In Brief
Humphreys et al. find that the virulence effector EspG of enteropathogenic and enterohaemorrhagic Escherichia coli inhibits pathogen phagocytosis by inhibiting activation of the WAVE regulatory complex (WRC). EspG uncouples the small GTPases Arf1 and Arf6 from Rac1, thus initiating WRC-dependent actin polymerization.

Highlights
- WAVE regulatory complex (WRC) and Arf and Rac1 direct phagocytosis of EPEC and EHEC
- Virulence effector EspG inhibits the WRC to counteract pathogen phagocytosis
- EspG blocks Arf6 signaling to ARNO that activates Arf1
- EspG blocks Arf1 collaboration with Rac1 and WRC activation
Inhibition of WAVE Regulatory Complex Activation by a Bacterial Virulence Effector Counteracts Pathogen Phagocytosis

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SUMMARY

To establish pathogenicity, bacteria must evade phagocytosis directed by remodeling of the actin cytoskeleton. We show that macrophages facilitate pathogen phagocytosis through actin polymerization mediated by the WAVE regulatory complex (WRC), small GTPases Arf and Rac1, and the Arf1 activator ARNO. To establish extracellular infections, enteropathogenic (EPEC) and enterohemorrhagic (EHEC) Escherichia coli hijack the actin cytoskeleton by injecting virulence effectors into the host cell. Here, we find that the virulence effector EspG counteracts WRC-dependent phagocytosis, enabling EPEC and EHEC to remain extracellular. By reconstituting membrane-associated actin polymerization, we find that EspG disabled WRC activation through two mechanisms: EspG interaction with Arf6 blocked signaling to ARNO while EspG binding of Arf1 impeded collaboration with Rac1, thereby inhibiting WRC recruitment and activation. Investigating the mode of EspG interference revealed sites in Arf1 required for WRC activation and a mechanism facilitating pathogen evasion of innate host defenses.

INTRODUCTION

Professional phagocytes cells represent the first line of host defense against bacterial pathogens. To eradicate pathogenic bacteria, professional phagocytes employ myriad host cell-surface receptors that bind the target bacterium directly (e.g., bacterial surface sugars) or indirectly through host-derived opsonins (e.g., antibodies, complement) (Celli and Finlay, 2002; Sarantis and Grinstein, 2012). Receptor binding triggers polymerization of actin filaments that guide the plasma membrane around the pathogen to facilitate bacterial uptake and destruction within an intracellular microbicidal phagolysosome compartment. The actin polymerization requires Rho GTPases Rac1 and Cdc42 that anchor by lipid prenylation to the membrane where they recruit and activate myriad cellular effectors responsible for directing cytoskeleton remodeling via the Arp2/3 complex (Caron and Hall, 1998; May et al., 2000).

Counteracting phagocytosis is a central paradigm in bacterial pathogenicity. For example, to inhibit opsonin-dependent trans-phagocytosis Staphylococcus aureus secretes protein A, which sequesters antibodies, while several pathogens use cell-surface capsule polysaccharides to inhibit deposition of complement (Celli and Finlay, 2002; Sarantis and Grinstein, 2012). However, phagocytes offset this strategy through myriad non-opsonic phagocyte receptors that directly bind bacteria and mediate cis-phagocytosis independent of opsonins. Nevertheless, uniting the diverse uptake mechanisms is the role of the actin cytoskeleton whose remodeling is required for phagocytosis (May et al., 2000). Consequently, pathogens have evolved sophisticated measures to interfere with the actin cytoskeleton and antagonize a spectrum of phagocytic mechanisms at the molecular level.

Enteropathogenic and enterohemorrhagic Escherichia coli (EPEC and EHEC) are major global human health threats causing gastroenteritis and bloody diarrhea, respectively (Hartland and Leong, 2013). To cause disease, they inject a cocktail of virulence effectors into host cells via a type 3 secretion system (T3SS) to enable cell-surface colonization on intestinal epithelia where the pathogen forms lesions characterized by the destruction of brush border microvilli. Here, the bacteria encounter macrophages that infiltrate sites of infection yet EPEC and EHEC are able to block their own phagocytosis through the injected virulence effectors (Santos and Finlay, 2015). Indeed, mutants of type 3 secretion are phagocytosed by macrophages (Goosney et al., 1999; Marchés et al., 2008). Four virulence effectors are known to contribute to anti-phagocytosis (Santos and Finlay, 2015); EspB interacts with the actin binding motor protein myosin-1c (Iizumi et al., 2007), EspF inhibits PI3 kinase signaling (Celli et al., 2001), EspH inhibits the Dbl subfamily of Rho guanine nucleotide exchange factors (GEFs) (Dong et al., 2010) and EspJ impedes phagocytosis through inhibition of Src kinase activity (Young et al., 2014).

EPEC and EHEC employ multiple mechanisms to disable phagocytosis. While it is clear that the pathogens target the actin cytoskeleton, we do not yet understand the identity of the cellular actin nucleation machinery governing pathogen phagocytosis.
and therefore the mechanisms of bacterial interference. Consequently, we first sought to identify the players underlying the actin filament polymerization that are targeted by virulence effectors.

RESULTS

EPEC Opposes Phagocytosis Directed by the WAVE Regulatory Complex

To address how pathogenic *Escherichia coli* resist engulfment by macrophages, we infected differentiated human THP1 macrophage cells with wild-type EPEC (EPECWT) or T3SS-deficient mutant EPEC (EPECΔT3SS) labeled with pHrodo, a pH-sensitive dye that fluoresces red in the low pH of phagosomes and signified pathogen uptake (Figure 1A). Intracellular bacteria were inaccessible to antibodies against EPEC that marked extracellular bacteria. Only ~27% of EPECWT were found intracellular within phagosomes of host cells (actin) and labeling of extracellular bacteria with antibodies demonstrated that the majority of bacteria had counteracted phagocytosis (Figures 1A and 1B). In contrast, very few extracellular EPECΔT3SS were observed as ~92% of bacteria had been phagocytosed. Equivalent results were observed in RAW267.4 mouse macrophage cells (Figure S1A), confirming that EPEC fights phagocytosis using T3SS effectors.

Given the central role of Arp2/3-dependent actin polymerization in phagocytosis (May et al., 2000), it seemed likely that EPEC targeted activators of Arp2/3. The WAVE regulatory complex (WRC) is one such activator that is known to drive pathogen macropinocytosis in epithelial cells (Humphreys et al., 2012b, 2013) and has been implicated in phagocytosis by Dictyostelium (Seastone et al., 2001), mammalian granulocytes (Plis et al., 2012), and neutrophils and macrophages (Park et al., 2008). The WRC is a heteropentameric complex comprising Cyfip, Nap/Hem, Abi, and HSPC300 or their homologs (Gautreau et al., 2006), which must be activated directly by Rac1 and non-hydrolysable GTP

EPEC Effector EspG Disables WAVE Regulatory Complex Activation

Given the prominent role of small GTases in EPECΔT3SS phagocytosis, we reasoned that EPEC effectors might directly target these players to resist uptake. This strategy is a central virulence strategy employed by many pathogens that deactivate small GTases through effectors possessing GTase-activating activity that promote GTase hydrolysis (Dean, 2011). Even so, EPEC encodes no known GTase-activating protein (GAP) of Rac1 or Arf1 and therefore likely uses an alternative mechanism. One possibility included the EPEC effector EspG that was previously shown to act as a molecular scaffold by simultaneously binding the Rac1 effector p21 activated kinase (PAK) and GAP-1 (Hayashi et al., 2010). Though whether EspG directly activates PAK, while EspG interaction with Arf sterically hinders Arf GAPs, thereby maintaining the GTase-bound form of Arf, which still has portions of its switch 1 and 2 domains exposed to permit interactions with some, but not all, of its cellular effectors (Selyunin et al., 2011, 2014).

First, we examined whether EspG antagonizes WRC-dependent cytoskeleton remodeling by infecting THP1 macrophages with pHrodo-labeled EPECWT or an isogenic strain with null mutations in espG and its close homolog espG2, henceforth EPECΔespG (Figure 1D). In contrast to wild-type bacteria, EPECΔespG bacteria were incapable of resisting phagocytosis, and ~85% were observed intracellularly mirroring the phagocytosis of EPECΔT3SS (Figures 1B and 1D). Furthermore, this EspG role appears conserved as ΔespG strain of the related pathogen EHEC was also susceptible to phagocytic uptake while wild-type EHEC were resistant (Figure S1D).

We next investigated the mechanism of WRC interference by EspG. The phosphoinositide PIP3 is known to activate the WRC through Rac1 and Arf GTases (Lebensohn and Kirschner, 2009; Koronakis et al., 2011), and PIP3 is a major regulator of pathogen phagocytosis (Cox et al., 1999; Celli et al., 2001; Quidort et al., 2006), but how EPEC counteracts PIP3-driven pathways is unclear. We first reconstituted WRC-dependent actin polymerization driven by PIP3 using a motility assay in cell-free extracts as previously described (Hume et al., 2014). Silica microspheres coated with a phospholipid bilayer containing equal amounts of phosphatidylcholine (PC) and phosphatidylinositol (PI) plus 2% PIP3 (PII3) were added to cell-free extract containing fluorescent rhodamine-labeled actin and non-hydrolysable GTase. When PIP3-driven motility was examined in extract containing purified recombinant EspG, actin comet tail formation was abrogated and there was no actin assembly on the membrane surface that propelled the beads through the extract (Figure 1E).

To demonstrate that EspG was blocking WRC activation by Rac1 and Rac1, PC:PI microspheres (i.e., without PIP3) were added to cell-free extract containing fluorescent rhodamine-labeled actin and non-hydrolysable GTase. In the control, PIP3 microspheres triggered actin polymerization and generated actin-comet tails (of ~14 μm) on the membrane surface that propelled the beads through the extract (Figure 1E). When PIP3-driven motility was examined in extract containing purified recombinant EspG, actin comet tail formation was abrogated and there was no actin assembly on the membrane surface (+EspG). This mirrored the phenotype observed with a Rac1 inhibitor (EHT1864) (Figure 1E) indicating EspG inhibition of the WRC.
EspG (Figure 1F). WRC-dependent actin comet tail formation (~15 μm) was observed in extracts containing PC:PI microspheres co-anchored with Arf1QL and Rac1QL, but actin polymerization was abolished in extract containing EspG.

To further examine how EspG disables WRC-dependent actin polymerization, we scaled up the motility assays to isolate the components recruited to the membrane co-anchored with Arf1QL and Rac1QL in the presence or absence of EspG (Figures...
EspG Targeting of Arf1 Antagonizes WRC-Mediated Phagocytosis

EspG is a multifunctional virulence effector and may interfere with WRC-mediated cytoskeleton remodeling in several ways: EspG binds active Arf GTPases and deactivates Rab GTPases with WRC-mediated cytoskeleton remodeling in several ways: EspG is a multifunctional virulence effector and may interfere with WRC-mediated cytoskeleton remodeling in several ways: EspG is a multifunctional virulence effector and may interfere with WRC-mediated cytoskeleton remodeling in several ways: EspG is a multifunctional virulence effector and may interfere with WRC-mediated cytoskeleton remodeling in several ways.

EspG also activates PAK (Selyunin et al., 2011) that modulates actin filament polymerization, e.g., by deactivating cofilin (Edwards et al., 1999). Consistent with a possible role for PAK1, the presence of EspG at membranes co-anchored with Arf1QL and Rac1QL was co-incident with enhanced PAK1 recruitment (Figure 1H). To resolve how EspG disables WAVE complex activation, we first purified recombinant EspG mutants (Figure 2A) incapable of deactivating Rabs (EspGΔR; mutation Q293A) (Dong et al., 2012), binding PAK (EspGΔP; D205A, R208A) (Germane and Spiller, 2011), or binding Arf and PAK (EspGΔΔ; I152S, P351A, P355A) (Selyunin et al., 2011) before assessing interference with WRC-dependent actin-based motility directed by Arf1QL and Rac1QL (Figure 2B). Like EspGWT, mutant derivatives EspGΔR and EspGΔP both abrogated actin-comet tail formation showing that EspG interaction with PAK and its Rab GAP activity were dispensable for WRC inhibition. This was not the case for EspGΔΔ, which had no effect on WRC activity as actin comet tails (of ~14 μm as control) were formed and the microspheres moved through the extract. Thus, EspG targeting of Arf1 and not PAK or Rabs blocked WRC activation.

Consistent with this view, only EspGΔΔ was deficient in binding Arf1QL-anchored membranes in buffer (Figure 2C). Moreover, when EspGWT was pre-incubated with membranes co-anchored with Arf1QL and Rac1QL to form an Arf1-EspG

Figure 2. EspG Interaction with Arf GTPase Blocks the WRC

(A) Cartoon depicting key residues (white) in EspG (black) responsible for interaction with Rabs (green), PAK (blue), or Arf (purple).

(B) WRC-dependent actin-based motility via membrane-anchored Arf1QL and Rac1QL (depicted in cartoon) alone (control) or in the presence of EspG, namely, wild-type (WT) or mutants in binding Rab (EspGΔR), PAK (EspGΔP) or both Arf and PAK (EspGΔΔ). Molecular weight markers in kilodaltons (left).

(C) Interaction of EspG derivatives with membrane-anchored Arf1QL in buffer.

(D) Proteins recruited by membrane-anchored Arf1QL and Rac1QL (colored circles) in the presence or absence of EspG derivatives as (B) analyzed by SDS-PAGE. Molecular weight markers in kilodaltons (left).

(E) Immunoblotting of samples from (D) with indicated antibodies (right).

(F) Phagocytosis of EPEC ΔespG expressing a control vector or the vector encoding espG variants described in (B). Error bars represent ± SEM. Asterisks indicate a significant difference from control (black bars). ns, not significant. See also Figure S2.
complex before incubation in extract, WRC-dependent actin comet tail formation was not observed (data not shown). Furthermore, chemical inhibitors of PAK (i.e., IPA3) have been shown to inhibit activation by EspG (Selyunin et al., 2011), yet WRC-dependent actin-based motility was observed in PAK-inhibited extract (Figure S2B), reaffirming that EspG inhibits the WRC independently of PAK.

To further examine EspG inhibition of the WRC, we assessed WRC recruitment by Arf1QL and Rac1QL from cell extract in the presence of the EspG derivatives (Figures 2D and 2E). WRC recruitment to the membrane was obstructed by EspGWT, EspGΔN, and EspGΔA but not EspGΔAΔP as exemplified by the SDS-PAGE (Figure 2D, green arrows) and immunoblotting of Hem (Figure 2E). All EspG variants except EspGΔAΔP were recruited to Arf1QL and Rac1QL co-anchored membranes. Interestingly, EspGΔA but not EspGΔAΔP recruited PAK1 indicating that PAK recruitment was dependent upon the Arf1-EspG interaction and localization of the virulence effector at the membrane.

In parallel, we assessed phagocytic uptake of EPECΔespG expressing either EspGWT, EspGΔN, EspGΔA, EspGΔAΔP, or the empty vector as a control (Figures 2F and S2A). THP1 macrophages phagocytosed ~82% of EPECΔespG encoding the vector alone, while bacteria expressing EspGWT resisted WRC-dependent uptake that was reduced to ~35% (Figures 2F and S2A), mirroring the resistance imposed by wild-type EPEC (Figure 1). Similarly, EPECΔespG expressing EspGΔA or EspGΔAΔP also antagonized phagocytosis. In contrast, ~79% of bacteria expressing EspGΔAΔP were incapable of resisting the WRC and were phagocytosed to the same extent as the EPECΔespG vector control strain. Thus, EspG-mediated interaction with Arf GTPases, and not PAK or Rabs, combats WRC-directed pathogen phagocytosis.

Next, we examined whether EspG could counteract the activities of other pathogens dependent upon the WRC. In contrast to extracellular pathogens EPEC and EHEC, Salmonella Typhimurium is an intracellular pathogen that invades host epithelial cells by activating the WRC-Rac1-Arf1 axis (Humphreys et al., 2012b, 2013). When we examined Salmonella invasion in HeLa cells expressing HA-tagged EspG, pathogen uptake was reduced by ~64% relative to control, which was not observed in cells expressing EspGΔAΔP (Figure 3A), thus providing further evidence of EspG interference of WRC-dependent cytoskeleton remodeling.

EspG Incapacitates Arf6 and ARNO Upstream of WRC Activation
EspG is known to bind Arf6 in an analogous fashion to Arf1 (Selyunin et al., 2011), though no function has been ascribed for this host-pathogen interaction. To trigger WRC-dependent invasion, Salmonella Typhimurium hijacks Arf6 to recruit and activate the Arf1 GEF ARNO of the cytohesin family (Humphreys et al., 2012b, 2013; Stalder et al., 2011). We therefore speculated that EspG might also incapacitate WRC activation by inhibiting Arf6 upstream of ARNO-mediated activation of Arf1.

First, we examined whether the cytohesin family (i.e., ARNO) facilitated phagocytosis. THP1 cells treated with the cytohesin inhibitor secinh3 impeded the relative uptake of EPECΔespG from ~82% to ~30% (Figures 3B and 3C). ARNO is known to facilitate macropinocytosis (Humphreys et al., 2012b, 2013), but the uptake of EPECΔespG was not affected by the macrophagocytosis inhibitor eipa (Figure 3B). Secinh3 but not eipa also inhibited the phagocytosis of EPECΔespG opsonized with human serum (Figure S2C). Thus, ARNO directs phagocytosis of EPEC, which was counteracted by EspG.

We next examined the mechanism by which EspG antagonized ARNO by reconstituting Arf6-driven activation of WRC as previously reported (Humphreys et al., 2013). PC-PI microspheres anchored with recombinant myristoylated Arf6 activated with GTPγS were incubated in extract with or without (–) recombinant ARNO (Figure 3D). Arf6 only triggered actin assembly in the presence of ARNO, but this was abrogated in extract containing EspGWT but not EspGΔAΔP. To determine how EspG impeded Arf6-dependent actin polymerization, PC-PI microspheres were isolated from extract then analyzed by immunoblotting (Figure 3E). To trigger WRC activation, Arf6 must recruit ARNO (Humphreys et al., 2013). Indeed, Arf6 alone (– ARNO) recruited very little Arf1 and Hem, which was enhanced upon addition of recombinant ARNO (+ ARNO). However, in the presence of EspG, Arf6 was incapacitated as the recruitment of ARNO as well as downstream players Arf1 and Hem were impeded. This was not the case with the Arf binding mutant EspGΔAΔP that had no effect on the Arf6 cascade.

Finally, to investigate whether EspG directly inhibits ARNO via binding to Arf6, we examined interactions in buffer with purified components and PC-coated microspheres (Figures 3F and 3G) that minimize known ionic interactions between the ARNO pleckstrin-homology domain and acidic phospholipids such as PI (Maia et al., 2000). ARNO weakly bound PC microspheres alone, but its recruitment was potentiated by Arf6 (Figures 3F and 3G). In the presence of EspGWT, the virulence effector was recruited through Arf6 that blocked interaction with ARNO (Figures 3F and 3G). In contrast, the Arf binding mutant EspGΔAΔP was not recruited by Arf6 and was incapable of impeding ARNO (Figures 3F and 3G). Thus, EspG directly disables Arf6-dependent actin polymerization by blocking signaling to its cellular effector ARNO.

The Molecular Basis of WRC Interference by EspG
Activated Arf1 mediates interaction with cellular effectors via its switch 1 (residues 40–51) and 2 (68–81) domains (Nie et al., 2003). EspG exhibits an unusual Arf binding interface that is rotated away from the switch 2 site (Figure 4A) where it interacts with the switch 1 and the alpha-1 helix (29–37) positioned outside of the canonical switch regions (Selyunin et al., 2011). Consequently, Arf1 bound to EspG can still bind cellular effectors that interact with its switch 2 domain such as the Arf binding GAT domain of GGA vesicle adaptors (Selyunin et al., 2014; Kuai et al., 2000) as depicted in Figure 4A and confirmed experimentally in Figure S3A.

We took advantage of the distinct binding modes of EspG and GAT3 to investigate the mechanism of Arf1-mediated WRC activation and EspG interference. First, we examined actin-based motility in extracts containing equivalent concentrations of either EspG or GAT3 (Figure 4B). EspG was more potent at inhibiting WRC than GAT3, which impeded robust comet tail formation but still permitted initiation of actin assembly and small comet
tails (magnified inset) of ~8 μm relative to ~14 μm observed with the control. Indeed, while Arf1QL and Rac1QL recruited both EspG and GAT3 to the membrane (Figure 4C, GST), EspG but not GAT3 impeded WRC recruitment (Figure 4C; Hem, Figure S3B, green arrows). It is not known how Arf1 activates the WRC but the results suggested that EspG inhibits recruitment of Arf1 effectors that bind switch 1. This hypothesis was further substantiated by immunoblotting of the switch 1-binding protein AP-1 (Austin et al., 2002; Ren et al., 2013) that was recruited in the presence of GAT3 but not EspG (Figure 4C, AP-1).

EspG interaction with Arf1 is key to inhibiting WRC activation (Figure 2). Thus, we set out to resolve the molecular basis of EspG interference further by purifying an array of Arf1QL derivatives incorporating mutations within the alpha-1 helix (Y35Q), switch 1 (T45I, I49T), or switch 2 (I74T, Y81H) domain (Figure 4A), which have been implicated in interactions with EspG or its cellular effectors (Selyunin et al., 2011; Kuai et al., 2000). We examined EspG and GAT3 interactions with PC:PI microparticles anchored with each Arf1QL mutant derivative in buffer (Figures 3D and 3E, and comprehensively shown in Figures S3B and S3C). EspG bound control Arf1QL but interaction with anchored Arf1QL-I49T was evidently weaker (Figure 3D). The remaining Arf1 mutations had no effect. In contrast, GAT3 bound each Arf1QL variant except the switch 2 mutant Arf1QL-I74T (Figure 3E).
EspG Targets Arf1 Residues Essential to Cooperation with Rac1 and WRC Activation

As the Arf1 residue Y35, and to a lesser extent I49, likely underlie EspG inhibition of the Arf1-Rac1-WRC axis, we next examined whether these sites in Arf1 were key for collaboration with Rac1 in WRC recruitment and activation at the membrane. In contrast to Arf1QL or Rac1QL alone, only membranes co-anchored with both GTPases (ctrl) triggered recruitment of the WRC (Figures 5A and 5B) and robust actin comet tail formation (Figures 5C and S4A) demonstrating that small GTPase cooperation was required for WRC activation. Interestingly, Arf1QL alone recruited AP-1 (Figure 5B), a marker for classical Arf1 effectors that binds the alpha-1 helix and switch 1 domain of Arf1 (Austin et al., 2002; Ren et al., 2013). However, when Arf1QL combined with Rac1QL (ctrl) the presence of AP-1 was diminished relative to Arf1QL alone, while WRC recruitment was enhanced uncovering a remarkable switch in effector interplay by Arf1 when collaborating with Rac1 (Figure 5B). Thus, when working in synergy with Rac1 the results indicate that Arf1 recruits and activates the WRC via its alpha-1 helix and switch 1 domain in place of classical effectors such as AP-1.

When we examined the Arf1QL derivatives mutated in the alpha-1 helix, switch-1 or switch-2 domain, they all collaborated with Rac1 by recruiting the WRC (Figures 5A and 5B and comprehensively shown in Figures S4C and S4D). Given this observation, we were surprised to find that certain Arf1 mutations had a substantial impairment in WRC activation (Figures 5C and S4B). Like Arf1QL, mutants Arf1QL-T45I, -I74T, and -Y81H formed robust actin comet tails (exemplified by I74T in Figure 5C). In contrast, the motility of membranes anchored with Arf1QL-Y35Q or -I49T was markedly impaired (Figure 5C). We noticed that a small proportion of actin shells (~10%) surrounding the Arf1QL-Y35Q and Arf1QL-I49T membranes broke symmetry to form stumpy comet tails of ~2 μm (exemplified by green arrows in Figure 5C), indicating weak activation of the WRC. We speculated that the weak activation of the WRC by Arf1QL-Y35Q would be resistant to interference by EspG, which binds Y35 (Figure 4D). Sure enough, while EspG blocked the formation of comet tails generated by the switch 2 mutant Arf1QL-I74T, stumpy comets were still formed by Arf1QL-Y35Q even in the presence of EspG (Figure 5C).

Finally, as both Arf1 residues Y35 and I49 mediated interaction with EspG (Figure 4D), we examined WRC recruitment and actin-based motility at membranes anchored with a double mutant (Figures 5A–5C). Arf1QL-Y35Q/I49T was incapable of collaborating with Rac1QL as the WRC was neither recruited (Figures 5A and 5B) nor activated (Figure 5C). Thus, EspG targets specific residues in the alpha-1 helix and switch 1 domain of Arf1 that control +GAT3

Rac1QL

Arf1QL

+-EspG

Sw

Switch1 Switch

EspG

GAT3

Arf1

Y35Q

α

1-helix

GAT3EspGctrl

Rac1QL

Arf1QL

GAT3

EspG

Hem

AP-1

GST

Arf1

Rac1

T45I

I49T

I74T

Y81H

E

ctrl Y35Q I49T-

PCPI

EspG

Arf1



A B C

D

PCPI

- ctrl Y35Q I49T I74T

EspG

Arf1



E

PCPI

- ctrl Y35Q I49T I74T

GAT3

Arf1



Figure 4. Molecular Basis of WRC Interference by EspG

(A) Cartoon depicting Arf1 interaction with EspG or GAT3 of GGA3 with key residues and domains in Arf1 shown.

(B) WRC-dependent actin-based motility directed by Arf1QL and Rac1QL in extract alone (control), or with GAT3 or EspG. Insets magnify actin-comet tails. Scale bars 5 μm.

(C) Immunoblotting of proteins recruited by membrane-anchored Arf1QL and Rac1QL from extract alone (ctrl), or from extract containing EspG or GAT3, or both in combination. Anti-GST antibodies detected GAT3 and EspG.

(D) Interaction of EspG with PCPI membranes alone (−) or with membranes anchored with Arf1QL (ctrl) or Arf1QL derivatives containing indicated mutations.

(E) Experiment performed as (B) with GAT3.

See also Figure S3.
DISCUSSION

To avoid phagocytosis bacterial pathogens employ a wide range of strategies. For example, many pathogens secrete immunoglobulin proteases to cleave antibodies and impede FcR-mediated uptake (Sarantis and Grinstein, 2012). However, not all phagocytic mechanisms are driven by opsonization emphasizing the need for other inventive virulence strategies. We show that EPEC and EHEC circumnavigate this problem by inhibiting WRC signaling to the actin cytoskeleton whose remodeling is at the very center of phagocytosis. The role of Rac1 and Arf6 in phagocytosis is well established (Niedergang et al., 2003; Zhang et al., 1998; Caron and Hall, 1998), yet the contribution of the WRC (Park et al., 2008) and Arf1 is less clear (Bemmiller et al., 2006; Sendide et al., 2005). WRC activation by Arf1 and Rac1 is known to mediate lamellipodia formation and Salinanella macropinocytosis into host cells (Humphreys et al., 2012a, 2013). Here, we show a crucial role for the WRC in pathogen phagocytosis and establish that collaborating WRC activators Arf1 and Rac1 are required. Structural homologs cyfip and Hem of WRC are thought to oppose the plasma membrane where small GTPases are anchored (Chen et al., 2010). As Rac1 is known to bind cyfip (Kobayashi et al., 1998), it is possible that Arf1 collaborates with Rac1 by binding Hem (depicted in Figure 6) or through an unidentified Arf1 effector acting as an intermediate. By using EspG to probe the relationship between Arf1 and Rac1, we identify key residues in the Arf1 alpha-1 helix (Y31) and switch-1 domain (I49) that underpin its collaboration with Rac1 in WRC recruitment and activation at the membrane. Interestingly, we found that the equivalent residues in Arf6 (Y31 and V45) were dispensable for Arf6 activation of ARNO (Figure S5), and, although the EspG interactions with Arf6-Y31Q were attenuated as previously reported (Selyunin et al., 2011), the interactions at the membrane were sufficient to block ARNO-dependent signaling to the WRC mediated by either Arf6-Y31Q or Arf6-V45A (Figure S5). Thus, the sites on Arf6 that permit signaling to ARNO and interference by EspG are distinct from those in Arf1 that cooperate with Rac1 in WRC signaling at the membrane, and perhaps reflect differences in the primary sequence of Arf1 and Arf6 (70% identity).
cytohesin-1 in opsonin-dependent phagocytosis (Beemiller et al., 2006; Sendide et al., 2005). ARNO is activated at the plasma membrane by Arf6 (Stalder et al., 2011; Cohen et al., 2007), a long established regulator of phagocytosis (Niedergang et al., 2003; Zhang et al., 1998). EspG directly abrogated Arf6 recruitment and activation of ARNO thereby impeding Arf1 activation and describing a role for the Arf6-EspG interaction.

In summary, by targeting both Arf6 and Arf1 our work establishes a dual mechanism by which a single virulence effector uncouples two arms of the WRC regulatory pathway and ultimately inhibits phagocytic uptake to evade innate host defenses (depicted in our model in Figure 6).

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains**

EPEC EU2348/69 and EHEC EDL933 (TUV93-0 Shiga toxin deficient derivative) strains were used. Isogenic mutant EPEC ΔespG1/ΔespG2 (Prof. Feng Shao) and EHEC ΔespG (Dr. Ken Campellone) were kind gifts. For infections, bacteria were cultured as previously described (Smith et al., 2010).

**Plasmids**

The following plasmids were generated by Invitrogen Gateway methodology: pET15b-espG, pGEX2T-espG, pcDNA-HA-espG (encoding effector domain residues 48–398) and pET15b-espG, pGEX2T-espG, pcDNA-HA-espG (encoding effector domain residues 48–398) and pTrc99FA-espG (full length). Plasmids pET20b-Arf1, pET20b-Arf6, pET15b-Rac1, pGEX2T-ARNO-2G, and pGEX2T-GGA3-CAT were described previously (Humphreys et al., 2013). Point mutations were introduced by site-directed mutagenesis into pET15b-espG, pGEX2T-espG (EspGΔ4 residues D205A, R208A; EspGΔ6 Q293A; EspGΔ6 Δ5152S, P53A, P535A), pET20b-Arf1 or pET20b-Arf6 (mutations indicated in the text). GST- and His-tagged proteins were expressed in E. coli Rosetta (Novagen) at 18°C before affinity purification (Humphreys et al., 2012b).

**Antibodies**

Antibodies were purchased from Abcam (Rac1, ab33186; Arf1, ab58578; Arf6, ab51650; ARNO, ab56510; PAK1, ab154284), Sigma (Abi1, A5106; Actin, A2066; CytoHem-1, P0092; Nap1, N3786; AP1, A4200), GE Healthcare Life Sciences (GST, 27457701), QIAGEN (His, 34660) or were raised against recombinant peptides in rabbits by Diagnostics Scotland (WAVE2, amino acids 180–241).

**Mammalian Cell Culture and Transfection**

The human monocyte-like cell line THP1s (kind gift from Prof. Gordon Dougan) and mouse macrophage-like RAW264.7 (ATCC-TIB71) cells were cultured in RPMI-1640 or DMEM in the case of HeLa cells (ATCC-CCL-2) supplemented with 2 mM L-glutamine, 10% heat-inactivated fetal calf serum (FCS), 200 μg/mL streptomycin, and 100 U/mL penicillin (Schwende et al., 1996). THP1s were differentiated into mature macrophage-like cells by stimulation with 100 ng/mL Phorbol 12-myristate 13-acetate (PMA) for 3 days and then cultured for an additional day without PMA before phagocytosis assays.

**Phagocytosis Assays**

Prior to infection EPEC and EHEC strains were harvested by centrifugation, washed in phosphate-buffered saline (PBS) then incubated with pH-Rodo (Thermo Fisher Scientific) before washing with Tris (pH 7.4)-buffered saline. Approximately 2 × 10⁶ mammalian cells seeded onto glass coverslips were
infected with pH-Rodo-labeled bacteria (1 hr, 37°C, 5% CO₂) before washing with PBS and fixation using 4% paraformaldehyde. Fixed cells were incubated with rabbit anti-intimin (EPEC/EHEC outer membrane protein) antibodies, washed with PBS, and then incubated with anti-rabbit Alexa Flour 350 antibodies and cells visualized phalloidin-FITC-488 (Thermo Fisher Scientific) in PBS supplemented with Tx100. Phagocytosis was quantified by counting the number of extracellular bacteria labeled with intimin antibodies relative to intracellular bacteria showing pH-Rodo fluorescence using automated Velocity software (Improvision). When appropriate, cells were incubated with PBS and fixation using 4% paraformaldehyde. Fixed cells were incubated with anti-rabbit Alexa Flour 350 anti-}

**Salmonella Invasion of Non-phagocytic Host Cells**

Wild-type *Salmonella enterica* serovar Typhimurium SL1344 were used to assay invasion into non-phagocytic cells as previously described (Humphreys et al., 2013). Salmonella encoding pM975 that expresses GFP via the SPI2 promoter when bacteria are within Salmonella containing vacuoles (SCVs) (Schlumberger et al., 2007) were used to infect HeLa cells (10 min), and the number of fluorescent bacteria was counted per cell microscopically.

**In Vitro WRC-Dependent Actin-Based Motility**

Preparation of porcine brain extract, actin-based motility by phospholipid-coated beads, and isolation of bead membrane-associated proteins have been described in detail (Hume et al., 2014). When indicated, extract or buffer containing recombinant EspG derivatives, QAT3, or ARNO was used. Quantification of comet tail length was performed on 50 comet tails per experiment using Velocity measurement software (Improvision). All experiments were performed at least three times.

**Statistics**

All experiments were performed at least three times. Geometric means were calculated, and significance was determined by Student’s t test or one-way ANOVA followed by a post hoc Dunnett’s comparison. *p < 0.05 was considered significant.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes five figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.09.039.

**AUTHOR CONTRIBUTIONS**

D.H., V.S., and V.K. performed experiments and wrote the paper.

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