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Structural Mechanism of WASP Activation by the Enterohaemorrhagic *E. coli* Effector EspF_U

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Abstract

During infection enterohaemorrhagic *Escherichia coli* (EHEC) usurp the actin cytoskeleton of eukaryotic cells by injecting the EspF_U protein into the host cytoplasm^{1, 2}. EspF_U controls actin by activating members of the Wiskott-Aldrich Syndrome Protein (WASP) family^{1–5}. Here we show that EspF_U binds the autoinhibitory GTPase binding domain (GBD) in WASP proteins and displaces it from the activity-bearing VCA domain. This interaction potently activates WASP and N-WASP *in vitro* and induces localized actin assembly in cells. In the solution structure of the GBD-EspF_U complex, EspF_U forms an amphipathic helix that binds the GBD, mimicking interactions of the VCA in autoinhibited WASP. Thus, EspF_U activates by competing directly for the VCA binding site on the GBD. This mechanism is distinct from that used by the eukaryotic activators Cdc42 and SH2 domains, which globally destabilize the GBD fold to release the VCA^{6–8}. Such diversity of mechanism in WASP proteins is distinct from other multi-modular systems, and may result from the intrinsically unstructured nature of the isolated GBD and VCA elements. The structural incompatibility of the GBD complexes with EspF_U and Cdc42/SH2, plus high affinity EspF_U binding, enable EHEC to potently hijack the eukaryotic cytoskeletal machinery.

Pathogenic bacteria often target the host actin cytoskeleton during infection to assist entry, cell-to-cell spreading, and intimate attachment to host membranes^{9–13}. EHEC (serotype O157:H7) is a food-born human pathogen that causes severe diarrhea and hemolytic uremic syndrome¹⁴. During infection, EHEC binds eukaryotic host intestinal epithelial cells and secretes Tir (Translocated Intimin Receptor) and EspF_U (also known as TccP), into the host cell^{1, 2}. The secreted Tir localizes in the host plasma membrane, and upon clustering by binding to the bacterial outer surface protein intimin, recruits EspF_U via its C-terminal cytoplasmic domain³. EspF_U in turn hijacks members of the Wiskott-Aldrich Syndrome Protein (WASP) family, which locally stimulate the actin nucleation factor, Arp2/3 complex^{1–5}. Together, these interactions induce the formation of an actin-rich membrane protrusion beneath the site of EHEC attachment, termed an actin pedestal^{1, 2, 15}.

WASP proteins activate Arp2/3 complex through a C-terminal VCA domain¹⁶. In free WASP, this activity is blocked through intramolecular interactions with a central GTPase binding domain (GBD). Structural and biochemical studies have shown that the GBD sequesters an

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amphipathic helix in the C region of the VCA that is necessary for activation of Arp2/3 complex, resulting in autoinhibition^{7, 17, 18}. WASP can be allosterically activated by a number of intracellular ligands, including Cdc42 and (for phosphorylated WASP) SH2 domains^{8, 19}. These ligands bind to WASP and N-WASP in a manner that is structurally incompatible with the autoinhibited fold of the GBD. Thus, they activate by globally destabilizing the GBD, consequently releasing the VCA^{6, 8, 20, 21}.

EspF_U is composed of an N-terminal signal sequence necessary for translocation into the eukaryotic host, followed by 2–7 nearly identical 47 residue repeats (Fig. 1A)²². Each full repeat consists of an N-terminal 27 residue hydrophobic segment and a C-terminal 20 residue proline-rich segment. Previous studies showed that EspF_U can bind the N-WASP GBD and activate N-WASP toward Arp2/3 complex in actin assembly assays *in vitro*^{1, 2, 4}. A similar sequence is found in the related bacterial effector EspF, which also can activate N-WASP (Fig. S1)²³.

To understand EspF_U-mediated activation, we examined fragments containing the full 47-residue fifth repeat (R47, Fig. 1, Fig. S1) and an N-terminal 33-residue fragment lacking most of the proline-rich sequence (R33). In Arp2/3-mediated pyrene-actin assembly assays, R47 and R33 significantly and similarly enhanced the activities of autoinhibited N-WASP and WASP proteins (N-WASP_C, and GBD-VCA, respectively; Fig. 1B, Fig. S2). Titration of R47 into N-WASP_C produced a monotonic increase in actin assembly rate (Fig. 1C and Fig. S3), which reached a plateau value similar to that of the N-WASP VCA. Thus, the endpoint of the titration produces a state which biochemically resembles free N-WASP VCA. Fitting the data to a single-site binding isotherm yields a K_{Act} of 24 nM, approximating the K_D of R47 for N-WASP_C, 35 nM (Fig. S4). EspF_U binds N-WASP > 100-fold more strongly than does Cdc42²¹, resulting in much greater potency in actin assembly assays (Fig. 1C). This much higher affinity should enable EspF_U to hijack N-WASP away from its endogenous regulators *in vivo*²¹.

Cdc42 and SH2 domains activate WASP by physically displacing the VCA from the GBD^{7, 8}. EspF_U R33 disrupts GBD-VCA interactions similarly (Fig. S5), indicating that it also binds the GBD in a manner that is incompatible with the autoinhibitory interactions. The combined data show that EspF_U R33 is functionally equivalent to Cdc42 and SH2 domains: all three ligands activate WASP/N-WASP by releasing the VCA from the GBD.

The WASP GBD is largely unstructured on its own, but folds to a well-defined conformation upon binding VCA⁷. ¹H/¹⁵N and ¹H/¹³C amide and methyl TROSY NMR spectra showed that EspF_U R33 or a five-repeat fragment, R₅, also induced folding of the GBD (Fig. S6). Reciprocally, the GBD induces folding of the unstructured R33 (Fig. S6B, Fig. S7). Titrations of R33 and R₅ into GBD saturated at 1:1 and 0.2:1 EspF_U:GBD stoichiometry, respectively, showing that each repeat can bind N-WASP. The endpoint spectra are strikingly similar, indicating that the GBD folds to the same structure on each repeat, and that the molecular nature of allosteric activation can be understood through analysis of the R33 complex.

We determined the solution structure of the WASP GBD in complex with EspF_U R33 using NMR spectroscopy (Fig. 2, Fig. S8; Table S1). The complex can be considered in three stacked structural layers⁷. The first layer is composed of a short N-terminal i+4 β hairpin (β 1, 252–253; β 2, 257–258) and α 1 helix (265–274) from the GBD. The second layer lies behind the first and consists of GBD helices α 2– α 4 (α 2, 278–281; α 3, 284–296; α 4, 300–306), which have a roughly planar C-shaped arrangement. The third layer is an amphipathic α 5 helix (3–14) and an extended arm (16–20) from EspF_U. These elements contact the back of the GBD and are roughly antiparallel to α 3 and α 2, respectively (Fig. 2A, structural details in Fig. S9). WASP residues 242–249 and EspF_U residues 21–33 appear to be largely disordered in solution, based

on the absence of long range ^1H - ^1H NOEs and low heteronuclear $\{^1\text{H}\}$ - ^{15}N NOEs (Fig. S10). The EspF_U relative, EspF, is expected to bind the GBD through an analogous amphipathic $\alpha 5$ helix, but may not use an extended arm, as these C-terminal residues are not conserved (Fig. S1).

To better understand the physical basis of EspF_U activity, we examined a series of single repeat mutants based on the structure. First, we fused these proteins C-terminally to the WASP GBD through a short linker. Since the GBD and R33 are only folded when bound to each other, the thermodynamic stability of the fusions reports on the relative strengths of interaction between the two elements. We mutated three conserved hydrophobic residues, V4, L8 and L12, which lie at the center of the interface between $\alpha 5$ and the GBD (Fig. S9). The L12A (R33^{VL*}) and V4A/L8A (R33^{**L}) mutants have substantially reduced stability relative to wild type in the GBD-R33 fusions, demonstrating the importance of $\alpha 5$ in the interaction of N-WASP with EspF_U. Truncations from R33 to R30 or R27 decrease stability by ~ 2 kcal/mol. Thus, residues beyond Ala30 contribute thermodynamically to GBD-R33 interaction, despite the absence of discrete structure in these regions. Similar contributions to affinity through disordered regions have been observed in other systems as well²⁴. Truncation of all (R14) or part (R18) of the EspF_U C-terminal arm decreases the stability further, indicating that the arm contributes significantly to binding of the GBD to EspF_U. These unfolding studies mirror direct binding measurements. R33 and R18 bind N-WASPC with K_D values of 35 and 4000 nM, respectively (Fig. 1D), corresponding to a difference in free energy of 2.9 kcal/mol. This value agrees closely with the stability difference between the two GBD-fusions (2.6 kcal/mol, Fig. 1D). Finally, these differences in binding correspond well to the activities of the EspF_U mutants in N-WASP mediated actin assembly assays, where activity decreases in concert with strength of interaction (Figs. 1B, D).

To test how the biophysical properties of EspF_U-N-WASP interaction relate to biological function, we examined the ability of EspF_U derivatives to induce localized actin assembly in mammalian cells. To uncouple Tir-mediated recruitment of EspF_U from EspF_U function in pedestal generation after recruitment, we replaced the C-terminal cytoplasmic domain of Tir with two repeats of EspF_U (giving Tir Δ C-R₂). We expressed Tir Δ C-R₂ in mouse fibroblast-like cells, where it inserted into the plasma membrane, and then clustered it by adding *E. coli* expressing intimin. Immunostaining revealed foci of clustered Tir and N-WASP coincident with bound bacteria (Fig. 3A). Similarly, F-actin strongly colocalized with Tir. In contrast, a control Tir derivative (Tir Δ C) lacking cytoplasmic elements did not recruit N-WASP or produce actin pedestals (Fig. 3). Mutation of Leu12 to Ala in both EspF_U repeats (giving Tir Δ C-R₂^{VL*}) also abolished both actin assembly and N-WASP recruitment (Fig. 3A), consistent with the decreased binding of this mutant to N-WASP *in vitro* (Fig. 1). To examine the activity of EspF_U truncation mutants, we modified this assay to analyze derivatives of one EspF_U repeat unit. As predicted by *in vitro* data (Fig. 1), the triple V4A/L8A/L12A mutant, Tir Δ CR47^{***}, was also unable to assemble actin (Fig. S12). Truncation of R47 to R33 decreased activity, likely due to the loss of most of the proline-rich region, which in EspF is known to bind SH3-containing proteins to promote actin assembly²³ (Fig. 3B). R30 and R27 showed even lower actin assembly levels, consistent with the diminished N-WASP_C binding by these proteins compared to R33 (Fig. 1). Finally, R14, which bound N-WASP_C only very weakly (Fig. 1), did not promote actin assembly above background levels. Together, these data show that the ability of EspF_U derivatives to promote actin assembly *in vivo* reflects the physical interactions between EspF_U and WASP *in vitro*.

The GBD-R33 and GBD-C complexes are remarkably similar (Fig. 2). In both cases, ligand binding causes the GBD to fold into a β -hairpin and four helices. The backbone r. m. s. d. between the average GBD coordinates in the two structures is only 0.95 Å (residues 247–310). This structural similarity is reflected in high chemical shift similarity: the average GBD

chemical-shift differences are 0.015 ppm for HN, 0.062 ppm for H^α, 0.043 ppm for H^β, 0.21 ppm for C^α, and 0.30 ppm for C^β. The most significant chemical shift differences between the two complexes occur in α2, which contacts the extended arm of EspF_U in the GBD-R33 complex, but is solvent exposed in the GBD-C complex. The WASP C helix and EspF_U helix bind the same site on the GBD in the same orientation through analogous hydrophobic contacts involving similar sequences (Fig. 1A, Fig. S9). EspF_U makes additional contacts to the GBD through its C-terminal arm, which are not seen in the bound C region helix in autoinhibited WASP. These contacts contribute to the much higher WASP affinity of EspF_U compared to its eukaryotic counterparts²¹. The combined structural and biochemical data lead to a clear and simple mechanism for WASP/N-WASP activation by a single repeat element of EspF_U: displacement of the VCA through competitive binding to a common site on the folded GBD.

We have described the autoinhibited GBD-VCA structure as composed of three layers, analogous to the three layers of the GBD-R33 structure, with the C region of the VCA substituting for R33⁷. Three structurally distinct mechanisms for relieving autoinhibition of WASP/N-WASP are now known that involve attacks on distinct regions of this fold (Fig. S13). Cdc42 globally destabilizes the GBD by binding to the first layer of structure. Similarly, following phosphorylation of the conserved Tyr291/256 in WASP/N-WASP, SH2 domains can also destabilize the GBD by binding to this site in the second layer. EHEC have evolved the simplest and structurally most direct means of releasing the VCA: EspF_U binds to the GBD-VCA interface, displacing the VCA from the folded GBD by structural competition. Of the three known allosteric mechanisms, only this latter one is akin to those found in most other autoinhibited multi-domain systems, where activation is driven by rearrangements of intact functional modules. The diversity of WASP regulation likely originates from the intrinsically unstructured nature of the GBD, which enables its broad binding specificity. Since the folded GBD is structurally incompatible with Cdc42/SH2 binding, EspF_U can displace the VCA while simultaneously preventing interactions with eukaryotic activators, thus sequestering WASP proteins away from their normal signaling pathways. This, coupled with very high affinity binding, allows EHEC to subvert the host actin cytoskeleton to its own ends.

Methods Summary

Protein Expression and Purification

EspF_U and WASP proteins were expressed in bacteria as either His₆- or GST-fusions, and purified by affinity and conventional chromatographies. Bovine Arp2/3 complex and rabbit skeletal muscle actin were purified as described^{25, 26}.

Biochemical Assays

All biochemical assays were performed in KMEI buffer (10 mM imidazole pH 7.0, 50 mM KCl, 1 mM MgCl₂, and 1 mM EGTA), or KMEI plus 5 mM β-mercaptoethanol at 25 °C. *Actin Assembly*. Actin assembly assays were performed as described²⁵, with 4 μM actin (5% pyrene labeled) and 10 nM Arp2/3 complex. Filament barbed end concentrations were calculated as described²⁷. *Chemical Denaturation*. GBD-EspF_U fusion proteins (10 μM) were denatured with guanidine hydrochloride. Tryptophan fluorescence emission (λ_{ex} = 295 nm, λ_{em} = 320 nm, 355 nm) was fit to a six parameter equation²⁸ to yield ΔG_{unfolding}. *Isothermal Titration Calorimetry*. EspF_U proteins were titrated into N-WASP_C in a Microcal VP-ITC microcalorimeter. Baseline was corrected for EspF_U addition to buffer. Data were fit to a single site binding model.

Mammalian Cell Culture

Mouse fibroblast-like cells were transfected with HA-Tir-EspF_U fusions as described¹. Transfected cells were treated with *E. coli* expressing intimin for 3.5 hours (for Tir-R₂ fusions)

or antibodies recognizing the extracellular domain of EHEC Tir (gift from A. Donohue-Rolfe, Tufts University School of Veterinary Medicine), followed by *S. aureus* particles (Pansorbin; Calbiochem) (for Tir fusions with a single EspF_U repeat).

Immunofluorescence Microscopy

Monolayers were fixed and permeabilized as described²⁹. Cells were treated with HA.11 or α -N-WASP (gift from S. Snapper), washed and treated with Alexa488 goat anti-mouse antibody (1:200; Molecular Probes) or Alexa568 goat anti-rabbit antibody (1:150; Molecular Probes) respectively. F-actin was stained with Alexa568-phalloidin (1:100; Molecular Probes).

NMR Spectroscopy and Structure Calculation

The structure of the complex of GBD (residues 242–310) and R33 (residues 1–33 in Fig. 1, corresponding to residues 268–300 in EspF_U (Fig. S1)) was determined in iterative fashion based on NOE, dihedral and hydrogen bond restraints using ARIA 2.1³⁰.

Additional Methods

Protein Expression and Purification

To simplify nomenclature, EspF_U residues are numbered 1–47 below and throughout the text (cf. Fig. 1), corresponding to residues 268–314 of the full length protein (Fig. S1). WASP GBD (residues 242–310), EspF_U R33 (residues 1–33), R33 mutants, GBD-R33 (WASP 242-310 - GSGSGSHM- EspF_U 1-33) and other GBD-repeat fusions were cloned into a modified pGEX-2T vector with a Tev protease cleavage site at the C-terminus of GST. EspF_U R₅ (residues 80–314) was cloned into a pET15b vector. EspF_U R47 (residues 1–47) was cloned into a modified pET19b vector with a His₁₀-ubiquitin-Tev protease site at the N-terminus. All proteins were expressed in *E. coli* strain BL21(DE3). Uniform ¹⁵N/¹³C labeling of proteins was carried out in M9 minimum media supplemented with ¹⁵NH₄Cl and ¹³C-glucose as the sole nitrogen and carbon sources. Samples of U-[¹⁵N,²H], Ile-[¹³C^δH₃], Leu-[¹³CH₃,¹²CD₃], Val-[¹³CH₃,¹²CD₃] labeled proteins were obtained from bacterial growth in M9 D₂O media containing 3 g/l of U-[²H]-glucose, 1 g/l of ¹⁵NH₄Cl, and 50 mg/l of 2-keto-3-methyl-d₃-3-d₁-4-¹³C-butyrate and 50 mg/l 4-[¹³C,¹H]-3,3-²H- α -ketobutyrate (the latter two, added 1 hr before induction)³¹. The 3,3-[¹H] or the 3-[¹H] positions of commercially available α -ketobutyrate or 2-keto-3-methyl-butyrate were exchanged to ²H prior to use, according to the procedure of Gardner et al.³². EspF_U R14 and R18 peptides were synthesized at the UT Southwestern Protein Chemistry Technology Center and quantified by amino acid analysis.

Cells expressing GBD and GBD-EspF_U fusions were lysed by sonication in GST buffer (20 mM Tris pH 8.0, 100 mM NaCl, 1 mM DTT, and 1 mM EDTA) with protease inhibitors. Cleared lysates were applied to glutathione sepharose 4B (GE Healthcare). Following wash, GST-tagged proteins were eluted in GST buffer containing 10 mM reduced glutathione. Proteins were concentrated, cleaved with Tev protease to separate the GST tag, diluted three-fold into QA buffer (20 mM Tris pH 8.0, 1 mM DTT, and 1 mM EDTA) and loaded onto an anion exchange column (Mono Q, GE Healthcare) equilibrated in QA buffer. The column was developed with a linear gradient from 0% to 50% QB buffer (Buffer A plus 1 M NaCl) in 10 bed volumes. Protein-containing fractions were further purified by size exclusion chromatography (Superdex 75 column, GE Healthcare). GST-GBD was purified similarly, except that the Tev protease cleavage was omitted. EspF_U R33 and its mutants were purified similarly, except that in the anion-exchange step the protein was collected in the flow through. His₁₀-ubiquitin tagged EspF_U R47 was retained from cleared bacterial lysate on a Ni-NTA column (Qiagen), eluted with an imidazole gradient, desalted into QA buffer and cleaved with Tev protease to remove the His₁₀-ubiquitin tag. Subsequent anion exchange and size exclusion chromatographies were the same as described for EspF_U R33. His₆-EspF_U R₅ was purified by

Ni affinity chromatography and buffer exchanged into SA buffer (50 mM sodium phosphate pH 6.0, 1 mM DTT, and 1 mM EDTA). Thrombin (GE Healthcare) cleaved EspF_U R₅ was purified by cation exchange (Mono S, GE Healthcare) in a gradient from 300 mM to 600 mM NaCl over 10 column volumes. Fractions containing EspF_U R₅ were pooled and further purified by gel filtration chromatography (Superdex 200, GE Healthcare). WASP GBD-VCA, N-WASP_C (residues 193–501), bovine Arp2/3 complex and rabbit skeletal muscle actin were purified as described previously^{25–27}.

Pull-Down Assays

GST-GBD was immobilized on Glutathione-sepharose 4B beads (~75 nmole/ml beads, GE Healthcare). Beads (60 μ l) were incubated with 30 μ M EspF_U R33 and 15 μ M WASP VCA separately in a final volume of 230 μ l and washed three times. The same volume of beads containing VCA-bound GST-GBD was further incubated with 200 μ M R33 and washed four times. Flow through and beads were analyzed by SDS-PAGE stained with Coomassie Blue.

Mammalian Cell Culture and Immunofluorescence Microscopy

To create transfection plasmids expressing Tir-EspF_U fusions (all N-terminally HA-tagged), DNA fragments encoding EspF_U R14, R30, R33, R47, R₂ (repeats 4–5) and point mutants were cloned into the KpnI and BamHI sites of pHN-Tir Δ C¹. Mouse fibroblast-like cells were cultured in six-well plates in DMEM plus 10% FBS and transfected with 1 μ g of plasmid per well for 12–16 h using Lipofectamine Plus reagent (Invitrogen)¹. Cells were reseeded onto 12-mm glass coverslips to achieve 50–75% confluency after an additional 24 h of growth. Transfected cells expressing Tir Δ C-R₂ proteins were treated with an *E. coli* expressing intimin for 3.5 hours. Cells expressing Tir Δ C fused to a single EspF_U repeat were treated with a 1:200 dilution of antibodies raised against the extracellular domain of EHEC Tir (anti-TirM) for 30 min, washed to remove excess antibody, and then treated with *S. aureus* particles (Pansorbin; Calbiochem) for a further 2.5 h. In all cases, monolayers were fixed in PBS+4% paraformaldehyde for 30 minutes and permeabilized with 0.1% Triton-X-100 as described²⁹. Cells were then treated with HA.11 (diluted 1:500 in PBS+1% BSA) or HA.11 and α -N-WASP (1:500) for 30 minutes prior to washing and addition of Alexa488 goat anti-mouse antibody (1:200; Molecular Probes) or Alexa568 goat anti-rabbit antibody (1:150). F-actin was identified by staining with Alexa568-phalloidin (1:100; Molecular Probes).

NMR Spectroscopy and Structure Calculation

The complex between GBD and R33 was isolated by gel filtration chromatography (in 50 mM sodium phosphate pH 6.8, 100 mM NaCl, 1 mM DTT, and 1 mM EDTA), and concentrated to 1–1.5 mM for all NMR experiments. Backbone and sidechain chemical shift assignments were obtained using standard triple-resonance methods in ¹⁵N/¹³C-labeled samples^{33, 34}. A total of 1552 distance restraints were obtained from 2D ¹H-NOESY, 3D ¹⁵N-edited NOESY (τ_{mix} = 100 ms), and 4D ¹³C/¹³C-edited NOESY (τ_{mix} = 75 ms)³⁵ spectra. One hundred intermolecular NOEs were identified in a ¹³C-filtered NOESY spectrum (τ_{mix} = 100 ms) of ¹³C, ¹⁵N-GBD in complex with unlabeled R33 recorded in D₂O³⁶. A total of 165 unambiguously assigned NOEs define the GBD-R33 interface. Backbone dihedral angles were restrained based on chemical shift analyses in the program TALOS³⁷ (maximum of 30° or the standard deviation observed in the TALOS database matches). Hydrogen bond restraints (HN–O distance, 1.5–2.8 Å; N–O distance, 2.4–3.5 Å) were applied to amide groups that were protected for > 1 hour in a sample of a ¹⁵N-GBD-R33 fusion protein freshly dissolved in D₂O NMR buffer. Structures were calculated and iteratively refined using the program ARIA 2.1³⁰. Intermolecular NOEs were assigned manually, in iterative fashion, from the ¹³C-filtered NOESY spectrum, and assigned distance restraints within ARIA 2.1. Other NOEs were assigned automatically within ARIA 2.1. In the final iteration, structures were refined in

explicit water to improve sidechain packing and hydrogen bonding³⁸. All spectra were processed and analyzed using NMRPipe³⁹ and NMRView⁴⁰. The twenty final lowest energy conformers were analyzed with ProCheckNMR⁴¹. Structure figures were generated with Ribbons⁴² and PyMOL⁴³.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 2. Structures of the WASP GBD in complex with different ligands

A, B. Complex of WASP GBD and EspFU R33. Structure is colored: layer 1 (WASP 250–276), yellow; layer 2 (WASP 277–310), blue; layer 3 (EspFU 2–20), green. In B, GBD is shown as surface representation, R33 is shown as a ribbon with sticks for sidechains that contact the GBD. Views are related by 180° rotation about a vertical axis. **C, D.** Autoinhibited WASP (GBD-C⁷). GBD colored as in A; C region of WASP VCA is red. Representation and views are as in panels A and B.

Figure 3. EspF_U induces actin pedestal formation

A. Cells expressing the Tir Δ C, Tir Δ C-R₂ or Tir Δ C-R₂^{VL*} (with N-terminal HA-tags) were challenged with *E. coli* expressing intimin, and stained with anti-HA antibody (green) and Alexa568-phalloidin (red, left panels) or anti-N-WASP antibody (red, right panels). Colocalization is yellow in merged images. The % of transfected cells harboring at least five F-actin foci was quantified (Actin Assembly Index). Bacteria were visualized by DAPI staining. **B.** Cells expressing Tir-repeat proteins were challenged with anti-Tir antibody and *S. aureus* particles and Actin Assembly Index was determined. In A and B data represent mean $\pm$ SD from at least two samples with 20-50 cells examined per sample.