The Diaphanous-Related Formins Promote Protrusion Formation and Cell-to-Cell Spread of *Listeria monocytogenes*

*Ramzi Fattouh,1,2* Hyunwoo Kwon,4,6 *Mark A. Czuczman,1,4* John W. Copeland,7 *Laurence Pelletier,4,6* Margot E. Quinlan,8 *Aleixo M. Muise,1,2,3,5* Darren E. Higgins,9 and John H. Brumell1,3,4,5

1Cell Biology Program, 2Division of Gastroenterology, Hepatology, and Nutrition, Department of Paediatrics, and 3SickKids IBD Centre, Hospital for Sick Children, 4Department of Molecular Genetics and 5Institute of Medical Science, University of Toronto, 6Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, and 7Department of Cellular and Molecular Medicine, University of Ottawa, Canada; 8Department of Chemistry and Biochemistry and Molecular Biology Institute, University of California–Los Angeles; and 9Department of Microbiology and Immunobiology, Harvard Medical School, Boston, Massachusetts

The Gram-positive bacterium *Listeria monocytogenes* is a facultative intracellular pathogen whose virulence depends on its ability to spread from cell to cell within an infected host. Although the actin-related protein 2/3 (Arp2/3) complex is necessary and sufficient for *Listeria* actin tail assembly, previous studies suggest that other actin polymerization factors, such as formins, may participate in protrusion formation. Here, we show that Arp2/3 localized to only a minor portion of the protrusion. Moreover, treatment of *L. monocytogenes*-infected HeLa cells with a formin FH2-domain inhibitor significantly reduced protrusion length. In addition, the Diaphanous-related formins 1–3 (mDia1–3) localized to protrusions, and knockdown of mDia1, mDia2, and mDia3 substantially decreased cell-to-cell spread of *L. monocytogenes*. Rho GTPases are known to be involved in formin activation. Our studies also show that knockdown of several Rho family members significantly influenced bacterial cell-to-cell spread. Collectively, these findings identify a Rho GTPase–formin network that is critically involved in the cell-to-cell spread of *L. monocytogenes*.

**Keywords.** *Listeria monocytogenes*; diaphanous formins; mDia1, mDia2, mDia3; protrusion; *Listeria* cell-to-cell spread; Arp2/3; HeLa cells.

*Listeria monocytogenes* is an enteric human pathogen and the causative agent of listeriosis, a rare but potentially life-threatening infection in newborns, elderly persons, and immunocompromised individuals [1]. As part of its life cycle, *L. monocytogenes* replicates within the cytosol of host cells, where it exploits host cell actin assembly machinery to spread to neighboring cells [2]. The bacterial factor ActA recruits and activates host cell actin-binding proteins, including the actin-related protein 2/3 (Arp2/3) complex [3–6]. Actin polymerization at the bacterium–actin interface propels these microbes through the cytoplasm [7, 8], resembling what has been described to be a “comet with a long tail” [9]. The actin filaments that make-up the so-called comet tail were found to be short and highly cross-linked [10], as is characteristic of Arp2/3-mediated actin filament construction. Ultimately, sustained actin polymerization pushes the bacterium into the host cell plasma membrane, causing membrane distension; formation of bacterial-associated finger-like membrane extensions, termed “protrusions”; and cell-to-cell spread [9].

Interestingly, analysis of electron micrographs revealed that *L. monocytogenes* protrusions comprised 2 populations of actin filaments. In the region that is proximal to the base of the bacterium, there is an array of cross-linked short filaments, whereas the remainder of the protrusion is composed of long, parallel filaments [10, 11]. The latter observation implicates the involvement of actin assembly proteins other than the Arp2/3 complex in the formation of protrusions by *L. monocytogenes*. Among the host
factors able to directly catalyze the formation of actin filaments are members of the formin family, which possess conserved formin-homology domains 1 (FH1) and FH2 [12]. In contrast to the actin filaments assembled by the Arp2/3 complex, formins drive polymerization of unbranched actin filaments [12]. Formins are ubiquitously expressed in mammalian cells and are known to play essential roles in various fundamental cell processes, including the formation of filopodia, lamellipodia, and cellular protrusions [12–15]. Moreover, efficient protrusion formation and intercellular spread of Shigella flexneri and Rickettsia parkeri, bacteria with life cycles similar to that of L. monocytogenes, were shown to depend on actin polymerization by formins or formin-like proteins [16, 17]. Collectively, these findings support the notion that formins may play an important role in L. monocytogenes–induced protrusion formation and cell-to-cell spread. However, which ones are involved and what factors may regulate them are not known.

Here, we investigated the involvement of formins in cell-to-cell spread by L. monocytogenes. Our findings demonstrate that the Diaphanous-related formins are involved in L. monocytogenes–induced protrusion formation and, in conjunction with members of the Rho GTPase family, are important facilitators of dissemination of these bacteria.

METHODS

Cell Culture and Bacterial Strains

HeLa epithelial cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Hyclone) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Wisent) without antibiotics at 37°C and 5% CO₂. Cells were seeded in 24-well tissue culture plates on glass coverslips at 2.5 × 10⁴ cells/well and 5.0 × 10⁶ cells/well for small interfering RNA (siRNA) and plasmid transfection experiments, respectively, involving direct visual analysis. Wild-type L. monocytogenes 10403S [18] and 10403S ΔactA [19] were used for infection studies as indicated.

Antibodies, Reagents, and Constructs

Details about these materials are available in the Supplementary Materials.

siRNA and Endoribonuclease-Prepared siRNA (esiRNA) Treatment

A complete listing of the siRNA and esiRNA oligos used in this study are specified in Supplementary Table 2, and details about these analyses are available in the Supplementary Materials. The efficiency of knockdown was confirmed for select factors (Supplementary Figure 1).

Bacterial Infection

L. monocytogenes were grown for approximately 16 hours in brain-heart infusion (BHI) broth at 30°C without shaking, subcultured 1:10 in BHI without antibiotics, and grown at 37°C for 2 hours with shaking (to an OD₆₀₀ of approximately 0.3, which is equivalent to approximately 3 × 10⁸ colony-forming units/mL). Bacterial inocula were prepared by pelleting at 10 000 × g for 1–2 minutes, washing twice, and resuspending in phosphate-buffered saline (PBS). Bacterial inocula were then diluted in DMEM without FBS. Cells were washed twice in PBS and infected at a multiplicity of infection of 100. Bacteria were centrifuged onto cells at 225g for 3 minutes at room temperature, and infected cells were incubated at 37°C with 5% CO₂. Sixty minutes after infection, extracellular bacteria were removed by extensive washing with PBS. Gentamicin was added to the medium to achieve a final concentration of 10 µg/mL, which was maintained throughout the duration of the experiment.

Immunofluorescence

Details about this analysis are available in the Supplementary Materials.

Microscopy and Image Preparation

Details about these techniques are available in the Supplementary Materials.

Protrusion and Comet Tail Analysis

Images of so-called primary infected cells (ie, host cells containing >50 intracellular bacteria) were acquired, and the following parameters were analyzed using Volocity: (1) number of host cells, (2) total number of bacteria, (3) number of protrusions and actin (ie, comet) tails, and (4) lengths of protrusions and actin tails. A protrusion was defined as a bacteria-associated extension of the plasma membrane that stained positive for ezrin and actin. A comet tail was defined as a bacteria-associated actin tail that stained negative for ezrin. Only protrusions and comet tails that were >1 µm long were included in the analysis.

Plaque Assay

A total of 2.0 × 10⁵ HeLa cells were seeded per well in 6-well tissue culture plates. Cells were transfected with siRNA 24 hours later. Cells were infected with 4.0 × 10⁴ bacteria 48 hours after transfection. After 1 hour of infection, cells were washed 3 times with PBS and were overlaid with a 0.7% agarose–DMEM mixture containing 50 µg/mL gentamicin. At 96 hours after infection, a second agarose-medium overlay containing 6% neutral red (Sigma; N2889) and 50 µg/mL gentamicin was added. After 8 hours, plaques were imaged using a Fluorchem E scanner (Proteinsimple). Plaque diameter was measured using Adobe Photoshop, and the area was calculated as 3.14159 × [diameter/2]². Plaque area was normalized to control siRNA/esiRNA-treated cells infected with wild-type bacteria for each experiment.

Western Blotting and Real-Time Reverse-Transcription Polymerase Chain Reaction Analysis

Details about these analyses are available in the Supplementary Materials.
Data Analysis
Data were analyzed using GraphPad Prism v5.0 for Mac OS X (GraphPad Software). Data are expressed as mean ± standard error of the mean. Results were analyzed using analysis of variance with a post hoc test (described in detail in the figure legends) or an unpaired t test. Differences were considered statistically significant at P values of <.05.

RESULTS
Formins Are Required for Protrusion Formation
As a result, the silencing capacity is augmented, and off-target effects are reduced [23]. Knockdown of mDia1, mDia2, and mDia3 led to a substantial reduction in plaque area, indicative of diminished cell-to-cell spread (Figure 4A and 4B).

We also examined the influence of mDia1, mDia2, and mDia3 knockdown on protrusion formation. Depletion of mDia1 and mDia3 significantly reduced the average protrusion length, while knockdown of mDia2 had only a minor, statistically insignificant effect, compared with control esiRNA–treated cells (Figure 4C). No differences were observed with respect to protrusion frequency, comet tail length, or comet tail frequency between any of the mDia formin-depleted groups and the control group (Figure 4C and data not shown).

mDia1, mDia2, and mDia3 Selectively Localize to Protrusions and Are Required for Efficient Cell-to-Cell Spread of L. monocytogenes
Given that protrusion formation is critical to L. monocytogenes cell-to-cell spread and that treatment with a general formin inhibitor influenced protrusion length, we sought to specifically identify the formins that may be involved in cell-to-cell spread of L. monocytogenes. To this end, we screened the approximately 15 mammalian formins for localization to protrusions. We transfected HeLa cells with epitope-tagged constructs of formin proteins and examined their association with protrusions in HeLa cells 8 hours after infection. Of the 15 formins we examined, only the mDia proteins were enriched along protrusions (>95% of protrusions), while none of the other formins were present above background levels on these structures (<5% of protrusions; Figure 3A and data not shown). In addition, mDia1-3 were rarely, if ever, observed to associate with comet tails (<5% of comets; Figure 3B and data not shown).

Next, we investigated whether the mDia formins were required for L. monocytogenes cell-to-cell spread in a plaque assay, using esiRNA. esiRNAs are a pool of siRNA-like oligonucleotides that cover a larger region of the messenger RNA being targeted for depletion. The different siRNAs that constitute this pool are present in comparable quantities and share the same on-target gene. As a result, the silencing capacity is augmented, and off-target effects are reduced [23]. Knockdown of mDia1, mDia2, and mDia3 led to a substantial reduction in plaque area, indicative of diminished cell-to-cell spread (Figure 4A and 4B).

mDia1, mDia2, and mDia3 were enriched along protrusions (>95% of protrusions), while none of the other formins were present above background levels on these structures (<5% of protrusions; Figure 3A and data not shown). In addition, mDia1-3 were rarely, if ever, observed to associate with comet tails (<5% of comets; Figure 3B and data not shown).

Next, we investigated whether the mDia formins were required for L. monocytogenes cell-to-cell spread in a plaque assay, using esiRNA. esiRNAs are a pool of siRNA-like oligonucleotides that cover a larger region of the messenger RNA being targeted for depletion. The different siRNAs that constitute this pool are present in comparable quantities and share the same on-target gene. As a result, the silencing capacity is augmented, and off-target effects are reduced [23]. Knockdown of mDia1, mDia2, and mDia3 led to a substantial reduction in plaque area, indicative of diminished cell-to-cell spread (Figure 4A and 4B).

Formins Facilitate Listeria Spread • JID 2015:211 (1 April) • 1187
The actin-related protein 2/3 (Arp2/3) complex is primarily associated with comet tails. A, HeLa cells were transfected with LifeAct-RFP (F-actin probe), infected with *Listeria monocytogenes* for 8 hours, and immunostained with DAPI (to visualize bacteria) and antibodies to Arp p34 and ezrin. Representative confocal images are shown of a protrusion (inset 1: actin+ ezrin+) and comet tail (inset 2: actin+ ezrin−). Arrowheads denote the bacterium associated with the protrusion/comet tail identified by the dashed line. Scale bar, 10 µm for low magnification and 3 µm for I1 and I2. B, The length along the protrusion/comet tail demonstrating Arp p34 enrichment (based on signal thresholding) was quantified and expressed as a percentage of the total protrusion/comet tail length. Arp p34 staining was measured on at least 50 protrusions and comet tails. Data show individual measurements along with average values (± standard error of the mean). Statistical analysis was performed using an unpaired *t* test. ***P < .001.
Rho GTPases Participate in *L. monocytogenes* Cell-to-Cell Spread

Several reports have demonstrated that the mDia subfamily of formins function, at least in part, as Rho GTPase effectors. Thus, in an effort to identify the involvement of Rho GTPases in *L. monocytogenes* cell-to-cell spread, we screened a limited group of Rho GTPases that have been previously implicated in mDia activation. Using siRNA, we investigated the influence of individually depleting select GTPases on *L. monocytogenes* cell-to-cell spread by plaque assay. Of the GTPases examined, knockdown of Rac1, Cdc42, RhoA, RhoC, and RhoD led to considerable decreases in plaque size (Figure 5). Treatment with siRNA against RhoB, RhoG, and Rac2 had no effect on plaque size.

DISCUSSION

The mechanisms used by *L. monocytogenes* to drive intracellular motility within an infected host cell are well understood. Comparatively less is known regarding the processes and factors involved in the formation of protrusions and cell-to-cell spread. Given that the host Arp2/3 complex is important for...
L. monocytogenes comet tail formation and intracellular motility, we examined the localization of this complex to protrusions. In agreement with a recent report, we noted that in contrast to comet tails, where Arp2/3 is localized along the majority of the tail, this complex is enriched along a small portion of the protrusion [24], indicating to us that other host actin nucleating factors may contribute to protrusion formation.

The notion that formins are involved in the dissemination of L. monocytogenes is in line with previous studies that have demonstrated the importance of formins or formin-like factors
in the spread of various other human pathogens. Indeed, FHOD1 has recently been shown to facilitate robust actin-based motility of host-cell extracellular-associated vaccinia viral particles, leading to protrusion formation and dissemination to neighboring cells in vitro [25]. Notably, recruitment of FHOD1 and its involvement in actin tail formation was shown to be dependent on Rac1 activity [25]. In addition, the surface cell antigen 2 (Sca2) of Rickettsia is critically required for actin-tail formation and virulence [26]. Of significance, Sca2 functions, similarly to eukaryotic formins, as a profilin-dependent barbed-end elongation factor that directly mediates polymerization of host actin into unbranched filaments [17] to propel bacteria throughout the host cell. In the case of S. flexneri, studies performed by Heindl et al showed that, upon depletion of mDia1 and mDia2, the frequency of protrusions was decreased relative to controls and that this effect was associated with impaired spread [16]. Codepletion of mDia1 and mDia2 did not further influence protrusion formation. In contrast, we did not observe a difference in the number of protrusions formed following treatment of L. monocytogenes–infected HeLa cells with either an FH2-inhibitor or an esiRNA against mDia1–3. These findings suggest that, in the context of L. monocytogenes, other factors in addition to formins may participate in protrusion elongation and that the initiation of these structures may not be impaired by formin inhibition. As neither SMIFH2 treatment nor mDia1–3 esiRNA influenced comet tail length or number, perhaps it is not unexpected that L. monocytogenes was able to contact the plasma membrane and initiate protrusion assembly to the same extent as in control-treated cells. Interestingly, although it did not reach statistical significance,
inhibition of mDia1 or mDia2 showed a trend toward decreased Shigella actin tail length within the host cell body (ie, comets), indicating that, in addition to their involvement in protrusions, these formins may also participate in the assembly of Shigella comet tails [16]. Therefore, mDia proteins may influence Shigella cell-to-cell spread by regulating protrusion frequency and, perhaps, comet tail length, while in the case of L. monocytogenes, mDia proteins may facilitate cell-to-cell spread by mediating protrusion elongation.

At present, the possibility that mDia inhibition indirectly influenced L. monocytogenes cell spread in some fashion, such as by affecting cell-cell interactions, cannot be excluded. However, the reduction in protrusion length following targeted depletion of mDia1, mDia3, and, to a small extent, mDia2, coupled with the observation that mDia formins localize to protrusions, argues against the contribution of indirect effects. Moreover, decreased protrusion length has been previously shown to correlate with reduced cell-to-cell spread [27].

Yeast 2-hybrid-based analyses conducted over a decade ago identified mDia family members as downstream effectors of the Rho family of small GTPases [28, 29]. Binding of activated Rho proteins to the Rho GTPase–binding domain of mDia formins induces these formins to nucleate and polymerize actin [12]. Since these discoveries, a network of interactions between these 2 groups of proteins has been described, and various processes were shown to involve cooperation between Rho GTPases and mDia proteins [29–34] including, stress fiber formation, vesicle movement, and filopodia and lamellipodia protrusion [13, 22, 30, 34–38]. To begin to discover the potential network of Rho GTPases that may be involved in L. monocytogenes cell-to-cell spread, we screened a subset of the Rho GTPase family by using a conventional plaque assay. Several of the GTPases screened proved to be required for efficient cell-to-cell spread. Our observation that Cdc42 knockdown led to diminished L. monocytogenes spread is, seemingly, in disagreement with a previous report [39]. Rigano et al noted that efficient protrusion formation and spread of Listeria required the effector protein internalin C (InIC), which mediated the downregulation of activated Cdc42. Of significance, there were several well-recognized differences among the methods used that could account for these discordant results. Overexpression of a dominant-negative construct was used by Rigano et al to interfere with Cdc42, and their studies were conducted in the context of a polarized cell-line (Caco-2 BBE1 cells), whereas we used an siRNA-based approach and a nonpolarized cell line (HeLa cells). Alternatively, it is conceivable that Listeria drives local inhibition of Cdc42 at sites where InIC or other virulence factors are localized. But there may be a general requirement for Cdc42 (eg, for trafficking processes) at other cellular sites that are required for productive spread. On the basis of the experimental approach used here, we cannot rule out the possibility that some of the GTPases we examined may affect cell-to-cell spread through involvement in 1 or more events upstream and/or downstream of protrusion formation (eg, invasion, comet tail formation, and uptake of L. monocytogenes protrusions by neighboring uninfected host cells). It is worth noting that the findings of some studies, but not all, suggest that select Rho GTPases may play a role in promoting L. monocytogenes invasion, although perhaps not intracellular motility [40–45]. Noteworthy, the evidence suggests that involvement of Rho GTPases in these processes may depend, in part, on the cell type examined and the mechanism of invasion used by L. monocytogenes.

It is becoming increasingly clear that the formation of membrane protrusions by L. monocytogenes and related pathogens is an active process and not simply a byproduct of the forces generated by actin-based cytosolic propulsion. Indeed, Escherichia coli expressing IcsA, the molecule that is necessary and sufficient for Shigella actin-based motility, was adequate to promote protrusion formation in a manner that was largely related to the direction but not the speed of intracellular travel [46]. This finding is evidence that a primary objective of intracellular motility may be to contact the membrane and not necessarily to provide the forces required to project through it. This is not to say that the
force of contact is not an important factor for protrusion formation, but rather that it is not likely to be the sole contributing factor determining whether a protrusion develops. In this regard, disruption of ezrin, a key membrane-cytoskeleton linker that accumulates at *L. monocytogenes*-induced protrusions, specifically reduces the number and morphology (shortened and collapsed) of protrusions, resulting in impaired cell-to-cell spread [21]. Myosin-X has also been shown to play a critical role in the development of protrusions [27]. As a consequence of Myo10 depletion, *Shigella* protrusions were stunted, and cell-to-cell spread was dramatically diminished. Myo10 was observed to concentrate at the base of *Shigella* and to cycle along its sides within a protrusion as the protrusion lengthened. Similarly, Myo10 accumulated in *L. monocytogenes*-induced protrusions, and knockdown of this protein also impaired bacterial cell-to-cell spread [27]. In addition, InlC of *L. monocytogenes* has been shown to facilitate protrusion formation by regulating cell membrane tension [47]. InlC was found to interfere with the interaction between the mammalian adaptor protein Tuba and the actin regulator N-WASP2, which together regulate cortical actin tension at apical junctions. Disruption of the Tuba-N-WASP interaction by binding of InlC to Tuba, via a C-terminal SH3 domain, resulted in a loss of membrane tension, creating slack that permits protrusion formation by *L. monocytogenes*. Disruption of components of the host cell actin network disassembly machinery has also been reported to impair *L. monocytogenes* spread. Specifically, codepletion of actin-interacting protein 1 and coflin 1 dramatically reduced the velocity of *Listeria* in protrusions, led to altered protrusion morphology, and decreased bacterial spread to adjacent uninfected host cells [24]. Collectively, these findings strongly suggest that bacterial effector proteins in conjunction with host cell factors actively drive the formation of bacterial-associated protrusions.

The observations presented here identify a potential network of formins and Rho GTPases that aid in active protrusion formation (Figure 6). *L. monocytogenes* forms ActA- and Arp2/3-dependent comet tails within the host cell cytoplasm [2, 5, 6]. Similar to the involvement of N-WASP and FHOD1-Rac1 pathways in the spread of vaccinia virus [25], processes related to actin-based motility and/or contact with the cell membrane may activate Rho GTPases, which, in turn, recruit and induce the activation of formins, such as mDia1–3. Subsequently, these mDia proteins contribute to the elongation of actin filaments in protrusions, independent of Arp2/3, thereby promoting *L. monocytogenes* cell-to-cell spread. Thus, our findings show that *L. monocytogenes* exploits the mDia subfamily of formins to promote effective cell-to-cell spread. Characterization of the precise array of host factors involved specifically in the protrusion phase may help to identify novel strategies through which the spread and resulting pathologies of intracellular pathogens such as *L. monocytogenes* can be abrogated.

**Supplementary Data**

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

**Acknowledgements.** We thank Mike Woodside and Paul Paroutsis for their support with microscopy.

**Financial support.** This work was supported by the Arthritis Society of Canada (grant RG11/013 to J. H. B.); the US Public Health Service (grant A053669 from the National Institutes of Health to D. E. H.); the Hospital for Sick Children (Pembidao Chair in Cell Biology to J. H. B.); the Canadian Institutes of Health Research, in partnership with the Canadian Association of Gastroenterology and the Crohn’s and Colitis Foundation of Canada (postdoctoral fellowship to R. F.); the Research Training Committee, Hospital for Sick Children (studentship to M. A. C.); the University of Toronto.

---

**Figure 6.** Schematic model outlining the putative involvement of a Rho GTPase–formin network in the formation of *Listeria monocytogenes*-induced protrusions and cell-to-cell spread. A. Illustrates the network of Rho GTPase–Diaphanous-related formin (mDia) interactions potentially involved in *L. monocytogenes* cell-to-cell spread. Interactions between Rho GTPases and mDia proteins (ie, dashed lines) are based on previously published studies [35]. B. Following entry and escape from the host cell phagosome, *L. monocytogenes* uses its ActA protein (not shown) to recruit the host cell actin polymerization complex, Arp2/3 (i). Arp2/3-mediated actin polymerization provides the propulsive force that drives *L. monocytogenes* intracellular motility, facilitating contact with the plasma membrane (ii). Activation of Rho GTPases leads to binding and activation of the mDia formins that, in turn, catalyze the formation of unbranched actin filaments to assist in the formation of *L. monocytogenes* protrusions (iii).
(Open Fellowship to M. A. C.); the Canada Graduate Scholarships–Master’s Program, Natural Science and Engineering Research Council of Canada (scholarship to M. A. C.); a Canada Research Chair in Centrosome Biogenesis and Function (tier 2; to L. P.); and the Ontario Research Fund Global Leadership Round in Genomics and Life Sciences (for support of the esIR-NA pipeline).

**Potential conflicts of interest.** All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

**References**

7. Sanger JM, Sanger JW, Southwick FS. Host cell actin assembly is necessary and likely to provide the propulsive force for intracellular movement of *Listeria monocytogenes*. Infect Immun 1992; 60:3609–19.