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Activating mutations of N-WASP alter *Shigella* pathogenesis

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ABSTRACT

The pathogenesis of *Shigella* requires binding to the host protein N-WASP. To examine the roles of structural conformation and phospho-regulation of N-WASP during *Shigella* pathogenesis, mutant N-WASP constructs predicted to result in a constitutively open conformation (L229P and L232P) or either a phospho-mimicking (Y253E) or phospho-disruptive (Y253F) structure were constructed. Pyrene actin assays demonstrated that the N-WASP L229P and L232P constructs are constitutively active. Despite the increase in actin polymerization seen *in vitro*, cell lines expressing N-WASP L229P and L232P supported shorter actin tails when infected with *Shigella*. *Shigella* actin tails were unchanged in cells expressing N-WASP phospho-regulation mutant proteins. *Shigella* invasion, intracellular, and intercellular motility were not altered in cells expressing N-WASP L229P or L232P. However, plaque numbers were increased in cells expressing N-WASP L229P and L232P. These data demonstrate that N-WASP structural conformation is an important regulator of *Shigella* pathogenesis in distinct segments of its lifecycle.

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Introduction

Shigella flexneri is a bacterial pathogen of the human intestine that usurps control of the actin cytoskeletal machinery for its pathogenesis [1]. Intracellular *Shigella* movement is facilitated by directing host cell actin polymerization exclusively at one pole of the bacteria – a process known as actin-based motility [1]. The force generated by the polymerizing actin is sufficient to propel *Shigella* through the cytoplasm and into neighboring cells [1].

Shigella regulates the host actin cytoskeleton through its interactions with the host protein N-WASP [1]. N-WASP is a member of the WASP family of cytoskeletal regulators, which interact with various signaling molecules to transduce Arp2/3-mediated actin polymerization [2]. In its native conformation, intramolecular forces hold WASP and N-WASP in an auto-inhibited closed, hairpin-like structure [3,4]. Binding of activated Cdc42 to N-WASP disrupts this closed conformation, presumably opening up the molecule with a release of auto-inhibition, permitting binding to the Arp2/3 complex and inducing actin polymerization [2]. Once in an open conformation, N-WASP can also be modulated by phospho-regulation. Phosphorylation of tyrosine residue Y253 of N-WASP leads to enhanced actin polymerization activity *in vivo* and *in vitro* [5].

Steps controlling activation and regulation of N-WASP following *Shigella* IcsA binding are largely unknown. Cdc42 is not absolutely required for *Shigella*-mediated actin-based motility [6]. The role of IcsA and other bacterial/host molecules in the relief of N-WASP auto-inhibition is largely unknown. Moreover, the role of phospho-regulation of N-WASP during *Shigella*-mediated actin-based motility remains controversial. An early study failed to observe phosphorylated tyrosine residues located at the site of *Shigella*-mediated actin polymerization [7]. However, recent data suggests that the phosphorylation status of N-WASP may play an important role in the ability of *Shigella* to form actin tails [8].

Here, we sought to assess the role of N-WASP regulation through structural conformation or phosphorylation on *Shigella*-mediated actin-based motility by generating specific mutations in N-WASP predicted to regulate structural conformation and phosphorylation, and assessing their behavior during infection.

Materials and methods

Bacterial strains and cell culture. Shigella flexneri serotype 2a strain 2457T was used for all studies and grown on tryptic soy broth (TSB) with agar containing Congo red as described [17]. Individual red colonies were grown overnight in liquid TSB and then diluted and grown in fresh media until mid-log phase on the day of infection. N-WASP$^{-/-}$ mouse embryonic fibroblasts [17] were maintained in

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Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% bovine calf serum (with iron supplement) and L-glutamine.

Generation of N-WASP mutations. Point mutations in rat N-WASP were generated using an overlapping PCR strategy with WT rat N-WASP as a template. The FLAG peptide DNA sequence (5′-atggactacaaggacgacgatgacaag-3′) was inserted before the stop codon at the C-terminal end of N-WASP. All constructs were sequenced to verify presence of single point mutations.

Actin polymerization assay. Pyrene actin assays were performed as previously described [23]. Pyrene actin, Cdc42, and the Arp2/3 complex were purified and Cdc42 was loaded with GTPγS. Assays were performed by adding 2 μM pyrene actin to a protein mixture of 22.4 nM Arp2/3 complex, 500 nM Cdc42 GTPγS, and 100 nM N-WASP. For 20 min the fluorescence change was measured at 386 nm with excitation at 366 nm using a luminescence spectrometer (LS50B, Perkin Elmer Life Sciences).

Actin tail length assay. Exponential phase bacteria were centrifuged at 1000 rpm for 15 min onto a semi-confluent monolayer of cells seeded on a 20 × 20 mm glass coverslip at an MOI of 100. Cells were incubated for 30 min at 37 °C with 5% CO_2. Cells were washed three times with PBS, and then incubated for 2 h in media containing 50 μg/ml gentamicin. Cells were washed three times with PBS, then fixed with 3.7% paraformaldehyde in PBS for 10 min and stored in 1% BSA in PBS. Following staining, images of 10 different cells per condition per experiment were taken and tail lengths were measured using ImageJ software (Open Source Software). Statistics were performed using the Mann–Whitney Test.

Immunofluorescence microscopy. Fixed cells were permeabilized with 0.2% Triton X-100 in PBS for 5 min and washed three times with 1% BSA in PBS. N-WASP was stained with mouse αFLAG antibody (SIGMA catalog #F1804) at 1:1000 for 1 h, followed by either FITC-conjugated or Cy3-conjugated donkey anti-mouse antibody at 1:200 for 1 h. F-actin was stained with rhodamine-labeled or FITC-conjugated phalloidin (1 μg/ml in 1% BSA in PBS; Molecular Probes) for 1 h. DNA was stained with DAPI (300 nM; Molecular Probes) for 10 min.

Intracellular motility assay. Exponential phase bacteria expressing GFP were centrifuged at 1000 rpm for 15 min onto a semi-confluent monolayer of cells seeded on a glass-bottom 35 mm dish at an MOI of 100. Cells were incubated for 30 min at 37 °C with 5% CO_2. Cells were washed three times with PBS, and incubated in media containing 50 μg/ml gentamicin. After 1 h, the cells were imaged on a microscope (IX-70; Olympus Optical) with an automated stage using a 100× objective. Images were taken in both the phase and GFP channels every 10 s for 15 min. Using Slidebook software (Intelligent Imaging Innovations, Denver, CO), the GFP channel was analyzed to track bacterial movement. Only bacteria moving in at least 10 consecutive frames were counted.

Shigella invasion. Exponential phase bacteria were centrifuged at 1000 rpm for 15 min onto a semi-confluent monolayer of cells seeded on a 20 × 20 mm glass coverslip at an MOI of 100. Cells were incubated for 60 min at 37 °C with 5% CO_2. Cells were washed three times with PBS, then fixed with 3.7% paraformaldehyde in PBS for 10 min and stored in 1% BSA in PBS. Cells were stained as described above, and counted microscopically (AX-70; Olympus Optical). Invasion rate is calculated as the number of cells containing internalized bacteria divided by the total number of cells counted multiplied by 100. At least one hundred cells from two different coverslips were counted for each experiment.

Results

Point mutations in N-WASP predicted to alter either structural conformation or phospho-regulation result in constitutive activation

A series of point mutations were generated that were predicted to alter either N-WASP structural conformation or phospho-regulation (Fig. 1A). A leucine to proline mutation in the homolog human WASP (L270P) leads to a change in the structure of WASP resulting in its open conformation and constitutive activation [9]. We predicted that a homologous mutation in N-WASP (L232P) would also lead to a constitutively open and active conformation given the conserved location in the α-helix (Fig. 1A). Similarly, we predicted that a L229P mutation in N-WASP, located ~1 turn away from L232 in the critical α-helix, would lead to a constitutively open and active conformation of N-WASP (Fig. 1A). Point mutations in N-WASP that alter the phospho-regulation of N-WASP and cytoskeletal processes have previously been described by Suetsugu et al. [5]. We generated both the phospho-mimicking tyrosine to glutamic acid (Y253E) and the phospho-disruptive tyrosine to phenylalanine (Y253F) mutations in N-WASP (Fig. 1A).

To assess whether the N-WASP L229P and L232P mutations alter baseline actin polymerization activity, we employed a pyrene actin assay. Wild type (WT) N-WASP when combined with Arp2/3 and pyrene actin does not lead to significant actin polymerization (Fig. 1B) [10]. However, when activated Cdc42 is added to N-WASP, Arp2/3 and pyrene actin, a significant increase in actin polymerization was observed (Fig. 1B). N-WASP L229P and L232P, in the absence of activated Cdc42, polymerizes actin at rates similar or greater than polymerization rates achieved with WT N-WASP in the presence of activated Cdc42 (Fig. 1B). Moreover, this constitutive activation of N-WASP L229P and N-WASP L232P can be further increased by the addition of activated Cdc42 (Fig. 1B). Similar results demonstrating constitutive activation of L229P and L232P were also obtained employing a modified bead assay (Supplementary Fig. 1) [11].

The pyrene actin assay was also employed to assess the activity of the N-WASP constructs containing phospho-regulatory mutations. Similar to results obtained with an *in vitro* bead assay using WASP Y291E [12], the homologous N-WASP Y253E exhibited an enhanced basal level of actin polymerization compared to WT N-WASP (Fig. 1C). This enhanced basal level of activation of N-WASP Y253E was also sensitive to Cdc42 stimulation (Fig. 1C). In contrast, the phospho-disruptive N-WASP Y253F does not exhibit an enhanced level of actin polymerization compared to WT N-WASP, but was sensitive to stimulation by activated Cdc42 (Fig. 1C).

N-WASP-deficient cell lines expressing N-WASP activation mutations have enhanced migration

To assess, *in vivo*, the role of each of the N-WASP structural conformation and phospho-regulation mutants on a processes that requires dynamic modulation of the actin cytoskeleton (e.g., cell motility), we generated stable cell lines where each mutant construct, or WT N-WASP, was expressed in N-WASP-deficient cells. The effect of each of the constructs on cell motility was determined employing a standard scratch assay. Cell lines expressing each of the structural conformation and phospho-regulation mutants closed wounds faster than the control cell line expressing WT N-WASP (Supplementary Fig. 2 and Fig. 1D).

Shigella-mediated actin tail formation does not absolutely require regulation of N-WASP structural conformation or phospho-regulation

To assess *Shigella*'s ability to utilize each of the mutant N-WASP constructs for actin-based motility, *Shigella* infections were performed in N-WASP-deficient cell lines that stably expressed each of the N-WASP mutants. Each cell line permitted bacterial invasion and supported *Shigella*-mediated actin tail formation, with N-WASP localizing appropriately to a bacterial pole (Fig. 2A).

We measured the length of actin tails in *Shigella*-infected cells expressing each of the various N-WASP mutants to determine the efficiency by which *Shigella* utilized the N-WASP constructs.

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