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Report

Salmonella-Directed Recruitment of New Membrane to Invasion Foci via the Host Exocyst Complex

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Summary

***Salmonella* attachment to the intestinal epithelium triggers delivery of bacterial effector proteins into the host cytosol through a type III secretion system (T3SS), leading to pronounced membrane ruffling and macropinocytic uptake of attached bacteria. The tip of the T3SS is made up of two proteins, SipB and SipC, which insert into the host plasma membrane, forming a translocation pore [1]. Both the N and C termini of SipC are exposed in the host cytosol and have been shown to directly modulate actin cytoskeleton assembly [2]. We have identified a direct interaction between SipC and Exo70, a component of the exocyst complex, which mediates docking and fusion of exocytic vesicles with the plasma membrane. Here, we show that exocyst components coprecipitate with SipC and accumulate at sites of invasion by *Salmonella typhimurium*. Exocyst assembly requires activation of the small GTPase RalA, which we show is triggered during *Salmonella* infection by the translocated effector, SopE. Knockdown of RalA or Sec5 results in reduced membrane ruffling at sites of attachment and impairs bacterial entry into host cells. These findings suggest that *S. typhimurium* enhances invasion efficiency by promoting localized membrane expansion, directly through SipC-dependent recruitment of the exocyst and indirectly via SopE-dependent activation of RalA.**

Results and Discussion

SipC Interacts with the Eukaryotic Exocyst Complex

SipC is thought to adopt a hairpin topology, with both N and C termini exposed in the host cytosol (Figure 1A). In addition to its function in protein translocation, the SipC C terminus has been shown to nucleate actin filament assembly, whereas the N terminus has filament-bundling activity [2]. To identify eukaryotic proteins that interact with SipC, we performed a yeast two-hybrid screen using the cytoplasmic C terminus (amino acids [aa] 200–410, SipC-C) of the protein as bait. This screen yielded multiple interactors encoding the N terminus of Exo70, a component of the eukaryotic exocyst complex, which targets and tethers exocytic vesicles to the plasma membrane prior to SNARE-mediated fusion [3]. The C terminus of Exo70 contains several highly conserved, positively charged amino acids that enable electrostatic interactions with phosphatidylinositol 4,5-bisphosphate and localization to the plasma membrane [4, 5]. The C terminus is also capable of interacting with the Arp2/3 complex [6], which is important for actin filament nucleation and branching. As a result, overexpression of Exo70 has been shown to induce the formation of filopodia in transfected cells [6, 7].

To verify the interaction between SipC-C and Exo70, HeLa cells transfected with a green fluorescent protein (GFP)-tagged Exo70 construct were lysed and incubated with purified recombinant 6×His-tagged SipC-C. After precipitation with nickel agarose (Ni-NTA), Exo70-GFP, but not GFP alone, was found to interact with recombinant SipC-C (Figure 1B, upper panels). This interaction was also observed with endogenous Exo70 from cell lysates (Figure 1C). No Exo70 was detected in control affinity precipitations with the 6×His-tagged SipC N-terminal domain (aa 1–120, SipC-N) or with Ni-NTA in the absence of SipC-C.

Exo70 is one of eight proteins that associate to form the exocyst complex. As shown in Figure 1B (lower panels), SipC-C precipitates also contained endogenous Sec5 and Sec8, indicating that SipC-C can also bind to Exo70 when it is assembled into the octameric exocyst complex.

Finally, to confirm that the interaction between SipC and Exo70 is direct, recombinant 6×His-tagged SipC-C or SipC-N prebound to Ni-NTA was incubated in vitro with purified recombinant Exo70. In agreement with the pulldown assays described above, we found that recombinant Exo70 associates specifically with SipC-C (Figure 1D). Taken together, these results suggest that SipC-C interacts directly with Exo70, thereby coupling the bacterial invasion machinery to the mammalian exocyst complex.

Recruitment and Assembly of the Exocyst Complex during *Salmonella* Infection

Exocyst components exist as subcomplexes that are brought together to mediate vesicle docking at specific sites on the plasma membrane. The complex present on carrier vesicles is thought to include Sec5, Sec6, Sec8, Sec10, Sec15, and Exo84, while Sec3 and Exo70 are thought to associate with the plasma membrane, where they provide spatial landmarks for vesicle targeting [8–10]. Delivery of exocytic vesicles to the plasma membrane leads to assembly of these subcomplexes into an intact, octameric vesicle-tethering complex. To determine whether both subcomplexes are recruited to sites of *Salmonella* entry into host cells, HeLa cells expressing GFP-tagged Sec5, Sec8, or Sec10 were infected with *S. typhimurium* for 30 min. Fixed cells were then immunolabeled for detection of the actin-rich membrane ruffles of invasion foci (phalloidin), the invading bacteria (anti-LPS), and the indicated exocyst protein (Figure 2 and Figure S1, available online).

In agreement with the biochemical data, endogenous Exo70 became highly enriched at sites of bacterial attachment, where it could be seen in close association with invading bacteria (Figure 2A). Similar enrichment was observed for the vesicle-associated subunits Sec10-GFP (Figure 2B), Sec5-GFP, and Sec8-GFP (Figure S1A). Deconvolution microscopy verified the close association of Sec5-GFP with *S. typhimurium* (Movie S1, Movie S2, and Figure S1B), and confocal microscopy of single optical sections confirmed that the apparent enrichment of exocyst components around attached bacteria was not a result of increased cell thickness at invasion foci (Figure S2A). These observations suggest that exocytic

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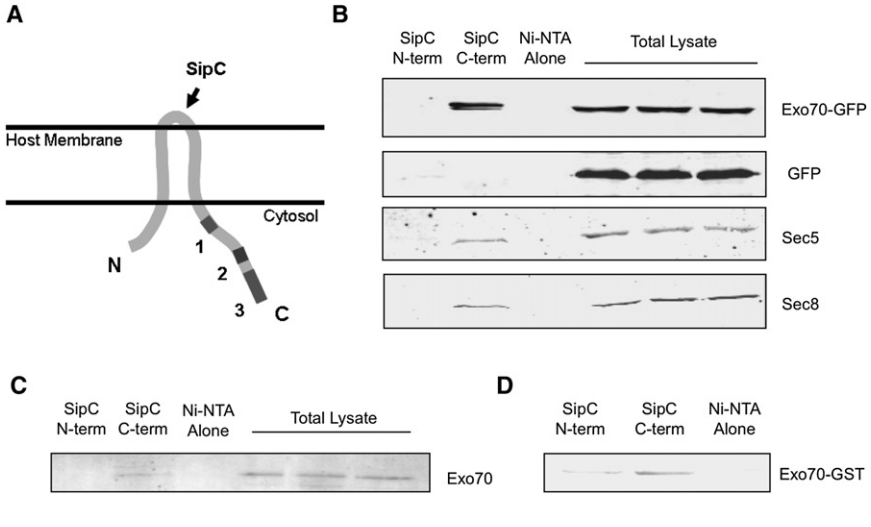

Figure 1. SipC Interacts Directly with Exo70 and Associates with Other Components of the Exocyst Complex

(A) Schematic representation of SipC topology with functional regions highlighted: (1) essential for actin polymerization, (2) putative coiled coil domain, (3) essential for effector translocation.

(B) Coprecipitation of Exo70-GFP and other exocyst components with the SipC C terminus. HeLa cell lysates were incubated with recombinant His6-SipC-N or SipC-C for 1 hr at 4°C. Complexes were pelleted with Ni-NTA and immunoblotted for individual exocyst components as shown.

(C) Coprecipitation of endogenous Exo70 with the SipC C terminus. Pulldown was performed as described above, immunoblotting for endogenous Exo70.

(D) Direct interaction of SipC-C with Exo70. Purified recombinant His6-SipC-N or SipC-C were incubated with recombinant Exo70-GST, pelleted with Ni-NTA, and immunoblotted for GST.

vesicles are targeted directly to sites of bacterial engagement with the plasma membrane. In rare instances, exocyst components could be observed on vacuoles containing *Salmonella* that were also labeled with the early endosomal marker EEA1, indicating that the exocyst association may persist during the early stages of vacuole biogenesis (Figure S2B).

Activation of RalA by the *Salmonella* Effector Protein SopE

Assembly of the exocyst requires activation of the small GTPase, RalA, which interacts directly with both the Exo84 and the Sec5 subunits [10]. Immunostaining of infected HeLa cells revealed that, in addition to the various exocyst subunits, endogenous RalA also became concentrated at sites of *Salmonella* invasion (Figure 2C). To determine whether *Salmonella* infection triggers the activation of RalA, we made use of a previously described pulldown assay that takes advantage of the high affinity of GTP-bound RalA for Sec5 [11, 12]. Infection with *S. typhimurium* led to a rapid and sustained increase in RalA activation. Treatment of cells with a known RalA agonist, epidermal growth factor, generated a quantitatively similar level of activation (Figure 3A).

It is well established that the *Salmonella* effector proteins SopE and SopE2 are able to activate the Rho family GTPases Rac and Cdc42 by acting directly as guanine nucleotide exchange factors. A third *Salmonella* effector, the inositol phosphatase SopB, activates Rac indirectly, through the activation of an upstream Rac regulator, RhoG [13]. To determine whether *Salmonella*-mediated activation of RalA similarly requires translocated effector proteins, cells were infected with either wild-type *S. typhimurium* SL1344 or a T3SS mutant (Δ*invG*) that cannot translocate bacterial effector proteins into the host cytosol [14]. While infection with the wild-type strain induced a robust activation of RalA, infection with the Δ*invG* strain had virtually no effect (Figure 3B), indicating that activation required one or more translocated effector proteins.

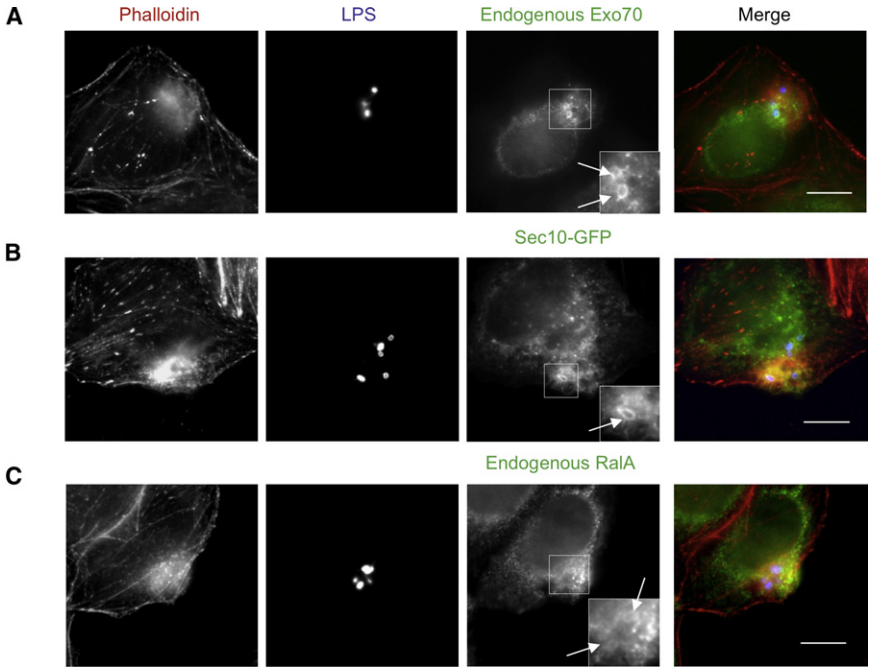

Figure 2. Exo70 and Other Components of the Exocyst Complex Are Recruited to *Salmonella* Invasion Foci

HeLa cells were either left untransfected (A and C) or transfected with a construct encoding Sec10-GFP one day prior to assay (B). Cells were infected for 30 min with *S. typhimurium*, then fixed and labeled with rhodamine-phalloidin for detection of filamentous actin (red) and with anti-LPS for detection of bacteria (blue). Labeling for endogenous Exo70 (A), Sec10-GFP (B), or endogenous RalA (C) is shown in green. The boxed areas are enlarged to show details of invasion foci. Arrows indicate close association of the protein with bacteria. The scale bars represent 10 μm.

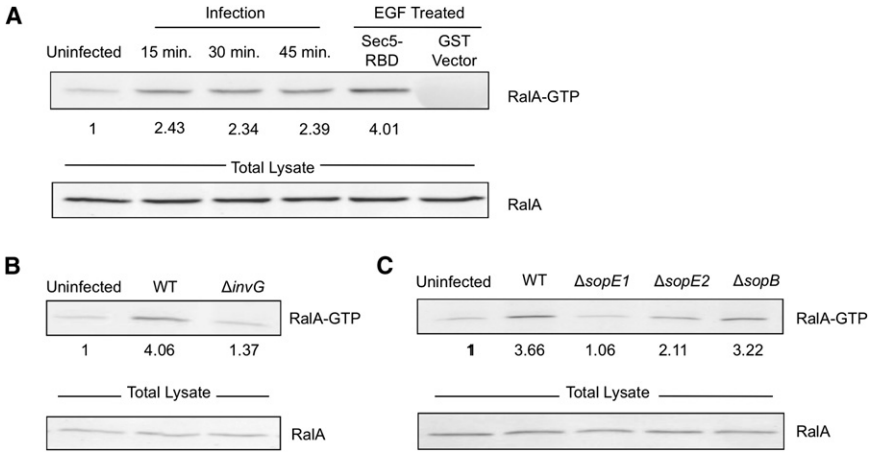

Figure 3. *Salmonella* Invasion Triggers RalA Activation in a SPI-1-Dependent Manner

(A) RalA is activated during *Salmonella* infection. HeLa cells were infected with *S. typhimurium* for the times indicated. Cell lysates were then incubated with the Ral binding domain (RBD) of Sec5 fused to GST. The bound proteins were pelleted with glutathione-sepharose, washed, and immunoblotted for detection of active RalA. As a positive control for RalA activation, cells were stimulated with epidermal growth factor (EGF). Unfused GST was used as a negative control (last lane). Band intensities were quantified by densitometry and normalized to the level of active RalA in uninfected cells. The relative activation is shown below each lane.

(B) RalA activation requires a functional T3SS. HeLa cells were infected with either wild-type *S. typhimurium* SL1344 (WT) or an isogenic strain lacking the T3SS component invG (*ΔinvG*) for 30 min. RalA activation was measured as in (A).

(C) RalA activation depends largely on SopE. HeLa cells were infected with either wild-type *S. enterica* serovar Dublin (WT) or a panel of isogenic strains lacking SopE (Δ*SopE*), SopE2 (Δ*SopE2*), or SopB (Δ*SopB*) for 30 min, and RalA activation was measured as described above.

To identify the effector(s) responsible for RalA activation, we tested a panel of isogenic strains lacking SopE, SopE2, or SopB. The strain lacking SopE (Δ*sopE*) induced no detectable activation of RalA, while the strain lacking SopB (Δ*sopB*) activated RalA to a level that was indistinguishable from that induced by the wild-type strain (Figure 3C). The strain lacking SopE2 was slightly attenuated in its ability to activate RalA, suggesting that while SopE is quantitatively more important, both SopE and SopE2 contribute to Ral activation during *Salmonella* infection.

The Exocyst and RalA Are Necessary for Efficient *Salmonella* Internalization

To determine whether recruitment of the exocyst complex is functionally important for bacterial entry, cells were depleted of Exo70, Sec5, or RalA with the use of siRNA, and invasion efficiency was quantified via a flow-cytometry-based assay (Figure 4A). All three knockdowns resulted in a significant inhibition of bacterial internalization by host cells (38 ± 10% for Sec5; 41.9 ± 4.5% for Exo70; 40 ± 4.5% for RalA). Moreover, the inhibitory effects were quantitatively similar, suggesting that all three proteins act in the same pathway. It should be noted that, although multiple siRNAs were tested for each target protein, we were never able to achieve complete knockdown of Sec5, Exo70, or RalA (Figure S4). It is therefore possible that the observed inhibitory effects underestimate the role of the exocyst in *Salmonella* entry. Importantly, the inhibition of internalization observed upon Sec5 depletion could be reversed by expression of an siRNA-resistant construct, demonstrating that this defect is not due to off-target effects (Figure 4C).

Given that RalA has other functions in addition to its role in exocyst assembly, we also tested the requirement for RalA interaction with Sec5 by using a Sec5 mutant (T11A) that fails to bind Ral [15]. As shown in Figure 4C, expression of Sec5 T11A in Sec5-depleted cells failed to restore invasion efficiency to wild-type levels, indicating that the primary role of RalA in *Salmonella* entry is in the regulation of exocyst assembly.

The exocyst has been implicated in the delivery of new membrane to sites of membrane expansion in a variety of contexts [6, 16–19]. Given that *Salmonella* invasion triggers localized membrane ruffling and macropinocytosis, we hypothesized that the bacteria use the SipC-mediated interaction with Exo70 to direct membrane traffic to sites of bacterial attachment. One prediction of this hypothesis is that inhibition of exocyst function should result in reduced ruffling at invasion foci due to impaired membrane expansion. Using quantitative fluorescence microscopy, we measured the size of the actin-rich foci associated with invading bacteria (Figure S3C). Knockdown of either Sec5 or RalA with siRNA led to a quantitatively similar reduction (35%–40%) in the size of *Salmonella* invasion foci relative to controls (Figure 4B). Simultaneous knockdown of both Sec5 and RalA did not have additive effects (Figure S3B), again indicating that these proteins act in the same pathway. Interestingly, impaired focus formation was readily observed in foci containing 1–4 bacteria but became less apparent as the number of bacteria increased, suggesting that cooperative interactions among attached bacteria are sufficient to overcome the requirement for exocyst function. In agreement with the invasion assay described above, the size of invasion foci was restored to wild-type levels by expression of siRNA-resistant wild-type Sec5, but not by Sec5 T11A (Figure 4D). Taken together, these observations suggest that interaction between RalA and the exocyst is necessary for efficient *Salmonella* internalization.

Concluding Remarks

Many Gram-negative bacterial pathogens, including *Salmonella*, *Shigella*, *Yersinia*, *Pseudomonas*, and *enteropathogenic Escherichia coli*, utilize Type III secretion systems to inject an array of virulence factors into host cells [20]. It is becoming increasingly clear that translocon components such as SipC and the homologous *Shigella* protein, IpaC, have effector functions in addition to their role in protein translocation. Both SipC and IpaC have been shown to stimulate actin remodeling directly, by nucleating actin filament assembly [2, 21]. IpaC can also promote actin reorganization indirectly by recruiting c-Src to its exposed C terminus, where the activity of the protein is necessary for efficient entry of *S. flexneri* into host cells [22].

To identify host proteins that interact with SipC, we conducted a yeast two-hybrid screen using the 200 amino acid SipC C terminus as bait. Here, we show that SipC interacts directly with the exocyst component, Exo70. Along with Sec3, Exo70 is thought to mark specific sites on the plasma

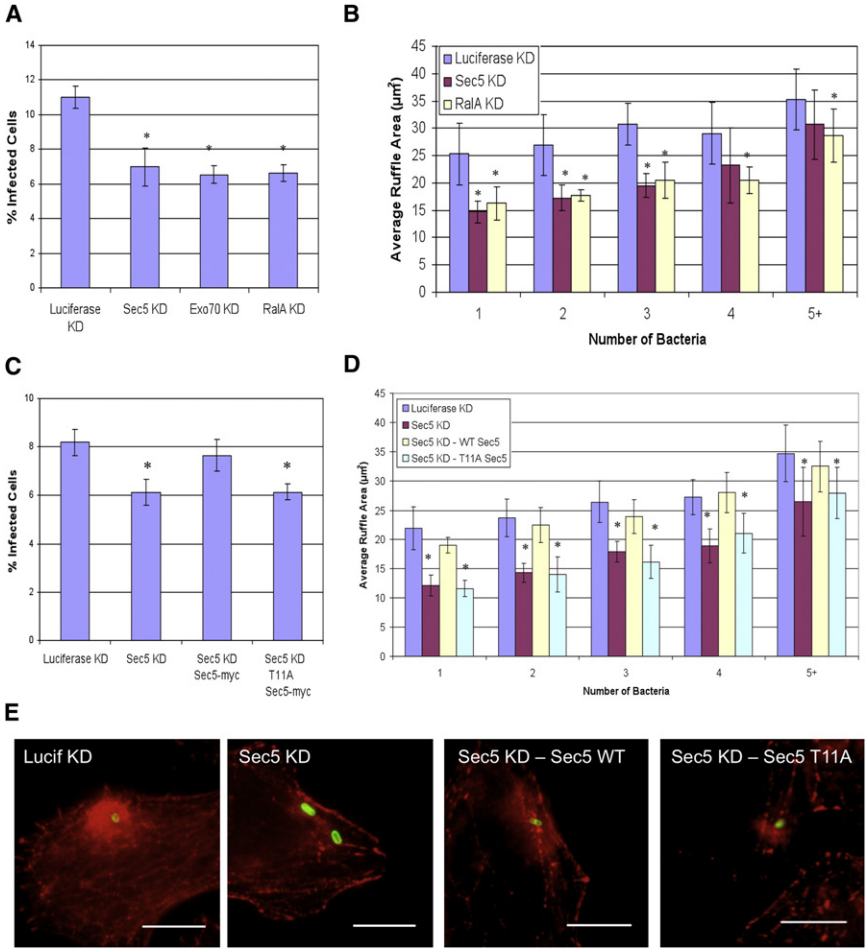

Figure 4. Knockdown of Either Sec5 or RalA Reduces Invasion Efficiency and Correlates with Reduced Membrane Ruffling at Invasion Foci

(A) Knockdown of Sec5, Exo70, and RalA inhibits *Salmonella* invasion. HeLa cells depleted of the indicated protein were infected for 30 min with *S. typhimurium* expressing GFP. After treatment with gentamicin to kill extracellular bacteria, cells were detached and relative GFP fluorescence was measured by flow cytometry. Data are represented as mean ± SEM.

(B) Knockdown of Sec5 or RalA reduces the surface area of invasion foci. HeLa cells were infected for 30 min with GFP-expressing *S. typhimurium*, fixed, and stained with rhodamine-phalloidin. The areas of individual invasion foci were measured as described in Supplemental Experimental Procedures. Over 50 invasion foci were measured per condition, and the data are binned according to the number of bacteria at each site. Data are represented as mean ± SEM.

(C) Expression of exogenous Sec5, but not a RalA-uncoupled mutant, rescues the internalization defect in Sec5-depleted cells. HeLa cells were depleted of endogenous Sec5 and subsequently transfected with the indicated construct. Cells were infected for 30 min with *S. typhimurium* expressing GFP, and bacterial internalization was measured by flow cytometry. Data are represented as mean ± SEM.

(D) Expression of exogenous Sec5, but not a RalA-uncoupled mutant, restores the size of invasion foci in Sec5-depleted cells. HeLa cells were depleted of endogenous Sec5 and transfected with the indicated rescue construct as described in (C). After 48 hr, cells were infected and the surface area of invasion foci quantified as in (B). Data are represented as mean ± SEM.

(E) Representative images of GFP-expressing *Salmonella* invading HeLa cells either mock-depleted (luciferase) or depleted of Sec5 and transfected with the indicated Sec5 rescue construct. Scale bars represent 5 μm.

membrane for the delivery of exocytic vesicles. A role for the exocyst in membrane expansion has been reported in other systems, including phagosome biogenesis [16], lamellipodia formation [6, 23], axonal growth cone expansion [19], yeast bud expansion [17], and cytokinesis [24]. Our data suggest that insertion of the translocon into the host membrane recruits Exo70, thereby promoting a redirection of exocytic vesicle traffic to sites of bacterial attachment. The localized delivery of these vesicles would then provide the additional new membrane required for macropinocytic uptake of the bound bacteria. This hypothesis is supported by our finding that the size of invasion foci induced by *S. typhimurium* is reduced in cells depleted of endogenous Sec5 or RalA and that this correlates with the observed impairment in bacterial uptake. However, Exo70 is unique among the eight exocyst subunits in its ability to activate the Arp2/3 complex and stimulate actin filament assembly. Therefore, we cannot exclude the possibility that Exo70 also acts in this capacity during *Salmonella* invasion.

The exocyst consists of two hemicomplexes, one that marks the vesicle docking site on the plasma membrane and another that assembles on the exocytic vesicle. Assembly of the two hemicomplexes, which is necessary for vesicle docking to the plasma membrane, requires local activation of RalA [10]. Here, we demonstrate that *Salmonella* infection stimulates the activation of RalA, predominantly through the translocated effector protein SopE, and, to a lesser extent, the highly related SopE2. These proteins are known to mimic eukaryotic guanine exchange factors: SopE has been shown to facilitate activation of both Rac and Cdc42 directly [25], while SopE2 appears to act more specifically on Cdc42 [26]. Because SopE exhibits fairly promiscuous substrate specificity in vitro [25], it is likely that SopE acts directly on RalA. However, our data do not exclude the possibility that SopE acts upstream of RalA to activate it indirectly.

Although the exocyst complex has been shown to be important for phagosome biogenesis [16], our findings provide the first evidence for pathogen-directed recruitment of the exocyst to facilitate bacterial invasion of host cells.

Experimental Procedures

The details of experimental methods are provided in Supplemental Experimental Procedures. In brief, the yeast two-hybrid screen was performed with the use of residues 200–410 of SipC as bait, and 3×10^6 colonies of a 7 day mouse embryonic library were screened. Ral activation assays were performed with the use of a GST fusion containing the Ral-binding domain of Sec5. Bacterial internalization was assayed by flow cytometry, with the use of *S. typhimurium* expressing GFP. Morphometric analysis of invasion foci was conducted from randomly selected fields of infected cells with the use of ImageJ software.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at doi:10.1016/j.cub.2010.05.065.

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