Role of the WASP family proteins for *Mycobacterium marinum* actin tail formation

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Mycobacterium marinum, a natural pathogen of fish and frogs and an occasional pathogen of humans, is capable of inducing actin tail formation within the cytoplasm of macrophages, leading to actin-based motility and intercellular spread. Actin tail formation by *M. marinum* is markedly reduced in macrophages deficient in the Wiskott–Aldrich syndrome protein (WASP), which still contain the closely related and ubiquitously expressed protein N-WASP (neuronal WASP). In fibroblasts lacking both WASP and N-WASP, *M. marinum* is incapable of efficient actin polymerization and of intercellular spread. By reconstituting these cells, we find that *M. marinum* is able to use either WASP or N-WASP to induce actin polymerization. Inhibition or genetic deletion of tyrosine phosphorylation, Nck, WASP-interacting protein, and Cdc42 does not affect *M. marinum* actin tail formation, excluding the participation of these molecules as upstream activators of N-WASP in the initiation of actin-based motility. In contrast, deletion of the phosphatidylinositol 4,5-bisphosphate-binding basic motif in N-WASP eliminates *M. marinum* actin tail formation. Together, these data demonstrate that *M. marinum* subversion of host actin polymerization is most similar to distantly related Gram-negative organisms but that its mechanism for activating WASP family proteins is unique.

mycobacteria | neuronal Wiskott–Aldrich syndrome protein | infection | bacterial motility

A highly regulated signal transduction cascade initiates Arp2/3 complex-mediated actin reorganization of the cytoskeleton through the members of the Wiskott–Aldrich syndrome protein (WASP) and WAVE families (reviewed in ref. 1). The ubiquitously expressed N-WASP (neuronal WASP) is a modular protein auto-inhibited by an intramolecular interaction in its inactive state. Binding of specific molecules including phosphatidylinositol 4,5-bisphosphate (PIP₂), Cdc42, Nck, and Grb2 to N-WASP disrupts this inhibition and unmasks the WASP homology 2 and acidic (WA) domain, allowing the Arp2/3 complex to initiate *de novo* actin polymerization (2–6). WASP, a protein found only in hematopoietic cells, is closely related to N-WASP, sharing 50% sequence similarity. The two proteins are similarly organized and are controlled by the same host cell inputs. Less related to N-WASP are the WAVE proteins that contain the WA domain output region but have different activating inputs (7).

Diverse pathogens have evolved mechanisms to hijack this Arp2/3-dependent pathway of actin polymerization for their own benefit (reviewed in ref. 8). For vaccinia virus, actin polymerization is required for viral egress from infected cells. Enterohemorrhagic and enteropathogenic *Escherichia coli* species' actin polymerization leads to pedestal formation, causing attachment and effacement lesions on gut epithelia. For *Listeria*, *Shigella*, *Burkholderia*, *Rickettsia*, and *Mycobacterium marinum*, actin polymerization on the surface of cytoplasmic bacteria results in actin tails, intracellular motility, and direct intercellular spread. Interestingly, these pathogens use independently evolved proteins to target different steps leading to Arp2/3 activation. *Listeria*, *Rickettsia*, and perhaps

Burkholderia mimic host cell N-WASP to activate the Arp2/3 complex directly, and actin tail formation by these species is independent of host cell N-WASP (9–14). The other pathogens depend on the WASP family for actin polymerization: *Shigella* and enterohemorrhagic *E. coli* (EHEC) have molecules that directly recruit host cell N-WASP (10, 15–18), whereas vaccinia and enteropathogenic *E. coli* (EPEC) initiate actin polymerization upstream of N-WASP in a manner similar to host cell receptor tyrosine kinases, involving host cell factors including Nck, Grb2, and WASP-interacting protein (WIP) (19–23). Understanding how pathogens exploit endogenous signal pathways yields insight into basic cell biology as well as mechanisms of microbial pathogenesis.

We recently identified *M. marinum* as the only mycobacterial species that clearly escapes from phagosomes and subsequently initiates actin tail formation (24). The Arp2/3 complex was found throughout the actin tail behind *M. marinum*, but WASP localized to the bacterial pole. Here, we determine the host cell requirements for efficient *M. marinum* actin polymerization and show the necessity for a member of the WASP family. Similar to *Shigella* and EHEC, *M. marinum* actin polymerization occurs independently of known N-WASP activators, including tyrosine phosphorylation, Nck, WIP, and Cdc42. However, unlike any other pathogen, work shown here suggests that the mechanism of *M. marinum* actin polymerization involves the lipid-binding basic motif of N-WASP (hereafter designated by “B”).

Materials and Methods

Host Cells. Macrophages were derived from the bone marrow of 129/Sv or WASP^{−/−} mice (25) and harvested after 7–21 days of culturing as described in ref. 24. Wild-type, N-WASP^{−/−} (10, 16), Nck^{−/−} (26), and WIP^{−/−} (27) embryonic fibroblast cell lines were maintained as previously described. All cells were seeded onto fibronectin-coated coverslips (Becton Dickinson) 1 day before infection.

Bacteria and Infection. Wild-type (strain M), GFP-expressing (24), or red fluorescent protein-expressing (28) *M. marinum* were cultured in Middlebrook 7H9 (Difco) supplemented with 0.2% glycerol/0.05% Tween 80/10% ADC enrichment (Fisher). Infection of macrophages for microscopy and intracellular growth was carried out as described in refs. 24 and 29. In brief, intracellular growth was assessed by infecting macrophages or fibroblasts with a multiplicity of infection (moi) of 3 and hypotonically lysing the cells 4 and 24 h

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Abbreviations: CRIB, Cdc42 and Rac interactive binding; EHEC, enterohemorrhagic *E. coli*; EPEC, enteropathogenic *E. coli*; GBD, GTPase-binding domain; PIP₂, phosphatidylinositol 4,5-bisphosphate; polyPro, polyproline; WA, WASP homology 2 and acidic; WASP, Wiskott–Aldrich syndrome protein; N-WASP, neuronal WASP; WH1, WASP homology 1 domain; WIP, WASP-interacting protein.

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postinfection for colony-forming unit quantitation. For fibroblasts, a moi of 60 was used, and infected cells were incubated for 48 h before microscopy. For quantification, intracellular bacteria were identified by phase contrast microscopy, and actin tails were visualized by fluorescence. Each experiment was done three times, with 50 cells counted per experiment. Unpaired *t* tests with Welch corrections were used to generate *P* values and determine statistical significance. Intercellular spreading assays were performed in confluent macrophage and fibroblast monolayers infected with fluorescent *M. marinum* as described in refs. 24 and 30. After infection, wells were overlaid with agar, and culture medium contained 40 $\mu\text{g}/\text{ml}$ amikacin. Media were changed every 2 days, and macrophage and fibroblast monolayers were examined for pattern of infection 5 or 8 days later, respectively. Quantification of fluorescent foci of infection was performed by using IPLAB analysis software (Scanalytics, Billerica, MA).

Expression Constructs and Transfection. The WAVE2 and many of the GFP-tagged murine N-WASP expression constructs have been described (16, 31, 32). GFP-tagged full-length and mini human WASP expression constructs were kindly provided by J. E. Dueber (University of California, San Francisco). N-WASP expression constructs (pEGFP-C1-N-WASP Y253F, pEGFP-C1 N-WASP polyProWA, pEGFP-C3 N-WASP WH1+WA, and pEGFP-C1 N-WASP $\Delta 226$ –267, pEGFP-C1 N-WASP $\Delta 200$ –226, and pEGFP-C1 N-WASP $\Delta 158$ –199) were generated by standard methods. All constructs were confirmed by sequencing, and expression of appropriate-size molecules was monitored by immunoblotting. All constructs that were scored here as negative for restoration of *M. marinum* actin tail formation are functional in other systems (unpublished data). Fibroblast cell lines were transfected 24 h after plating with FuGENE (Roche Diagnostics) according to the manufacturer's specifications. Transfected cells were infected 20 h after transfection.

Pharmacological Inhibitors. Infected macrophages and fibroblasts were treated with inhibitors 30 or 48 h postinfection, respectively. PP2 (4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine) (100 μM , Calbiochem) or genistein (250 μM) was added to infected cells for 1 h before fixation to study the effect of tyrosine kinase inhibition. Toxin B (1 $\mu\text{g}/\text{ml}$, Calbiochem) and cytochalasin D (1.3 μM , Sigma) were added to infected cells for 1.5 h before fixation.

Microscopy. Infected cells were fixed, permeabilized, and stained with Alexa Fluor phalloidin (Molecular Probes) as described in ref. 24. Indirect immunofluorescence was performed with anti-N-WASP (provided by J. Taunton, University of California, San Francisco), anti-WAVE2 (Santa Cruz Biotechnology), anti-Nck (Upstate Biotechnology, Lake Placid, NY, and BD Transduction Laboratories), anti-WIP (Santa Cruz Biotechnology), anti-phosphotyrosine (PY99, Santa Cruz Biotechnology; 4G10, Upstate Biotechnology), and species-appropriate Alexa Fluor secondary antibodies (Molecular Probes). Coverslips were mounted with Prolong antifade reagent (Molecular Probes). Images were acquired on a Nikon Eclipse TE300 microscope with an Evolution QEI cooled charge-coupled device (Media Cybernetics, Silver Spring, MD) with IPLAB acquisition software (Scanalytics).

Results

***M. marinum* Actin Tail Formation and Intercellular Spread in N-WASP^{-/-} Fibroblasts.** WASP localized to the pole of *M. marinum* from which the actin tail formed (Fig. 1A) (24). To determine the requirement for WASP in *M. marinum* actin tail formation, we infected WASP^{-/-} macrophages and found a significant, ≈ 2 -fold decrease in the number of infected cells in which actin tails were observed (Fig. 1A; WASP^{+/+}, $72.5 \pm 8.5\%$; WASP^{-/-}, $35.10 \pm 8.6\%$; *P* = 0.04), even though intracellular growth of *M. marinum*

was comparable in WASP^{+/+} and WASP^{-/-} macrophages by colony-forming unit quantification at 4 and 24 h postinfection (data not shown). Because the deficiency of WASP only partially ablated actin tail formation, we hypothesized that N-WASP might account for the residual actin tail formation. N-WASP was present in WASP^{-/-} macrophages at levels comparable to WASP^{+/+} macrophages (Fig. 1B). Indeed, we found that N-WASP localized to the pole of *M. marinum* with actin tails in WASP^{-/-} macrophages (Fig. 1A). *M. marinum* formed actin tails in N-WASP^{+/+} fibroblasts that lacked WASP (Fig. 1B), and N-WASP localized at the pole in these cells (Fig. 1C). Most significantly, there was an almost total decrease in actin tail formation in N-WASP^{-/-} fibroblasts that have neither WASP nor N-WASP (Fig. 1C), despite the fact that *M. marinum* grew similarly in N-WASP^{+/+} and N-WASP^{-/-} fibroblasts (data not shown). Less than 1% of N-WASP^{-/-} fibroblasts exhibited bacterial actin tails; this small residual bacterial motility was seen in two independently derived N-WASP^{-/-} fibroblast cell lines (data not shown).

To determine whether actin tail formation is required for intercellular spread of *M. marinum*, we examined spreading of fluorescent *M. marinum* within a confluent layer of macrophages or fibroblasts. Fluorescent foci of infection (FFI) in WASP^{+/+} and WASP^{-/-} macrophages were comparable in size (WASP^{+/+}, $78,726 \pm 11,794 \mu\text{m}^2$; WASP^{-/-}, $94,947 \pm 17,853 \mu\text{m}^2$; *P* = 0.45) and consisted of many cells, representing contiguously infected cells arising from a single originally infected macrophage. This finding suggests that the residual ability of *M. marinum* to form actin tails in WASP^{-/-} macrophages is sufficient for intercellular spread in macrophages. In contrast, FFI in the N-WASP^{+/+} monolayer were significantly larger than in the N-WASP^{-/-} monolayer (Fig. 1D; N-WASP^{+/+}, $17,092 \pm 1,314 \mu\text{m}^2$; N-WASP^{-/-}, $1,682 \pm 498 \mu\text{m}^2$; *P* = 0.002). FFI in the N-WASP^{-/-} monolayer most often involved only a single cell or a few cells.

Transient expression of either N-WASP or WASP restored actin tail formation in *M. marinum*-infected N-WASP^{-/-} fibroblasts (Fig. 2). In agreement with the immunofluorescence staining of WASP and N-WASP (Fig. 1), the GFP fusion N-WASP and WASP proteins localized to the pole from which the actin tail formed. However, transient expression of GFP-WAVE2 failed to restore actin tail formation by *M. marinum* in these cells (Fig. 2), and endogenous WAVE2 failed to localize to bacteria assessed by immunofluorescence (data not shown), demonstrating a specificity of *M. marinum* for WASP and N-WASP among the WASP/WAVE family.

Role of the Arp2/3 Complex, Tyrosine Phosphorylation, Nck, and WIP.

To determine how *M. marinum* recruits WASP and N-WASP, we embarked on a systematic examination of molecules known to interact with the WASP family and analysis of the N-WASP domains required for *M. marinum* actin polymerization. We verified the necessity of the WA output region of N-WASP that contains the verprolin homology, cofilin homology, and acidic domains by transfecting the N-WASP^{-/-} fibroblasts with an N-WASP construct lacking the WA domain (Fig. 3). Although N-WASP ΔWA associated with the surface of intracellular *M. marinum* (Fig. 5, which is published as supporting information on the PNAS web site), this construct did not restore actin tail formation (Fig. 3). The WA domain alone of N-WASP did not restore actin tails (Fig. 3). Together, these data demonstrate that the input region of N-WASP is critical for recruitment to *M. marinum* and that actin and the Arp2/3 complex binding to the WA domain of N-WASP are required for *M. marinum* actin tail formation.

To determine whether tyrosine phosphorylation has a role in *M. marinum* actin polymerization as it does in vaccinia and EPEC (19), we first examined phosphotyrosine staining in *M. marinum*-infected macrophages and fibroblasts. Anti-phosphotyrosine antibodies showed marked staining of adhesion sites and other cellular structures (Fig. 6, which is published as supporting information on the

Fig. 4. Nck and WIP are not involved in *M. marinum* actin tail formation. (A) Nck^{+/+} (Left) and Nck^{-/-} (Center) fibroblasts were infected with *M. marinum* (red) and stained with phalloidin (blue) and anti-Nck antibodies (green). In Right, the percentage of infected cells with actin tails for Nck^{+/+} and Nck^{-/-} fibroblasts is shown. (B) WIP^{+/+} (Left) and WIP^{-/-} (Center) fibroblasts were infected with *M. marinum* (red) and stained with phalloidin (blue) and anti-WIP antibodies (green). In Right, the percentage of infected cells with actin tails is shown. All quantification is the summary of three independent experiments. (Scale bars, 5 μ m.)

and mini WASP were sufficient to restore actin tail formation in N-WASP^{-/-} fibroblasts, albeit not as efficiently as full-length constructs (Fig. 3). The autoinhibited conformation of the WASP family requires interaction between the carboxyl terminus of the GBD, including amino acids 226–274 of N-WASP, and the WA domain (3, 4). EHEC EspFu binds to this region of N-WASP to initiate pedestal formation (18, 30). However, an N-WASP Δ 226–267 construct was fully capable of restoring actin tails in N-WASP^{-/-} fibroblasts, demonstrating that *M. marinum* does not target this region of the molecule. In contrast, two constructs lacking the N-terminal portion of the B-GBD, including the basic and Cdc42 and Rac interactive-binding (CRIB) motifs (Δ B-CRIB and Δ WH1-CRIB; Fig. 3), were unable to restore actin tail formation in the N-WASP^{-/-} fibroblasts (Fig. 3), focusing our attention on the area to which the host cell factors PIP₂ and Cdc42 bind.

Direct binding of GTP-loaded Cdc42 to the CRIB motif is a major mechanism by which the WASP family proteins are activated (2). To determine whether Cdc42 has a role in *M. marinum* actin polymerization, we transfected N-WASP^{-/-} fibroblasts with an N-WASP H208D construct that abolishes GTPase binding and with an N-WASP Δ 200–226 construct that lacks the CRIB motif. Both of these constructs restored actin tail formation by *M. marinum* (Fig. 3), demonstrating that the bacterium recruits and activates N-WASP independent of GTPases. Furthermore, we treated *M. marinum*-infected macrophages with *Clostridium difficile* toxin B, an inhibitor of the entire Rho family of small GTPases (Fig. 7, which is published as supporting information on the PNAS web site). Although toxin B treatment led to dramatic differences in overall actin distribution, *M. marinum* still formed actin tails at levels similar to untreated cells (Fig. 6; fraction of infected cells with tails: not treated, $84.5 \pm 1.7\%$; toxin B, $81.5 \pm 0.1\%$; $P > 0.27$; fraction of intracellular bacteria with tails: not treated, $23.1 \pm 3.2\%$; toxin B, $24.4 \pm 3.0\%$; $P > 0.85$). In contrast, when cytochalasin D was used to inhibit actin polymerization, the percentage of infected cells with actin tails significantly decreased, and the tails that were present were smaller (fraction of infected cells with tails compared with those that were untreated: cytochalasin D, $24 \pm 2\%$; $P = 0.0005$). Thus, WASP family binding of Cdc42 or a related GTPase is not required for *M. marinum* actin polymerization.

The host anionic lipid PIP₂ is capable of activating N-WASP or WASP by binding to the basic motif, and its main role in this pathway is to synergize with Cdc42 for highly efficient activation of N-WASP (3, 36, 37). Transfection with an N-WASP Δ 158–199 construct that lacks the basic motif was unable to be recruited to the bacterial surface (Fig. 5A) or restore actin tail formation in

N-WASP^{-/-} fibroblasts (Fig. 3), suggesting a role for this region in *M. marinum* recruitment of N-WASP and subsequent actin polymerization. This construct is functional; it does support actin tail formation by *Shigella* and actin pedestal formation by EHEC (Fig. 5B). Thus, in contrast to other pathogens that activate the WASP family, *M. marinum* actin tail formation requires the basic motif of N-WASP.

Discussion

A diverse group of intracellular pathogens, including *M. marinum*, enables efficient intercellular spread by activating actin polymerization on their surfaces within host cytoplasm. This motility is used for direct intercellular spread, bypassing host defense mechanisms directed against extracellular pathogens. Pathogens have evolved various methods for subverting the host cytoskeleton to generate Arp2/3-dependent actin polymerization. Unlike *Listeria*, which directly recruits the Arp2/3 complex (schematically represented in Fig. 8, which is published as supporting information on the PNAS web site), we show here that efficient *M. marinum* actin polymerization and intercellular spread depends on the WASP family. Unlike *Shigella*, which exploits only N-WASP, *M. marinum* can use either family member, depending on availability in accordance with cell type (Figs. 1 and 2). N-WASP-dependent tail formation by *M. marinum* depends on both the input and output regions, suggesting that this species, like the Gram-negative bacteria, has developed a mechanism of activating N-WASP that depends on the input region in the N-terminal part of the protein, leading to unfolding of the protein and unmasking of the WA output region required for Arp2/3-dependent actin polymerization. The observation that bacterial motility was not completely eliminated by the absence of WASP family proteins suggests the existence of a second, albeit inefficient, mode of actin polymerization by *M. marinum*.

Because its role was so predominant, we sought to elucidate the mechanism by which *M. marinum* activates WASP family proteins by using a variety of genetic, biochemical, and pharmacological reagents. *M. marinum* activation of N-WASP clearly differs from vaccinia and EPEC, which require tyrosine phosphorylation of bacterial molecules A36R and Tir, respectively, to initiate a Nck-dependent cascade for activation of N-WASP (Fig. 8) (19, 23). For both vaccinia and EPEC, the WIP-binding WH1 domain is involved in actin polymerization (16, 20), and WIP is thought to be downstream of Nck in the case of vaccinia but not in EPEC (20, 32). For *M. marinum*, tyrosine phosphorylation, Nck, and WIP are dispensable for actin tail formation (Figs. 4, 5, and 8). Furthermore, the

domains of N-WASP with which they interact, the WH1 and polyPro domains, respectively, can be removed without blocking *M. marinum* actin polymerization. However, those constructs are less efficient than full-length N-WASP (Fig. 3). It is possible that although not required for N-WASP-dependent tail formation, proteins that interact with the WH1 and polyPro domains contribute to amplification of N-WASP and actin recruitment. Indeed, WIP localizes to the pole behind which *M. marinum* tails form (Fig. 4). This observation is very similar to what has been shown for *Shigella* (20), where WIP binding to N-WASP is believed to be downstream of direct bacterial activation of N-WASP.

As illustrated in Fig. 8, *M. marinum* activation of N-WASP is more similar to the mechanisms described for *Shigella* and EHEC, which also are independent of tyrosine phosphorylation and bypass Nck or Cdc42 binding (Figs. 4 and 6). Both EHEC and *Shigella* express proteins that directly bind to N-WASP (18, 38). *Shigella* IcsA most likely binds to N-WASP at several sites in the input region, including the WH1 and GBD domains (38), whereas EHEC EspFu (16) directly disrupts the autoinhibitory interaction between the GBD and WA domain proteins by binding to the GBD (18, 32). None of the domains required for IcsA or EspFu initiation of actin polymerization is used by *M. marinum*. Thus, although superficially similar, the three microbes have evolved independent mechanisms for activating N-WASP-dependent actin polymerization.

One way in which the *M. marinum* mechanism differs from that of all other bacteria is that the basic motif of N-WASP is required for efficient N-WASP recruitment and actin polymerization by *M. marinum*. This region is known to bind to eukaryotic polyphosphoinositides through an ionic interaction mediated by its high concentration of lysines (3, 37). Unlike other prokaryotes that activate actin polymerization, mycobacterial cell walls are rich in phosphoinositides. One of these, the multiglycosylated lipoarabinomannan, is a potential virulence factor and modulates immune responses (reviewed in refs. 39 and 40). In addition, its precursor, phosphatidylinositol mannoside, also has been found to be associated with the bacterial surface (41). It is intriguing to speculate that mycobacterial phospholipids bind to the basic motif region of N-WASP. We cannot eliminate the possibility that *M. marinum* may be able to modulate levels of PIP₂ on the bacterial surface; however,

we have not found any association of the PKC δ pleckstrin homology domain, a PIP₂ reporter, with *M. marinum* (data not shown). PIP₂ is not an efficient activator of N-WASP on its own, and it is believed that its major role in host cell actin polymerization is in synergy with Cdc42 or other activators. However, many intracellular pathogens, including *Mycobacterium tuberculosis*, are known to control levels of host phosphoinositides for their own purposes (reviewed in ref. 42).

In conclusion, *M. marinum* hijacks eukaryotic signaling for Arp2/3 complex-dependent actin polymerization through activation of the WASP family independent of upstream signaling molecules, resulting in direct intercellular spread. This pathway is similar in principle to the mechanisms evolved by *Shigella* and EHEC and is another amazing example of convergent evolution by pathogens to exploit a host cell biological pathway for efficient infection. However, data presented here suggest that the *M. marinum* mechanism is distinct from those thus far described for other pathogens by requiring the basic motif of N-WASP for its activation. It is unknown whether actin-based motility is a characteristic unique to *M. marinum* among all mycobacteria, although we have not been able to demonstrate this phenomenon with either *M. tuberculosis* or *Mycobacterium ulcerans* (43). Further studies of actin polymerization by *M. marinum* are needed to explore its role in pathogenesis of infection and broaden understanding of actin cytoskeletal dynamics.

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