primers used are listed in Table S2. As detailed in *SI Methods*, cDNA encoding IRTKS and IRSp53 derivatives were amplified from the human cDNAs and cloned in the mammalian expression plasmids pKC425 (21) and pKC689 (21) to generate GFP-fusion proteins and TirΔC fusion proteins, respectively.

Assays for Protein-Protein Interaction. GST-$EspF_U$C, His-tagged EHEC Tir derivatives, and GST-tagged IRTKS derivatives were produced in *E. coli* strain BL21(DE3) and purified by affinity chromatography according to manufacturers' instructions. Screening of the phage-displayed SH3 domain library was performed as described previously (23). Yeast 2-hybrid assays were used to assess interaction among IRTKS, IRSp53, $EspF_U$, and EHEC Tir as previously described (36). Interaction between IRTKS and recombinant EHEC Tir was assessed in GST pull-down assays. Formation of a tripartite complex between GST-$SH3_{IRTKS}$, $EspF_U$-5R, and GBD_{WASP} was assessed by gel filtration chromatography.

RNAi Experiments. siRNA experiments were performed using stealth RNAi (Invitrogen). The sequences used were as described by Suetsugu et al. (26) (see *SI Methods*). Transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. To evaluate knock-down efficiency, total mRNA from RNAi-treated HeLa cells was isolated using TRIzol reagent (Invitrogen). A first-strand cDNA was synthesized from the mRNA using the SuperScript first-strand cDNA synthesis system for RT-PCR (Invitrogen).

Mammalian Cell Infections. Culture of bacteria before infection of mammalian cells, and culture and transfection of HeLa cells, were performed as previously described (14, 15). Transfection of mammalian cells for ectopic expression of proteins, and infection of mammalian cells with bacteria were performed as previously described (15, 37). Cells were treated with mouse anti-HA tag mAb HA.11 (1:500; Covance), mouse anti-IRSp53 mAb (1:100; Novus Biologicals), or mouse anti-IRTKS mAb (1:100; Novus Biologicals). Pedestal formation indices were determined as detailed in *SI Methods*.

ACKNOWLEDGMENTS. We thank Dr. Nathalie Cohet for help with quantitative PCR analyses, L. Soll for help with plasmid construction, T. Stradal and K. Rottner for helpful discussion and communication of unpublished results, and K. Campellone and D. Tipper for critical reading of the manuscript. This work was supported by National Institutes of Health Grant R01-AI46454 (to J.M.L.) and Fondation pour la Recherche Medicale (Paris, France) post-doctoral fellowship SPE20061208629 (to D.V.).

1. Kaper JB, Nataro JP, Mobley HL (2004) Pathogenic Escherichia coli. *Nat Rev Microbiol* 2:123–140.
2. Donnenberg MS, et al. (1993) Role of the eaeA gene in experimental enteropathogenic Escherichia coli infection. *J Clin Invest* 92:1412–1417.
3. Ritchie JM, Thorpe CM, Rogers AB, Waldor MK (2003) Critical roles for stx2, eae, and tir in enterohemorrhagic *Escherichia coli*-induced diarrhea and intestinal inflammation in infant rabbits *Infect Immun* 71:7129–7139.
4. Frankel G, Phillips AD (2008) Attaching effacing Escherichia coli and paradigms of Tir-triggered actin polymerization: getting off the pedestal. *Cell Microbiol* 10:549–556.
5. Hayward RD, Leong JM, Koronakis V, Campellone KG (2006) Exploiting pathogenic *Escherichia coli* to model transmembrane receptor signalling. *Nat Rev Microbiol* 4:358–370.
6. Deibel C, Kramer S, Chakraborty T, Ebel F (1998) EspE, a novel secreted protein of attaching and effacing bacteria, is directly translocated into infected host cells, where it appears as a tyrosine-phosphorylated 90 kDa protein. *Mol Microbiol* 28:463–474.
7. Kenny B, et al. (1997) Enteropathogenic E. coli (EPEC) transfers its receptor for intimate adherence into mammalian cells. *Cell* 91:511–520.
8. Campellone KG, Giese A, Tipper DJ, Leong JM (2002) 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. *Mol Microbiol* 43:1227–1241.
9. Gruenheid S, et al. (2001) Enteropathogenic E. coli Tir binds Nck to initiate actin pedestal formation in host cells. *Nat Cell Biol* 3:856–859.
10. Rohatgi R, et al. (2001) Nck and phosphatidylinositol 4,5-bisphosphate synergistically activate actin polymerization through the N-WASP-Arp2/3 pathway. *J Biol Chem* 276:26448–26452.
11. Campellone KG, Leong JM (2003) Tails of two Tirs: actin pedestal formation by enteropathogenic E. coli and enterohemorrhagic *E. coli* O157:H7. *Curr Opin Microbiol* 6:82–90.
12. Allen-Vercoe E, Waddell B, Toh MC, DeVinney R (2006) Amino acid residues within enterohemorrhagic Escherichia coli O157:H7 Tir involved in phosphorylation, alpha-actinin recruitment, and Nck-independent pedestal formation. *Infect Immun* 74:6196–6205.
13. Brady MJ, Campellone KG, Ghildiyal M, Leong JM (2007) Enterohaemorrhagic and enteropathogenic Escherichia coli Tir proteins trigger a common Nck-independent actin assembly pathway. *Cell Microbiol* 9:2242–2253.
14. Campellone KG, et al. (2006) Enterohaemorrhagic Escherichia coli Tir requires a C-terminal 12-residue peptide to initiate EspFU-mediated actin assembly and harbours N-terminal sequences that influence pedestal length. *Cell Microbiol* 8:1488–1503.
15. Campellone KG, Robbins D, Leong JM (2004) EspFU is a translocated EHEC effector that interacts with Tir and N-WASP and promotes Nck-independent actin assembly. *Dev Cell* 7:217–228.
16. Garmendia J, et al. (2004) TccP is an enterohaemorrhagic *Escherichia coli* O157:H7 type III effector protein that couples Tir to the actin-cytoskeleton. *Cell Microbiol* 6:1167–1183.
17. Ritchie JM, et al. (2008) EspFU, a type III-translocated effector of actin assembly, fosters epithelial association and late-stage intestinal colonization by E. coli O157:H7. *Cell Microbiol* 10:836–847.
18. Cheng HC, et al. (2008) Structural mechanism of WASP activation by the enterohaemorrhagic E. coli effector EspF(U). *Nature* 454:1009–1013.
19. Garmendia J, et al. (2006) Characterization of TccP-mediated N-WASP activation during enterohaemorrhagic Escherichia coli infection. *Cell Microbiol* 8:1444–1455.
20. Sallee NA, et al. (2008) The pathogen protein EspF(U) hijacks actin polymerization using mimicry and multivalency. *Nature* 454:1005–1008.
21. Campellone KG, et al. (2008) Repetitive N-WASP-binding elements of the enterohemorrhagic Escherichia coli effector EspF(U) synergistically activate actin assembly. *PLoS Pathog* 4:e1000191.
22. Mayer BJ, Saksela K (2004) SH3 domains. *Structure and Function of Modular Protein Domains*, ed Cesareni G, Gimona M, Sudol M, Yaffe M (Wiley-VCH, Weinheim, Germany), pp 37–58.
23. Karkkainen S, et al. (2006) Identification of preferred protein interactions by phage-display of the human Src homology-3 proteome. *EMBO Rep* 7:186–191.
24. Millard TH, Dawson J, Machesky LM (2007) Characterisation of IRTKS, a novel IRSp53/MIM family actin regulator with distinct filament bundling properties. *J Cell Sci* 120:1663–1672.
25. Lim KB, et al. (2008) The Cdc42 effector IRSp53 generates filopodia by coupling membrane protrusion with actin dynamics. *J Biol Chem* 283:20454–20472.
26. Scita G, Confalonieri S, Lappalainen P, Suetsugu S (2008) IRSp53: crossing the road of membrane and actin dynamics in the formation of membrane protrusions. *Trends Cell Biol* 18:52–60.
27. Suetsugu S, et al. (2006) Optimization of WAVE2 complex-induced actin polymerization by membrane-bound IRSp53, PIP(3), and Rac. *J Cell Biol* 173:571–585.
28. Cory GO, Cullen PJ (2007) Membrane curvature: the power of bananas, zeppelins and boomerangs. *Curr Biol* 17:R455–457.
29. Alto NM, et al. (2007) The type III effector EspF coordinates membrane trafficking by the spatiotemporal activation of two eukaryotic signaling pathways. *J Cell Biol* 178:1265–1278.
30. Marches O, et al. (2006) EspF of enteropathogenic Escherichia coli binds sorting nexin 9. *J Bacteriol* 188:3110–3115.
31. Lundmark R, Carlsson SR (2003) Sorting nexin 9 participates in clathrin-mediated endocytosis through interactions with the core components. *J Biol Chem* 278:46772–46781.
32. Viswanathan VK, et al. (2004) Comparative analysis of EspF from enteropathogenic and enterohemorrhagic Escherichia coli in alteration of epithelial barrier function. *Infect Immun* 72:3218–3227.
33. Luo Y, et al. (2000) Crystal structure of enteropathogenic Escherichia coli intimin-receptor complex. *Nature* 405:1073–1077.
34. Touze T, et al. (2004) Self-association of EPEC intimin mediated by the beta-barrel-containing anchor domain: a role in clustering of the Tir receptor. *Mol Microbiol* 51:73–87.
35. Padrick SB, et al. (2008) Hierarchical regulation of WASP/WAVE proteins. *Mol Cell* 32:426–438.
36. Liu H, et al. (2002) Point mutants of EHEC intimin that diminish Tir recognition and actin pedestal formation highlight a putative Tir binding pocket. *Mol Microbiol* 45:1557–1573.
37. Campellone KG, Leong JM (2005) Nck-independent actin assembly is mediated by two phosphorylated tyrosines within enteropathogenic Escherichia coli Tir. *Mol Microbiol* 56:416–432.
38. Campellone KG, et al. (2007) Increased adherence and actin pedestal formation by dam-deficient enterohaemorrhagic Escherichia coli O157:H7. *Mol Microbiol* 63:1468–1481.
39. Weiss SM, et al. (2009) IRSp53 links the enterohemorrhagic *E.Coli* effectors Tir and $EspF_u$ for actin pedestal formation. *Cell Host Microbe* 5:244–258.
40. Jerse AE, Yu J, Tall BD, Kaper JB (1990) A genetic locus of enteropathogenic *Escherichia coli* necessary for the production of attaching and effacing lesions on tissue culture cells. *Proc Natl Acad Sci USA* 87:7839–7843.