WAVE2 Signaling Mediates Invasion of Polarized Epithelial Cells by Salmonella typhimurium

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The bacterial pathogen Salmonella penetrates the intestinal epithelium by inducing its own phagocytosis into epithelial cells. The dramatic reorganization of the actin cytoskeleton required for internalization is driven by bacterial manipulation of host signaling pathways, including activation of the Rho family GTPase Rac1 and subsequent activation of the Arp2/3 complex. However, the mechanisms linking these two events remain poorly understood. Rac1 is thought to promote activation of the Arp2/3 complex through its interaction with suppressor of cAMP receptor/WASP family verprolin-homologous (SCAR/WAVE) family proteins, but this interaction is apparently indirect. Two different Rac1 effectors have been shown to bind WAVE2: IRSp53, the SH3 domain of which binds the WAVE2 proline-rich domain, and PIR121/Sra-1, which forms a pentameric complex containing WAVE, Abi1, Nap1, and HSPC300. However, the extent to which each of these complexes contributes to Arp2/3 complex activation in the context of Salmonella infection is unclear. Here, we show that WAVE2 is necessary for efficient invasion of epithelial cells by Salmonella typhimurium. We found that although Salmonella infection strongly promotes the formation of an IRSp53/WAVE2 complex, IRSp53 is not necessary for bacterial internalization. In contrast, disruption of the PIR121/Napi/Abi1/WAVE2/HSPC300 complex potently inhibits bacterial uptake. These results indicate that WAVE2 is an important component in signaling pathways leading to Salmonella invasion. Although infection leads to the formation of an IRSp53/WAVE2 complex, it is the association of WAVE2 with the Abi1/Nap1/PIR121/HSPC300 complex that regulates bacterial internalization.

Salmonella enterica serovar Typhimurium is one of the most common causes of food-borne infectious gastroenteritis in humans. Although intestinal epithelial cells are not inherently phagocytic, Salmonella species have evolved sophisticated mechanisms to induce their own internalization into host cells. Contact with apical microvilli activates a type III secretion system encoded by the Salmonella pathogenicity island 1 (SPI-1), through which bacterial effector proteins are injected into the host cell cytoplasm (1, 2). The combined action of multiple effector proteins leads to rapid, localized microvillar disassembly followed by the appearance of large, actin-rich membrane protrusions surrounding the bacteria, which eventually engulf them and complete the internalization process (3, 4).

Small GTPases of the Rho family are key regulators of actin cytoskeleton dynamics in eukaryotic cells (5). Several SPI-1 effectors can directly modulate Rho GTPase activity; SopE and SopE2 function as guanine nucleotide exchange factors, which activate Cdc42 and Rac1 by catalyzing the exchange of GTP for GDP (6–8). In contrast, SptP serves as a GTPase-activating protein to down-regulate the activity of Cdc42 and Rac1, presumably to terminate the internalization process (9).

Although enterocytes are among the physiological targets of Salmonella, most of our understanding of the cellular basis of Salmonella invasion has been derived from studies using non-polarized epithelial or fibroblastic cell lines. We have shown that in contrast to non-polarized cell lines, in which Cdc42 plays a prominent role (10), apical invasion of polarized epithelia by Salmonella specifically requires Rac1 (11), supporting the notion that host cell signaling pathways utilized by bacterial pathogens may differ between non-polarized cells and polarized epithelia.

Several other effectors promote bacterial internalization by directly modulating actin filament dynamics. SipC, a component of the translocation pore, both nucleates filament assembly and bundles assembled filaments (12), while SipA, a secreted protein, stabilizes filaments by inhibiting their depolymerization (13–15). However, these activities result in the formation of unbranched filaments, not the highly branched actin networks characteristic of phagocytic pseudopodia. Such branching is usually facilitated by the Arp2/3 complex, which promotes new filament growth from the sides of existing actin filaments. We have previously shown that the Arp2/3 complex is recruited to sites of bacterial invasion and is functionally indispensable to Salmonella entry in polarized epithelial cells (16).

The activity of the Arp2/3 complex is robustly stimulated by proteins of the WASP/WAVE family (17). The mammalian WASP/WAVE family consists of five members, WASP (Wiskott-Aldrich Syndrome protein), N-WASP, and three WAVE isoforms (WAVE1–3). Of these, N-WASP and WAVE2 are ubiquitously expressed. WASP and N-WASP are preferentially activated by Cdc42, the binding of which relieves an autoinhibitory

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1 The abbreviations used are: WASP, Wiskott-Aldrich Syndrome protein; WAVE, WASP family verprolin-homologous; N-WASP, neural WASP; MDCK, Madin-Darby canine kidney cells; HA, hemagglutinin; siRNA, small interfering RNA; VCA, verprolin homologous domain, cofilin-homologous domain, and acidic domain; VPH, verprolin homologous.
ported conformation and promotes the formation of filopodia. In contrast, WAVEs are preferentially activated by Rac1 and are necessary for the production of Rac1-induced lamellipodia (18–20). However, WAVEs do not bind Rac1 directly, and their mode of activation has been the subject of some controversy. One model is that Rac1 binds to an accessory protein, IRSp53, which then binds WAVE2 to form an active, trimeric complex (21). However, recent evidence suggests that WAVE2 exists at steady state as part of a large complex containing four other proteins, Nap1 (Nck-associated protein 1), PIR121/Sra-1, Abi1, and HSPC300 (22), that is recruited to the membrane through an interaction between PIR121/Sra-1 and Rac1 (23, 24).

Here, we demonstrate a requirement for WAVE2 in the apical invasion of polarized epithelial cells by *Salmonella typhimurium*. We show that Salmonella-induced Rac1 activation promotes the formation of an IRSp53/WAVE2 complex but that IRSp53 is not necessary for bacterial internalization. In contrast, sRNA-mediated knockdown of Abi1 dramatically impairs bacterial uptake, suggesting that the Abi1/Nap1/PIR121/HSPC300 complex is essential for activation of WAVE2 downstream of Rac1 during *Salmonella* invasion.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The T23 subclone of strain II MDCK cells, which stably express the tetracycline-repressible transactivator, has been described previously (25). Cells were grown in Dulbecco's modified Eagle's medium with 4.5 g/liter glucose, 10% fetal bovine serum, 1 mM sodium pyruvate, 2 mM L-glutamine, and antibiotics in a 37 °C CO2 incubator. Transfectants were additionally maintained in 20 ng/ml doxycycline (Sigma) to repress expression of the transgene. To establish polarized monolayers, cells were seeded on Transwell filters (Costar) coated with rat tail collagen (Upstate Biotechnology). 48–72 h later, cells were gently washed in Dulbecco's phosphate-buffered saline and replaced in complete Dulbecco's modified Eagle's medium lacking doxycycline (Sigma). To test the role of WAVE2 in bacterial invasion, we used two MDCK cells co-expressing Myc-tagged WAVE2 and HA-tagged IRSp53. To establish polarized monolayers of the epithelial cell line MDCK. Cells cultured on permeable filter supports were infected apically with wild-type *S. typhimurium* strain SL1344 for 20 min as described under "Experimental Procedures," and then WAVE2 localization was evaluated by immunofluorescence microscopy. As reported previously, invading bacteria can be recognized by the presence of a ring of filamentous actin that accumulates at the invasion site, which indicates the formation of a phagocytic cup (Fig. 1A). Immunolabeling of infected cells revealed that endogenous WAVE2 colocalized extensively with filamentous actin at sites of bacterial internalization (Fig. 1A). Quantitation of invasion foci (n = 50) revealed that 94% of the sites containing both bacteria and a ring of filamentous actin also contained endogenous WAVE2.

**RESULTS**

**WAVE2 Functions in Apical Salmonella Invasion**—To determine whether WAVE2 regulates Arp2/3 complex activation at apical invasion sites, we first examined the localization of endogenous WAVE2 during *Salmonella* entry in polarized monolayers of the epithelial cell line MDCK. Cells cultured on permeable filter supports were infected apically with wild-type *S. typhimurium* (strain SL1344) for 20 min as described under "Experimental Procedures," and then WAVE2 localization was evaluated by immunofluorescence microscopy. As reported previously, invading bacteria can be recognized by the presence of a ring of filamentous actin that accumulates at the invasion site, which indicates the formation of a phagocytic cup (Fig. 1A). Immunolabeling of infected cells revealed that endogenous WAVE2 colocalized extensively with filamentous actin at sites of bacterial internalization (Fig. 1A). Quantitation of invasion foci (n = 50) revealed that 94% of the sites containing both bacteria and a ring of filamentous actin also contained endogenous WAVE2.

**To test the role of WAVE2 in bacterial invasion, we used two different approaches. In the first, we generated stable MDCK cell lines inducibly expressing either wild-type WAVE2 or two different dominant interfering WAVE2 mutants under the control of a tetracycline-responsive promoter (25). Inducible expression is necessary to allow the cells to form a polarized epithelial monolayer without interference from the mutant WAVE2. The C-terminal VCA domain of WAVE2 (Fig. 1B) is required for Arp2/3 activation. The V (verprolin-homologous) domain binds monomeric actin, and its deletion (WAVE2 VPH) has been shown to inhibit Arp2/3 function (20). The C (cofilin-homologous) domain binds monomeric actin, and its deletion (WAVE2 VPH) has been shown to inhibit Arp2/3 function (20). The C (cofilin-homologous) domain binds monomeric actin, and its deletion
C-terminal VCA domain (WAVE2ΔVCA). Each of these mutants is presumably capable of interacting with upstream binding partners but cannot activate the Arp2/3 complex.

We then used these cell lines to examine the invasion efficiency of wild-type *S. typhimurium*, using a standard gentamicin resistance assay as described under “Experimental Procedures” (11). As shown in Fig. 1C, expression of wild-type WAVE2 led to a small but reproducible enhancement of bacterial internalization, relative to the same cells cultured in the presence of doxycycline (which suppresses transgene expression). In contrast, expression of either the WAVE2ΔVPH or the WAVE2ΔVCA mutant inhibited apical *Salmonella* invasion by ~50% (*p < 0.05*). Importantly, the expression of these proteins did not significantly alter bacterial adherence to the apical cell surface (Fig. 1D), verifying that the defect in bacterial invasion was due to impaired internalization rather than bacterial attachment.

To confirm that the observed impairment of *Salmonella* internalization was directly due to WAVE2 inhibition and not a secondary effect of WAVE2 mutant expression, we used RNA interference to knock down endogenous WAVE2 expression in polarized MDCK cells. As shown in Fig. 1E, transfection of cells with a scrambled siRNA duplex specific for canine WAVE2 resulted in a ~60% reduction in WAVE2 expression, relative to cells transfected with a scrambled siRNA duplex. The knockdown was specific as expression of the related protein N-WASP was unaffected. Knockdown of WAVE2 largely inhibited the formation of membrane ruffles in response to *Salmonella* infection (see supplemental data, Fig. S1). Quantitation of *Salmonella* uptake in these cells revealed that, in agreement with the dominant negative inhibition described above, selective knockdown of WAVE2 expression also reduced internalization by ~50% (*p < 0.01*). Taken together, these data suggest that efficient apical *Salmonella* invasion requires functional WAVE2.

*Salmonella Invasion Stimulates the Formation of an IRSp53/WAVE2 Complex*—Previous reports have shown that binding of GTP-Rac1 to the N terminus of IRSp53 promotes the formation of a Rac1/IRSp53/WAVE2 complex and that this interaction is necessary for membrane ruffling induced by an active Rac1 mutant (Rac1G12V) (21). Localization of endogenous IRSp53 in *Salmonella*-infected MDCK monolayers revealed that, like WAVE2, IRSp53 becomes concentrated at sites of bacterial internalization (Fig. 2A). Eighty-eight percent of invasion foci exhibited strong immunolabeling for endogenous IRSp53. To determine whether apical *Salmonella* infection induces the formation of an IRSp53/WAVE2 complex, we measured the extent of this interaction using a co-immunoprecipitation assay. For this purpose, an HA-tagged wild-type IRSp53 construct was transiently transfected into MDCK cells expressing Myc-tagged wild-type WAVE2 to achieve co-expression of the two proteins. Following apical infection with either wild-type *S. typhimurium* (SL1344) or an invasion-deficient (*hilA*) mutant strain (VV341), WAVE2 was immunoprecipitated from cell lysates, and the immunoprecipitates were probed with an antibody against the HA tag to detect co-precipitated IRSp53. Fig. 2B shows that, although a small amount of interacting IRSp53 can be detected at steady state, apical exposure to wild-type *S. typhimurium* for 30 min potently enhanced the binding of IRSp53 to WAVE2. In contrast, infection with the invasion-defective *hilA* strain did not significantly change the levels of co-precipitated IRSp53, indicating that expression of the bacterial invasion machinery is necessary to induce this interaction. These results suggest that the interaction of...
IRSp3 and WAVE2 is strongly enhanced by *Salmonella*-induced activation of Rac1.

**IRSp3 Is Not Necessary for Apical Uptake of *Salmonella***—To determine whether the interaction between IRSp3 and WAVE2 is required for *Salmonella* entry, we generated stable MDCK cell lines inducibly expressing either wild-type IRSp3 or an IRSp3 mutant lacking the SH3 domain, which cannot bind to the proline-rich domain of WAVE2 (Fig. 3A). As reported previously (21), we found that this mutant fails to bind WAVE2, although it retains the ability to bind Rac1 (see supplemental data Fig. S2, and data not shown). Apical *Salmonella* invasion was then measured in these two cell lines as described above. Surprisingly, expression of neither wild-type IRSp3 nor the IRSp3ΔSH3 mutant significantly affected bacterial uptake when compared with controls maintained in the presence of doxycycline (Fig. 3B). To further confirm this observation, we performed siRNA-mediated knockdown of endogenous IRSp3. We found that depletion of IRSp3 did not impair the formation of membrane ruffles in response to *Salmonella* invasion and that WAVE2 was still recruited to the sites of infection (Fig. 3C and supplemental data Fig. S1). Moreover, invasion assays indicated that knockdown of IRSp3 in MDCK cells also had no effect on *Salmonella* invasion efficiency (Fig. 3D). These findings indicate that although *Salmonella* invasion potently stimulates the formation of an IRSp3/WAVE2 complex, this interaction is not required for bacterial internalization.

**A Role for the Abi1/Nap1/P1R121/HSPC300 Complex in *Salmonella* Internalization**—In addition to its interaction with IRSp3, WAVE2 has recently been reported to exist as part of a pentameric complex also containing the proteins Abi1, Nap1, PIR121/Sra-1, and HSPC300 (22–24). In this context, PIR121/Sra-1 serves as the Rac1-binding subunit of the complex. Several of these proteins have been shown to modulate actin remodeling by regulating the stability and localization of WAVE in *Dictyostelium*, *Drosophila*, and mammalian cells (23, 24, 27, 28). To determine whether this complex is recruited to sites of bacterial invasion, we examined the localization of endogenous Abi1 in MDCK cells during *Salmonella* infection. Like WAVE2 and IRSp3, Abi1 was also observed in a concentrated ring surrounding invading bacteria at the apical plasma membrane (Fig. 4). Of 50 invasion foci examined, 86% contained endogenous Abi1. Because most of the Abi1 in cells has been shown to exist in a stable complex with Nap1, PIR121/Sra-1, HSPC300, and WAVE2 (24), this observation suggests that WAVE2 recruitment to invasion foci may occur in the context of this large protein complex.

To confirm a role for the Abi1/Nap1/PIR121/HSPC300 complex in the *Salmonella* internalization process, we used RNA interference to inhibit Abi1 synthesis in polarized MDCK monolayers. If it is the Abi1/Nap1/PIR121/HSPC300 complex that recruits WAVE2 to the sites of *Salmonella* invasion, knocking down one of the complex components should abolish WAVE2 localization to the phagocytic cups. Indeed, very few bacteria-induced ruffles were observed in cells in which Abi1 expression was induced in the presence of doxycycline (Fig. 5A). Not surprisingly, *Salmonella* internalization efficiency was impaired in those Abi1 knockdown cells. As shown in Fig. 5B, an 85% reduction of Abi1 expression led to an ∼50% (p < 0.01) reduction in apical *Salmonella* uptake, similar to that observed by knockdown of endogenous WAVE2. A more complete reduction in Abi1 levels could be achieved in non-polarized HeLa cells by transfecting with an siRNA-expressing plasmid (Fig. 5C) (24). Quantitation of bacterial entry in these cells revealed that internalization was impaired by ∼80% (p < 0.01) when compared with control cells transfected with an empty siRNA vector.

However, as reported by others (23, 24), we found that reduced Abi1 expression results in a corresponding down-regulation of WAVE2, presumably due to destabilization of the WAVE2/Abi1/PIR121/Nap1/HSPC300 complex (Fig. 5B). In three separate experiments, we observed an average 41% reduction in the levels of WAVE2 when Abi1 was knocked down. Therefore, to rule out the possibility that impaired bacterial invasion is simply due to the reduced level of WAVE2, we restored WAVE2 expression in Abi1 knockdown MDCK cells by co-expression of Myc-tagged human WAVE2. Fig. 5B demonstrates that WAVE2 expression was restored to endogenous levels in these cells despite the reduced levels of Abi1. However, restoration of WAVE2 expression did not rescue *Salmonella* internalization in Abi1 knockdown cells, suggesting that Abi1 is indispensable for WAVE2-mediated bacterial invasion. Taken together, our results suggest that, although *Salmonella* infection induces the formation of an IRSp3/WAVE2 complex, it is the WAVE2/Abi1/Nap1/PIR121/HSPC300 complex that directly mediates the actin reorganization required for apical *Salmonella* infection.
Reorganization of the actin cytoskeleton within host cells is a prerequisite for Salmonella entry. The current study defines a signaling pathway triggered by apical Salmonella infection in which Rac1 promotes recruitment of the WAVE2/Abi1/Nap1/PIR121/HSPC300 complex to sites of bacterial attachment, in which it activates the Arp2/3 complex. This leads to rapid actin reorganization culminating in bacterial internalization (Fig. 6). We have determined that functional WAVE2 is important for bacterial entry at the apical plasma membrane of polarized epithelial cells. Moreover, although apical Salmonella invasion robustly stimulates the interaction between IRSp53 and WAVE2, which has been shown to enhance WAVE2 activity in other cell types (21), this interaction appears to be dispensable for bacterial uptake. Importantly, we show that the WAVE2/Abi1/Nap1/PIR121/HSPC300 complex is also recruited to sites of bacterial attachment and that efficient Salmonella internalization requires the presence of Abi1. Together, these findings indicate that Salmonella utilize the WAVE2/Abi1/Nap1/PIR121/HSPC300 complex, rather than a complex of WAVE2 with IRSp53, to stabilize and recruit WAVE2 during the invasion process.

Upon contact with the plasma membrane, Salmonella secrete a panel of effector proteins into the host cell cytosol via the SPI-1-encoded type III secretion system, which not only promote the activation of Rac1 but also directly modulate actin cytoskeleton dynamics. Although the translocon component SipC can nucleate actin filament assembly at sites of bacterial attachment and the secreted effector SipA stabilizes assembled filaments, the combined action of these proteins results in the formation of long unbranched filaments in vitro (12). In contrast, the extension of membrane ruffles as observed during Salmonella entry typically requires the assembly of highly branched, dendritic actin networks, the formation of which is mediated by the Arp2/3 complex (4). Our data indicate that WAVE2 plays an essential role in activation of the Arp2/3 complex.
complex during apical *Salmonella* invasion of epithelial cells. A similar role for WASP has been demonstrated in macrophages during both FcγR-mediated phagocytosis (29) and the invasin-mediated internalization of *Yersinia enterocolitica* (30).

However, WAVE2 is not the only Arp2/3-activating protein that functions in *Salmonella* internalization. Unsworth et al. (31) found that both N-WASP and WAVE participate in *S. typhimurium* invasion of non-polarized HeLa cells, in which Cdc42 plays a critical role in bacterial internalization. Our previous observation that Cdc42 is not activated during apical infection of epithelial cells by *Salmonella* (11) suggests that N-WASP may not function in the epithelial context. However, since N-WASP can be activated by WASP-interacting SH3 protein independently of Cdc42 (32), it remains possible that N-WASP could have a role in apical invasion. This hypothesis remains to be tested.

Although it is well accepted that WASP and N-WASP become activated in response to direct binding of Cdc42, the mechanisms by which Rac1 promotes activation of WAVEs remain controversial. Miki *et al.* (21) reported that IRSp53 bound WAVE2 in a Rac1-dependent manner and that this interaction enhanced the ability of WAVE2 to activate the Arp2/3 complex. However, multiple groups have subsequently shown that both WAVE1 (22) and WAVE2 (23, 24) exist primarily as complexes with PIR121/Sra-1, Nap1, Abi1, and HSPC300. In the case of WAVE1, its association with this complex has been reported to

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**FIG. 6.** A model for *Salmonella*-induced cytoskeleton remodeling and bacterial internalization. Activation of Rac1 by the bacterial effector proteins SopE2/SopE2 recruits the Abi1/Nap1/PIR121/HSPC300/WAVE2 complex to sites of *Salmonella* invasion at the apical plasma membrane, where WAVE2 stimulates Arp2/3-mediated actin nucleation and actin filament branching. Cytoskeletal reorganization promotes formation and extension of the phagocytic pseudopods responsible for bacterial uptake.
inhibit its ability to activate the Arp2/3 complex. Binding of either GTP-bound Rac1 or Nck resulted in dissociation of WAVE1 as an active subcomplex with HSPC300 (22). However, several recent studies on WAVE2 have suggested that Rac1 activation does not induce complex disassembly; instead, the entire complex is recruited to the membrane surface, where active Rac1 is located. Moreover, *in vitro* assays indicate that WAVE2 retains full activity even in the context of the intact complex and that this activity is not further enhanced by the addition of Rac1 (24). These recent results all favor the view that activated Rac1, through the Abi1/Nap1/PIR121/HSPC300 complex, recruits WAVE2 to the sites in which actin remodeling will occur.

In the context of *Salmonella* infection, we hypothesize that local activation of Rac1, facilitated by the bacterially encoded guanine nucleotide exchange factors SopE/SopE2, promotes the recruitment of the WAVE2/Abi1/Nap1/PIR121/HSPC300 complex to sites of bacterial attachment. Activation of the Arp2/3 complex by WAVE2 would then lead to the formation of the protrusive lamellipodial extensions that characterize *Salmonella* invasion. Our observation that restoration of WAVE2 expression did not rescue bacterial entry in cells in which Ab1 expression was knocked down further demonstrates that WAVE2 cannot localize properly under these conditions and is therefore not functional. Although it is clear from our results that Rac1 activation also leads to the formation of an IRSp53/WAVE2 complex, this interaction does not appear to be critical to bacterial internalization. A mutant form of IRSp53 lacking the SH3 domain, which fails to bind WAVE2 (21), had no effect on *Salmonella* invasiveness. One possible interpretation of this result is that, in the context of *Salmonella* infection, the WAVE2/Abi1/Nap1/PIR121/HSPC300 complex is sufficient to activate Arp2/3 and does not require additional input from the IRSp53/WAVE2 complex. Alternatively, it is possible that some additional essential function normally supplied by IRSp53 is filled by one or more bacterial effector proteins. Further investigation will be required to resolve this issue.

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