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Cdc42 Facilitates Invasion but Not the Actin-Based Motility of *Shigella*

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Summary

The enteric pathogen *Shigella* utilizes host-encoded proteins to invade the gastrointestinal tract. Efficient invasion of host cells requires the stimulation of Rho-family GTPases and cytoskeletal alterations by *Shigella*-encoded IpaC [1]. Following invasion and lysis of the phagosome, *Shigella* exploits the host's actin-based polymerization machinery to assemble an actin tail that serves as the propulsive force required for spreading within and between cells [2, 3]. The *Shigella* surface protein IcsA stimulates actin-tail formation by recruiting host-encoded N-WASP to drive Arp2/3-mediated actin assembly [4–7]. N-WASP is absolutely required for *Shigella* motility, but not for *Shigella* invasion [4, 6]. Although Rho-family GTPases have been implicated in both the invasion and motility of *Shigella*, the role of Cdc42, an N-WASP activator, in this process has been controversial [8, 9]. In these studies, we have examined the role of Cdc42 in *Shigella* invasion and actin-based motility using Cdc42-deficient cells. We demonstrate that Cdc42 is required for efficient *Shigella* invasion but reveal a minor Cdc42-independent pathway that can permit *Shigella* invasion. However, the actin-based motility of *Shigella,* as well as vaccinia, proceeds unperturbed in the absence of Cdc42. These data further support the involvement of distinct host-encoded proteins in the steps regulating invasion and intercellular spread of *Shigella*.

Results and Discussion

Efficient *Shigella flexneri* Entry Is Cdc42 Dependent

We sought to characterize the role of Cdc42 in *Shigella* pathogenesis using Cdc42-deficient cells. $Cdc42^{-/-}$ and $Cdc42^{+/+}$ fibroblast-like cells (KO-FLCs and WT-FLCs, respectively) were derived from $Cdc42^{-/-}$ and $Cdc42^{+/+}$ ES cells, previously generated by gene targeting [10]. The absence of Cdc42 protein expression in KO-FLCs was confirmed by Western blotting analysis (data not shown).

To examine the ability of *Shigella* to invade FLCs in the absence of Cdc42, we utilized microscopic approaches and a gentamicin protection assay. In the microscopic approach, the rate of invasion in WT- and KO-FLCs was determined in three independent experiments by calculating the number of cells with intracellular bacteria (DAPI positive) among total FLCs. As shown in Figure 1A, the number of *Shigella*-infected cells was significantly less in KO-FLCs compared with WT-FLCs (5.0% $\pm$ 0.7% versus 15.8% $\pm$ 6.7%, respectively, $p < 0.04$). Similarly, using the gentamicin protection assay, we determined that the invasion rate of *S. flexneri* in KO-FLCs was markedly decreased when compared with WT-FLCs cells (Figure 1B, <15% of WT, $p < 0.001$). Together, these data demonstrate that efficient invasion of *Shigella* into FLCs is Cdc42-dependent, and the data are consistent with recent studies with dominant-negative Cdc42 constructs [4]. However, these data also demonstrate the existence of a minor Cdc42-independent entry pathway that can permit a reduced rate of *Shigella* entry.

Actin-Based Motility of *Shigella* Is Cdc42 Independent

After entry into the cell, *Shigella* rapidly escapes the phagosome into the cytoplasm, replicating freely, and moves within the cell by exploiting the host's actin polymerization machinery [2, 3]. The *Shigella* surface protein IcsA stimulates the formation of an actin-containing tail (comet) at one pole of the bacterial surface that serves as the propulsive force required to spread intra- and intercellularly [2, 11–14]. *Shigella* tail formation requires recruitment and activation of the host-encoded protein N-WASP that subsequently activates the Arp2/3 complex [4–7]. IcsA has been demonstrated by two groups to bind to (and presumably activate) N-WASP [4, 5]. Activated N-WASP binds the Arp2/3 complex, driving actin assembly and tail formation [4]. *Listeria* and vaccinia also employ actin-based motility for intracellular movement. Whereas vaccinia utilizes cell surface protein (A36R) to activate N-WASP via adaptor protein(s) (e.g., Nck and WIP), *Listeria* bypasses the requirement for N-WASP via ActA, which binds and activates the Arp2/3 complex directly [7].

The activation of N-WASP by *Shigella* IcsA has been likened to the mode in which Cdc42 activates N-WASP, leading to filopodia formation [4]. N-WASP is autoinhibited in its resting state, resulting from an intramolecular interaction between its C-terminal VCA region and the more N-terminal region that contains the GTPase binding domain (GBD) [15, 16]. Cdc42 binding to the GBD of N-WASP results in a release of the autoinhibited state, permitting Arp2/3 binding and actin polymerization [15].

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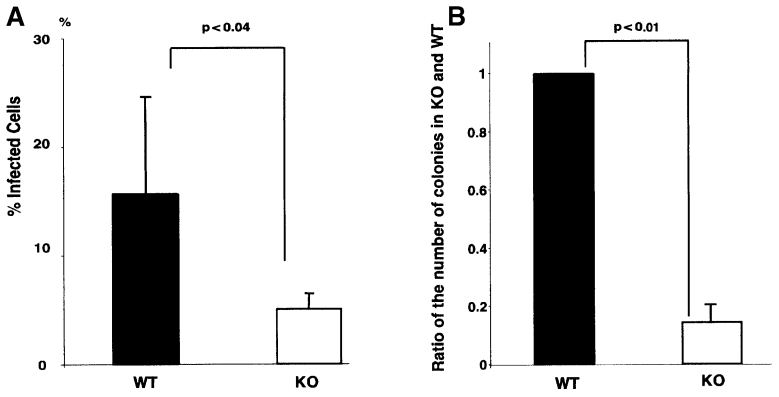

Figure 1. Efficient *Shigella* Invasion Requires Cdc42

(A) Microscopic assay: *S. flexneri* is located intracellularly in 15.8% ± 6.7% of WT-FLCs (black bar), but in only 5.0% ± 0.7% of KO-FLCs (white bar). The bar graph denotes the mean percentage of WT- and KO-FLCs with intracellular bacteria (± SD) following infection out of 100 total cells in three independent experiments.

(B) Gentamicin protection assay: the number of intracellular bacteria in KO-FLCs is <15% (white bar) of that in WT-FLCs (black bar). Following invasion and gentamicin treatment, lysates from WT- and KO-FLCs were plated on culture medium, and colonies were counted after 24 hr [23]. Each bar denotes the ratio of the number of gentamicin-resistant colonies in the indicated cell type divided by the number of colonies in WT-FLCs. For each cell type, the average of three independent experiments is shown (± SD).

Egile et al. have demonstrated that IcsA requires the N-terminal region for binding, a region containing the GBD [4]. They have postulated a mode of activation of N-WASP by IcsA that is similar to and independent of Cdc42. They demonstrated that IcsA, as shown previously by Rohatgi for Cdc42 [17], stimulates the ability of N-WASP to bind Arp2/3 and polymerize actin. To further support this hypothesis, they demonstrated that DN-Cdc42 (as well as DN-Rac and DN-Rho) had no effect on the ability of IcsA to stimulate tail formation [8]. Induction of actin polymerization in vitro also appeared not to require Cdc42, since IcsA-coated bacteria stimulated tail formation when only F-actin, N-WASP, Arp2/3, ADF, and capping protein were present [18].

Suzuki et al. have proposed an alternative model of N-WASP activation by IcsA that requires Cdc42 [5, 9]. These authors present in vitro and in vivo data suggesting that IcsA does not bind to a region overlapping the GBD, but rather binds to the VCA domain [5]. They propose a model whereby N-WASP autoinhibition is first relieved by Cdc42 binding, thus permitting IcsA binding and subsequent Arp2/3-dependent tail formation [9]. In their studies, *Xenopus* extracts that were depleted of all Rho-family GTPases by RhoGDI were unable to support actin-tail formation by IcsA. Cdc42 (but not Rac) rescued the ability of IcsA to stimulate tail formation. Moreover, when DN-Cdc42 was added to untreated extracts, there was a dramatic decrease in actin assembly.

Since the models presented by Egile et al. [4] and Suzuki et al. [9] appear to be mutually incompatible, we investigated directly the requirement for Cdc42 in *Shigella* invasion and actin-based motility utilizing Cdc42-deficient cells. Since a recent report indicated that vaccinia motility is Cdc42 independent [19], we also examined the requirement for Cdc42 in vaccinia actin-based motility. WT- and KO-FLCs were infected with each pathogen, and actin tails were visualized by phalloidin staining (*Shigella*, Figures 2A and 2D; vaccinia, Figures 2H and 2K). Bacterial and viral infection was confirmed by DAPI staining (*Shigella*, Figures 2B and 2E; vaccinia, Figures 2I and 2L). Actin tails were readily visualized in both WT- and KO-FLCs that were infected either with *Shigella* (Figures 2C and 2F, respectively) or vaccinia (Figures 2J and 2M, respectively). The percentage of infected cells with actin tails was equivalent in *Shigella*-infected WT- and KO-FLCs (WT: 72.3% ± 8.6%; KO: 65.7% ± 0.6%, $p > 0.3$, respectively; Figure 2G). The efficiency of tail formation in vaccinia-infected WT- and KO-FLCs was also indistinguishable (WT: 84.7% ± 10.1%; KO: 80.7% ± 5.0%, $p > 0.5$, respectively; Figure 2N). These data indicate that the induction of actin-tail formation by either *Shigella* or vaccinia does not require Cdc42.

Shigella Tail Length and Velocity Is Cdc42 Independent

Although not absolutely required for actin-tail assembly, Cdc42 could participate in the efficiency of tail extension or in the speed of movement of intracellular *Shigella*. In this regard, Suzuki et al. demonstrated that expression of constitutively active Cdc42 accelerated *Shigella* speed, while dominant-negative Cdc42 expression decreased *Shigella* motility [9]. To address this issue, we measured both the length of tails and bacterial speed in *Shigella*-infected WT-and KO-FLCs. Tail lengths were similar in *Shigella*-infected WT- and KO-FLCs (6.6 ± 5.4 μm and 5.9 ± 5.3 μm, respectively; Figure 3). Furthermore, the rate of *Shigella* movement as measured by time-lapse microscopy was also similar in WT- and KO-FLCs (7.66 ± 3.10 μm/min and 7.39 ± 2.74 μm/min, respectively). Figure 4 depicts such Cdc42-independent movement by *Shigella*.

Conclusions

Shigella entry into cells, movement within cells, and spread between cells all require the microbe to induce cytoskeletal changes in the host cell. The Rho-family GTPase Cdc42 has been implicated in both the signaling events leading to *Shigella* entry as well as those signaling events that mediate *Shigella* intracellular actin-based motility [8, 9]. Since these studies have yielded inconsistent results, we sought to definitively address the role of Cdc42 in these processes using Cdc42 KO cells.

We have demonstrated that Cdc42 is essential for the efficient entry of *Shigella* into mammalian cells, confirming recent work [8]. While the downstream signaling events that lead to the morphological changes associated with *Shigella* entry are unknown, it is known that formation of cell surface filopodia correlates with bacterial entry [1] and may be essential for entry. Although filopodia growth is known to occur via Cdc42 activation

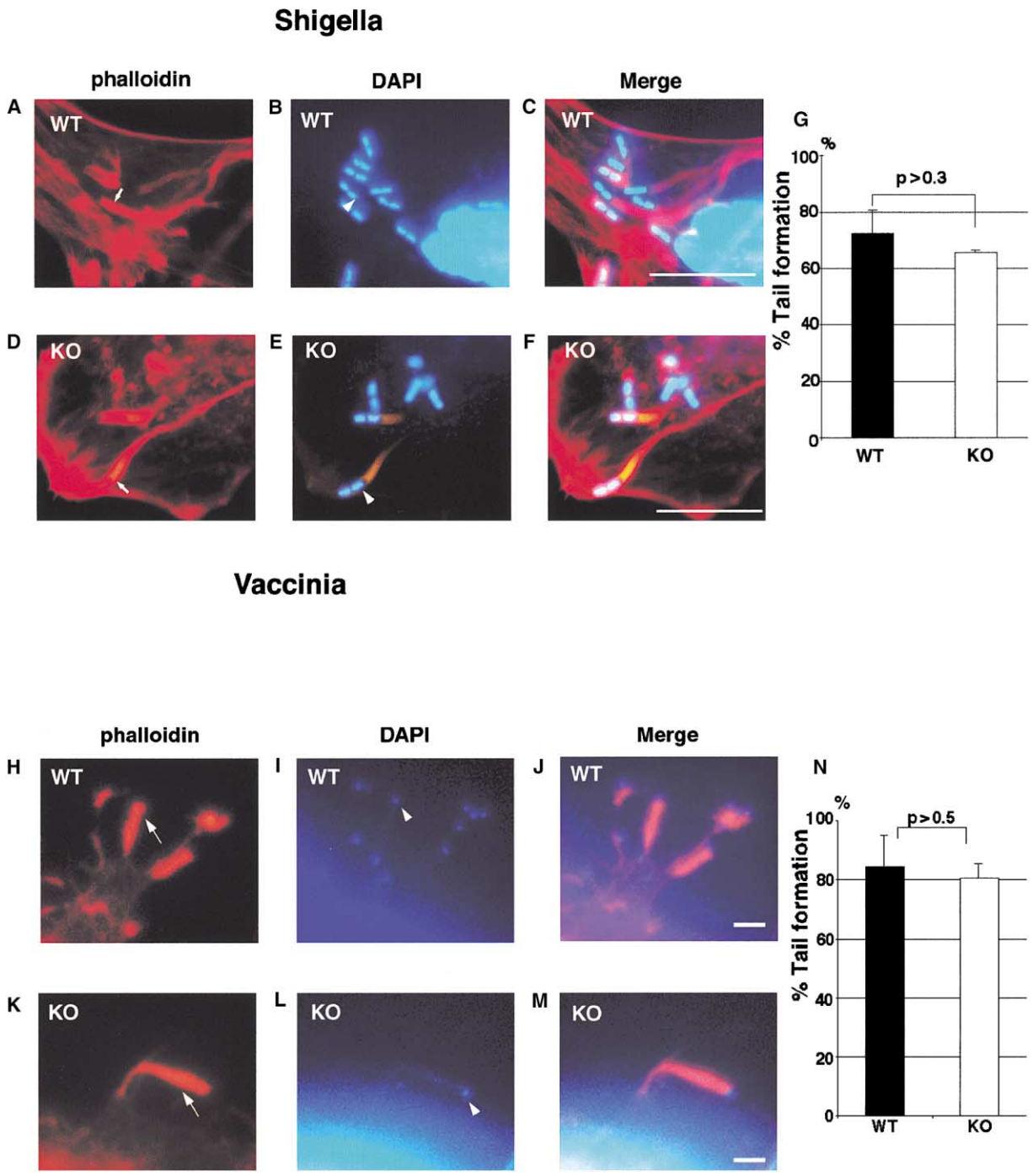

Figure 2. *Shigella*- and Vaccinia-Induced Actin Assembly Is Cdc42 Independent

(A–C) *Shigella*-infected WT-FLCs.

(D–F) *Shigella*-infected KO-FLCs.

(G) The bar graph denotes the mean percentage of *Shigella*-infected WT (black bar)- and KO (white bar)-FLCs in which actin tails were observed in three independent experiments.

(H–J) Vaccinia-infected WT-FLCs.

(K–M) Vaccinia-infected KO-FLCs.

(N) The bar graph denotes the mean percentage of vaccinia-infected WT (black bar)- and KO (white bar)-FLCs in which actin tails were observed in three independent experiments.

WT- (*Shigella*: [A–C]; vaccinia: [H–J]) and KO- (*Shigella*: [D–F]; vaccinia: [K–M]) FLCs were infected with *S. flexneri* or vaccinia virus [24], and actin tails (arrow) were visualized by phalloidin staining (*Shigella*: [A and D]; vaccinia: [H and K]). Bacterial and viral DNA (arrowhead) was revealed by DAPI staining (*Shigella*: [B and E]; vaccinia: [I and L]). Merged images of phalloidin- and DAPI-stained cells are denoted in the right panels (*Shigella*: [C and F]; vaccinia: [J and M]). No statistical difference in actin-tail formation was observed between WT- and KO-FLCs. The scale bar represents 10 μm for *Shigella* and 1 μm for vaccinia.

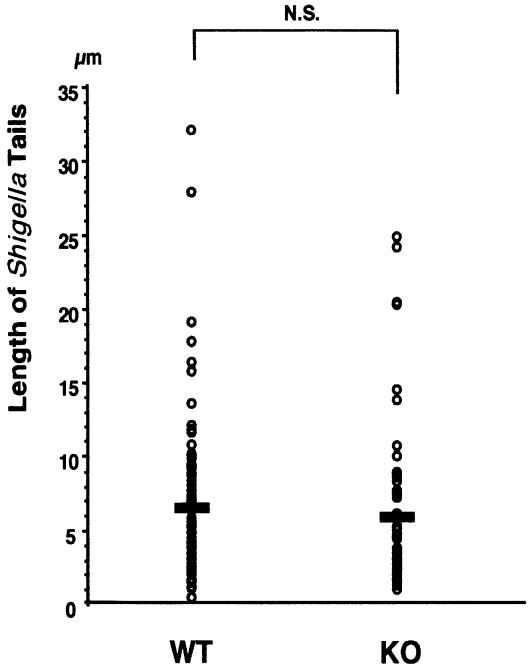

Figure 3. Cdc42 Does Not Regulate *Shigella* Actin-Tail Length

The length of actin tails was measured in *Shigella*-infected WT- (left bar) and KO-FLCs (right bar). Each circle represents the size of a single tail in either WT- or KO-FLCs. For each cell type, at least 20 tails were measured by random sampling in three independent experiments. The mean tail length is denoted by a horizontal line.

of N-WASP [16], N-WASP-deficient cells permit efficient *Shigella* entry [6]. Therefore, either filopodia are not required for *Shigella* entry or Cdc42 can activate filopodia formation via an N-WASP-independent mechanism. Consistent with the possibility that filopodia are not required for *Shigella* entry, Rac has been shown to be important for *Shigella* entry [8]. Lamellipodial extensions resulting from Cdc42-induced Rac activation may be the structure that permits *Shigella* entry. On the other hand, consistent with the possibility that Cdc42 induces filopodia via an N-WASP-independent mechanism during *Shigella* entry, we have recently demonstrated that filopodia can form in the absence of N-WASP [6]. Further experimentation will be required to distinguish between these possibilities.

It is noteworthy that, despite the absence of Cdc42, a significant fraction of *Shigella* is still able to invade host cells. This suggests the existence of a Cdc42-independent pathway for *Shigella* entry. Such a pathway might involve molecules, either homologous or not homologous with Cdc42, that functionally complement Cdc42. Alternatively, such a pathway might involve a nonspecific cellular uptake pathway, such as fluid phase macropinocytosis.

Shigella IcsA and host N-WASP are both required for *Shigella* actin-based motility [2, 4, 6, 12, 13]. Vaccinia utilizes a similar strategy for actin-based motility, utilizing its protein A36R and one or more cellular adaptor proteins to activate N-WASP [19, 20]. However, in contrast to *Shigella*, vaccinia utilizes actin-based motility solely for movement at the cell membrane [21, 22]. For both microbes, N-WASP activation drives Arp2/3-mediated actin assembly and tail formation. Our studies conclusively demonstrate in vivo that Cdc42 is not required for N-WASP activation by *Shigella* or vaccinia. Cdc42 deficiency is also not associated with a change in the average tail length formed or in the speed of *Shigella* in infected cells. These data support a model whereby IcsA can relieve N-WASP autoinhibition in a manner analogous to, yet independent of, Cdc42 [18]. Alternatively, we cannot rule out the possibility that Cdc42-homologous proteins or other Rho-family GTPases may be able to compensate for Cdc42 in actin-based motility.

Supplementary Material

Supplementary Material including the Experimental Procedures and time-lapse video microscopy of *Shigella*-infected WT-FLCs and

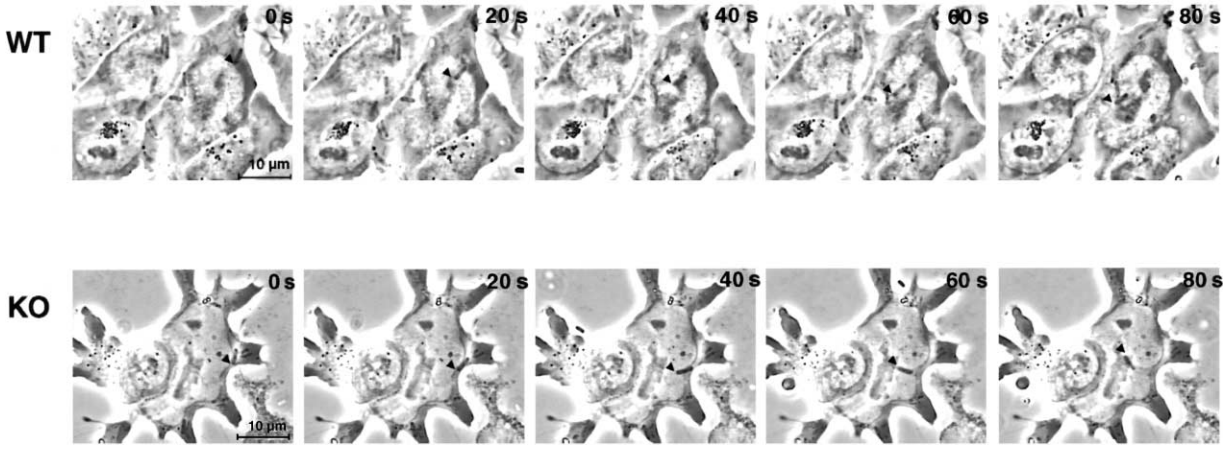

Figure 4. *Shigella* Movement Is Cdc42 Independent

The motility of intracellular *Shigella* was monitored in WT- and KO-FLCs at 1 hr after infection by a phase-contrast microscope equipped with a CCD video camera. Fields were observed and recorded every 5 s. Representative images are displayed at 20-s intervals. A bacterial pole is indicated in each panel by an arrow. The corresponding time-lapse videos (WT and KO) displaying intracellular motility of *Shigella* can be found on line.

KO-FLCs is available at http://images.cellpress.com/supmat/supmatin.htm.

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Note Added in Proof

Lommel et al. (Lommel, S., Benesch, S., Rottner, K., Franz, T., Wehland, J., and Kuhn, R. [2001]. Actin pedestal formation by enteropathogenic *Escherichia coli* and intracellular motility of *Shigella flexneri* are abolished in N-WASP-detective cells. EMBO Rep. *2*, 850–857) have also demonstrated that the actin-based motility of *Shigella* is N-WASP dependent.