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The Pathogen Protein $EspF_U$ Hijacks Actin Polymerization Using Mimicry and Multivalency

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Abstract

Enterohemorrhagic *E. coli* (EHEC) attaches to the intestine through actin pedestals that are formed when the bacterium injects the protein $EspF_U$ into host cells[1]. $EspF_U$ potently activates the host WASP (Wiskott-Aldrich syndrome protein) family of actin-nucleating factors, which are normally activated by the GTPase Cdc42, among other signaling molecules. Apart from its N-terminal type III secretion signal, $EspF_U$ consists of five and a half 47-amino-acid repeats. Here we find that a 17-residue motif within this $EspF_U$ repeat is sufficient for interaction with N-WASP. Unlike most pathogen proteins that interface with the cytoskeletal machinery, this motif does not mimic natural upstream activators: instead of mimicking an activated state of Cdc42, $EspF_U$ mimics an autoinhibitory element found within N-WASP. Thus $EspF_U$ activates N-WASP by competitively disrupting the autoinhibited state. By mimicking an internal regulatory element and not the natural activator, $EspF_U$ selectively activates only a precise subset of Cdc42-activated processes. Although one repeat is able to stimulate actin polymerization, we show that multiple-repeat fragments have dramatically increased potency. The activities of these $EspF_U$ fragments correlate with their ability to coordinate activation of at least two N-WASP proteins. Thus this pathogen has used a simple autoinhibitory fragment as a component to build a highly effective actin polymerization machine.

Many pathogens target their host's cytoskeletal machinery to facilitate attachment, entry, or cell-to-cell spreading[2, 3]. EHEC (O157:H7) causes severe gastrointestinal disease, and infection is dependent on attachment of the bacterium to intestinal epithelial cells via actin pedestals[1]. Pedestal formation is mediated by the pathogenic protein $EspF_U$ (also known as TccP)[4, 5], which is injected through a type III secretion system[6] into host cells where it stimulates actin polymerization by activating host WASP proteins (Fig. 1a). WASPs are activators of the Arp2/3 complex, but under basal conditions this function is regulated by an autoinhibitory interaction between the GTPase-binding domain (GBD) and the "C" helix in the catalytic WCA region[7]. Normal activation of neuronal WASP (N-WASP) requires disruption of autoinhibition (Fig. 1b), involving coordinated action of multiple endogenous

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activators, including Cdc42, the phospholipid PIP_2, and SH3-domain-containing proteins such as Nck[8, 9] (Fig. 1c). $EspF_U$ can independently activate N-WASP with a potency that is orders of magnitude higher than single endogenous activators[8] (Fig. 1c,d, Fig. S1).

A common strategy exploited by pathogens in targeting host cell processes is to produce a protein that mimics an endogenous activator of that process[6, 10, 11]. This mimic will generally be constitutively active compared to the endogenous protein, which shows regulated activity[12, 13]. Among pathogens that target actin polymerization, there are examples of effector proteins that mimic the active states of N-WASP[14] or N-WASP activators like Cdc42[15]. Previous studies have shown that $EspF_U$ interacts with the GBD[16, 17], the regulatory domain in N-WASP that participates in autoinhibition and binds to activated Cdc42[7]. Thus, we set out to test the hypothesis that $EspF_U$ may mimic an activated state of Cdc42.

$EspF_U$ has a type III secretion signal followed by five and a half 47-amino-acid repeats (Fig. 1e) (a six-and-a-half-repeat isoform of $EspF_U$ has also been reported[5]). The repeats are nearly identical and share little sequence homology with other proteins, except for the related EHEC protein EspF. EspF also activates N-WASP[18] but does not participate in pedestal formation[4, 19] (EspF contributes to the disruption of epithelial tight junctions).

We first mapped the minimal interacting fragments of both $EspF_U$ and N-WASP using pulldown-binding assays. We found that the N-WASP GBD could bind to a single $EspF_U$ repeat. We tested other truncations and found that the first 17 amino acids of each repeat constitute the minimal fragment with high-affinity for the GBD (Fig. 1f, Fig. S2). This sequence corresponds to a predicted α-helix[20] and it is repeated six times in the full-length protein. There are minor sequence differences among the six copies of this motif, but the repeats appear to be equivalent in binding and functional assays (data not shown).

The N-WASP GBD is a composite regulatory domain made up of two overlapping elements – a Cdc42-binding element and an autoinhibitory element that binds to the C helix[7]. Detailed mapping of the N-WASP side of the interaction showed that the C-terminal, autoinhibitory portion of the GBD interacts with $EspF_U$ and the N-terminal, Cdc42-binding portion does not. The minimal $EspF_U$-binding fragment of N-WASP is residues 228–270 (Fig. 1f, Fig. S2), which form the core of the interaction with the C helix in the autoinhibited structure (Fig. 2a)[7].

Since a small peptide from $EspF_U$ interacts with the autoinhibitory portion of the GBD, it seemed unlikely that $EspF_U$ was activating N-WASP by mimicking Cdc42. Instead, we postulated that this $EspF_U$ fragment might be mimicking the C helix and disrupting N-WASP autoinhibition by competitively binding to the GBD. We found that $EspF_U$ does indeed competitively disrupt the N-WASP GBD autoinhibitory interaction in a pulldown-binding assay (Fig. 2b). Sequence alignment of the C helix and the first 17 amino acids of the $EspF_U$ repeat revealed sequence homology at three positions where hydrophobic amino acids are known to be critical for interaction with the GBD (Fig. 2a,c)[21]. We carried out a systematic alanine scan of both the N-WASP and $EspF_U$ fragments and tested binding of the mutants to the GBD. The results showed that mutation of any of the conserved hydrophobic positions to alanine on either the C helix or the $EspF_U$ fragment completely disrupted interaction with the GBD (Fig. 2c).

Previous work has shown that the isolated WASP GBD is only partially folded and unstable, but that fusion of the C helix to the GBD leads to a stably folded, helical structure that approximates the autoinhibited state[7]. We made a fusion of the GBD to the minimal 17-amino-acid interacting fragment from $EspF_U$ found that this protein had very similar helical content and thermal stability to the GBD-C helix fusion (Fig. S3).

These results show that the N-terminal 17 amino acids of the $EspF_U$ repeat are mimicking the C helix (an autoinhibitory element) and this motif competitively disrupts autoinhibition of WASPs (Fig. 2d). A similar sequence motif is present in EspF and likely activates N-WASP using the same mechanism. We found that one $EspF_U$ repeat binds to the N-WASP GBD (amino acids 214–274) with a K_d of 18 nM (Fig. S4). This affinity is about 100-fold tighter than previously measured values for the corresponding WASP GBD-C helix interaction (*in trans*) [7] and this higher affinity explains how an intermolecular interaction can competitively disrupt an intramolecular interaction. The C helix also contributes to N-WASP's interactions with actin monomers and the Arp2/3 complex[22]. We found that $EspF_U$ can bind to these molecules as well (Fig. S4). In spite of this, $EspF_U$ cannot stimulate actin polymerization without N-WASP (data not shown).

Utilization of a competitive autoinhibitory fragment represents a novel strategy for a pathogen to use in hijacking host cell signaling. One possible advantage of this strategy is increased specificity: a constitutively active signaling mimic will activate all downstream endogenous signaling pathways, whereas a mimic of a regulatory element should only activate a small subset. To test this idea we investigated whether $EspF_U$ could activate the p21-activated kinases (PAKs) – another group of Cdc42-responsive proteins whose regulation is most closely related to WASPs'. Cdc42 activates PAKs by disrupting an autoinhibitory interaction (which is structurally homologous to that of WASP)[23]. However, the PAK homolog of the WASP C helix lacks the consensus sequence of three conserved hydrophobic amino acids. $EspF_U$ was unable to activate PAKs (Fig. 2e, Fig. S5), indicating specificity of $EspF_U$ towards WASP family proteins (Fig. 2d). This more targeted activation may be advantageous for the pathogen, given the broad range of downstream effects mediated by WASP activators like Cdc42.

We also investigated the functional significance of this autoinhibitory mimic being repeated six times in one $EspF_U$ molecule. We tested the relative abilities of different length $EspF_U$ fragments to stimulate actin assembly using an *in vitro* pyrene-actin polymerization assay. One repeat from $EspF_U$ can disrupt the N-WASP autoinhibitory interaction in a binding assay (Fig. S6), but has low ability to stimulate actin polymerization (comparable to a single endogenous activator) (Fig. 3a). A fragment of two repeats shows intermediate activity in the actin assay, but at least three repeats are necessary for high potency. Each additional repeat gives higher activity until full-length $EspF_U$ (residues 80–337, lacking the secretion signal), which has a maximal activity at the same level as unregulated N-WASP (Fig. 3a, Fig. S7). To see if this trend continued, we made an $EspF_U$ protein that has eight and a half repeats and found that it reaches the same maximal activity as the wildtype protein, but is even more potent at low concentrations (Fig 3a). At very high concentrations, $EspF_U$ proteins had a partial inhibitory effect on actin polymerization (data not shown), likely due to $EspF_U$ sequestering actin monomers and/or Arp2/3.

We constructed a minimized $EspF_U$ protein in which six copies of the 17-residue C-helix mimic are separated by glycine-serine linkers. This construct was still able to stimulate actin polymerization (albeit with lower potency than wildtype $EspF_U$), showing that the C-terminal portion of each $EspF_U$ repeat is not essential for activity (Fig. S8).

Next, we tested whether multiple repeats were also required in more complex assays of actin assembly – namely a reconstituted bead motility assay and an *in vivo* clustering assay. In the first assay, $EspF_U$ fragments are chemically coupled to polystyrene beads and these beads are incubated with a mixture of actin and actin-regulatory proteins and examined by microscopy for the ability to stimulate actin assembly and motility[24]. Beads coated with full-length $EspF_U$ triggered formation of an F-actin shell that breaks symmetry and induces transient polarized bead movement (Fig. 3b). Beads coated with three $EspF_U$ repeats showed similar behavior (albeit with a slightly more diffuse actin shell), while two-repeat beads formed shells

that were very diffuse, showed significantly reduced actin fluorescence, and dissembled within minutes. Beads coated with a single repeat showed minimal ability to polymerize actin (Fig. 3b). We varied the surface density of $EspF_U$ protein on the beads and found that this had little effect on the relative activities of the different fragments (data not shown). In particular, one-repeat beads had weak activity, even at maximal density. This shows that a high local concentration of a single repeat has minimal effect – multiple repeats in the same polypeptide chain are required for potent activation of actin polymerization.

To test the ability of $EspF_U$ fragments to form actin structures in cells, we used an antibody-induced clustering assay. Previous work has shown that N-WASP or N-WASP activators can form actin structures when clustered at the plasma membrane[25]. In our assay, the $EspF_U$ fragment is expressed as a fusion to the cytoplasmic tail of a transmembrane protein and then clustered using an antibody that recognizes the extracellular portion of the protein[26]. Upon clustering of full-length $EspF_U$, we observed co-localization of N-WASP and bright actin foci to the clusters, showing that clustering of $EspF_U$ is sufficient to trigger localized actin assembly (Fig. 3c, Fig. S9, S10). Furthermore, we tested three-, two- and one-repeat fragments and observed the same trend: three repeats showed similar activity to full-length, two repeats had intermediate activity and one repeat had lower activity (Fig. 3c). We used a computational image analysis tool (D. Vasilescu, unpublished) to detect and correlate antibody and actin clusters (Fig. S11). These quantitative results show that clustering of $EspF_U$ fragments with more repeats causes formation of actin clusters with higher frequency and intensity (Fig. 3d).

The requirement of multiple repeats for activation of actin polymerization suggests that $EspF_U$ needs to simultaneously bind and activate multiple N-WASP proteins to be highly active. To address this, we investigated the stoichiometry of the $EspF_U$ – N-WASP complex using analytical ultracentrifugation (AUC). By AUC (data not shown) and binding assays (Fig. S12), we found that each repeat in an $EspF_U$ fragment could simultaneously bind an isolated N-WASP GBD (for example, a three-repeat fragment bound to three GBDs). However, the model that $EspF_U$ has high potency because it can activate multiple N-WASPs does not explain why a two-repeat fragment has lowered activity in our actin assays. We found a likely answer to this question through stoichiometric analyses of complexes of a two-repeat protein with a larger, autoinhibited N-WASP construct. This AUC data showed that while the two-repeat fragment can bind one N-WASP easily, binding of a second is less favorable (possibly due to steric effects) and only happens in large excess of N-WASP (Fig. S13). Larger fragments of $EspF_U$, on the other hand, readily formed complexes with two or more autoinhibited N-WASP proteins (Fig. S13). Taken with our activation data, this suggests that activity of $EspF_U$ fragments correlates with their ability to coordinate the activation at least two N-WASP proteins (Fig. 3e). There are a number of other cases where oligomerization of WASPs or WASP activators leads to similarly increased activity – including the potent actin polymerization induced by GST-tagged N-WASP WCA[27] (presumably due to GST dimerization) and by oligomerized SNX9 in endocytosis[28].

We have shown here that the EHEC protein $EspF_U$ has evolved a unique mechanism to potently and specifically activate N-WASP-mediated actin polymerization through a repeated autoinhibitory mimic. This co-opting of a small internal regulatory unit for activation of a target protein represents a novel pathogenic strategy. Furthermore, multiple repeated copies of this motif are essential for potent activity, apparently because a single $EspF_U$ protein must coordinate simultaneous activation of multiple N-WASPs.

Methods Summary

$EspF_U$ expression and purification

$EspF_U$ fragments were amplified by PCR from EHEC genomic DNA (ATCC). All cloning and expression was done in *E. coli* BLR(DE3) strains lacking recombinase A.

Pyrene-actin polymerization assay

Arp2/3 was purified from bovine brain and actin was purified from rabbit muscle and pyrene-labeled, as previously described[29]. Actin polymerization was monitored as an increase in pyrene-actin fluorescence. Raw fluorescence data were normalized to the lower and upper baselines of each curve. Relative activities of $EspF_U$ fragments were determined through calculation of the time required to reach half-maximal fluorescence ($t_{1/2}$).

Bead motility assay

Actin and Arp2/3 were purified from *Acanthamoeba castellanii*, as previously described[30]. $EspF_U$ proteins were coupled to polystyrene beads and mixed with actin, Arp2/3, N-WASP, capping protein and profilin. The assay was adapted from a protocol developed by Orkun Akin & R. D. Mullins[24]. Images were acquired with a Nikon TE300 Eclipse inverted epifluorescence microscope with constant imaging settings between experiments.

In vivo clustering experiments

NIH 3T3 fibroblasts were co-transfected with actin-GFP and the corresponding CD16/7 fusion construct. Cells were treated with clustering antibodies, as previously described[26]. Fixed cells were imaged with an oil-immersion 60X NA 1.4 objective on a Zeiss LSM 510 Meta microscope. Imaging settings were constant between experiments, except for minor adjustments of detector gain to accommodate variation in expression level.

Quantitative detection of clusters was done with a computational tool developed by D. Vasilescu. Ten cell images from three independent experiments were quantified for each construct. Data were presented in two metrics: the percentage of antibody clusters that have associated actin clusters and the intensities of these associated actin clusters. The relative intensity value is the ratio of the sum of intensities of associated actin clusters to the sum of intensities of all antibody clusters.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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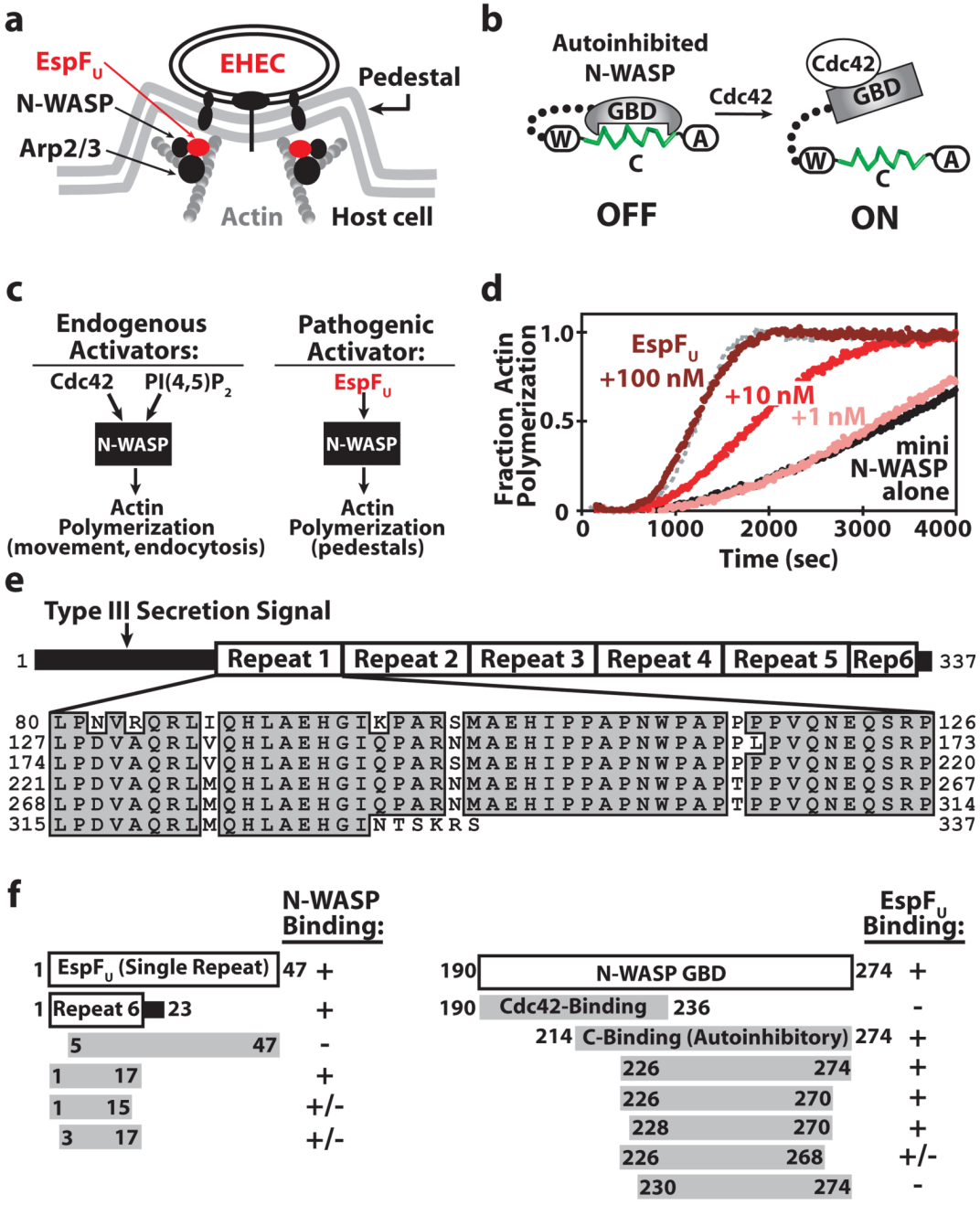

Figure 1. EspF$_U$ is a potent activator of N-WASP

a, EspF$_U$ is secreted from EHEC into a host cell where it stimulates actin polymerization through endogenous N-WASP and Arp2/3, leading to pedestal formation. **b**, N-WASP is basally autoinhibited, but can be activated by inputs like Cdc42. **c**, Multiple endogenous activators are necessary to potently activate N-WASP. EspF$_U$ accomplishes this with a single input. **d**, EspF$_U$ potently activates N-WASP in an *in vitro* pyrene-actin polymerization assay. Maximal rate (50 nM unregulated N-WASP WCA) shown in gray dotted line. All other reactions contain 50 nM mini N-WASP (B-GBD-linker-WCA). **e**, Sequence of EspF$_U$. **f**, Mapping minimal binding fragments of EspF$_U$ and N-WASP with a glutathione S-transferase (GST) pulldown assay.

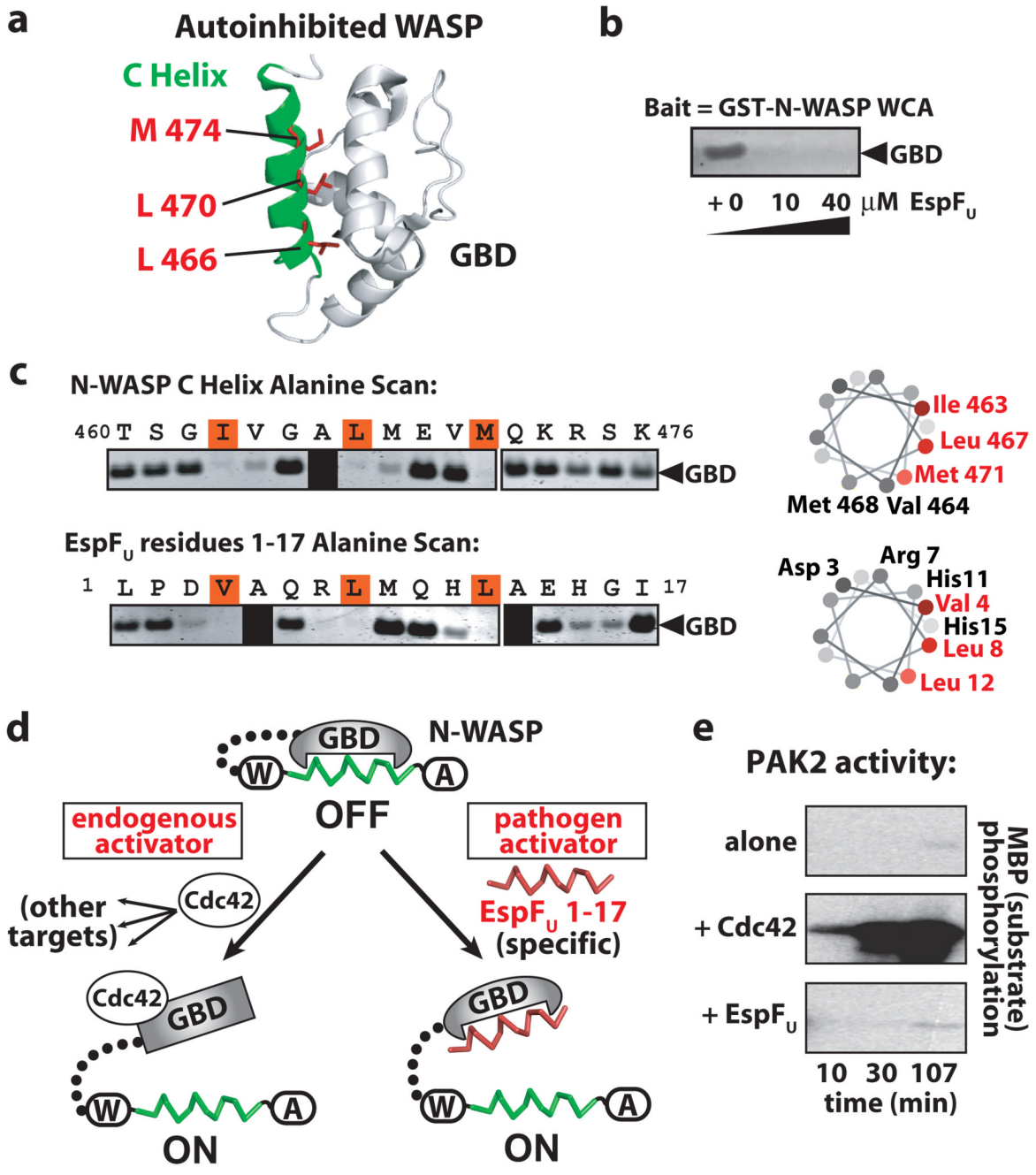

Figure 2. EspF$_U$ mimics the N-WASP autoinhibitory C helix

a, Structure of autoinhibited fragment of WASP[7], highlighting the C helix (green) and three critical side chains (red). **b**, EspF$_U$ can competitively disrupt the N-WASP autoinhibitory interaction (*in trans*) in a GST-pulldown assay. **c**, Alanine scanning mutagenesis shows similar pattern of critical residues in N-WASP C helix and EspF$_U$ (repeat 4, residues 1–17) (assayed by binding to GBD in a GST-pulldown assay). **d**, Model comparing N-WASP activation by Cdc42 and EspF$_U$. **e**, PAK kinase assay showing phosphorylation of myelin basic protein (MBP). PAK2 alone is autoinhibited – Cdc42 activates it, but EspF$_U$ does not.

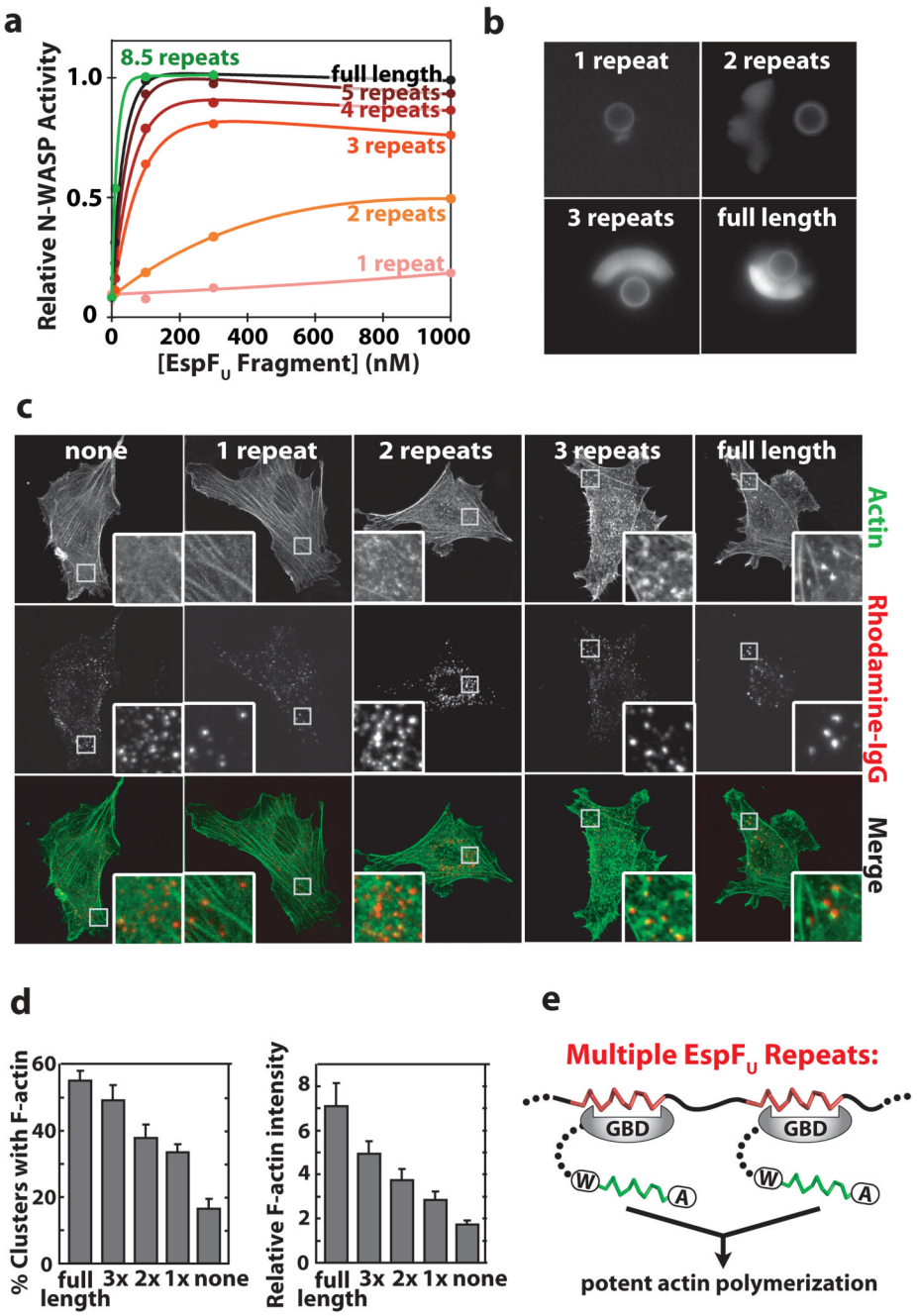

Figure 3. Multiple repeats of EspF$_U$ are required for potent activation of actin polymerization

a, Activities of EspF$_U$ fragments in a pyrene-actin polymerization assay. Data are fit to single exponential curves using MATLAB (MathWorks), with a small linear term subtracted to account for EspF$_U$ sequestration of actin and/or Arp2/3. **b**, Visualization of fluorescently-labeled actin around beads coated with EspF$_U$ fragments. Images were acquired four minutes after initiation. **c**, NIH 3T3 cells expressing GFP-actin and EspF$_U$ fused to a transmembrane protein (CD16/7), which is clustered with a rhodamine-tagged antibody[26]. Inset in each image is a portion of that cell (white box) shown at higher magnification. **d**, Results from quantification of clustered cells, shown in two metrics. Bars represent mean ± s.e.m. of 10 cell

images. **e**, Activity correlates with ability of $EspF_U$ fragments to bind at least two N-WASP proteins simultaneously.