

Arp2/3-Mediated Actin-Based Motility: A Tail of Pathogen Abuse

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Intracellular pathogens have developed elaborate mechanisms to exploit the different cellular systems of their unwilling hosts to facilitate their entry, replication, and survival. In particular, a diverse range of bacteria and viruses have evolved unique strategies to harness the power of Arp2/3-mediated actin polymerization to enhance their cell-to-cell spread. In this review, we discuss how studying these pathogens has revolutionized our molecular understanding of Arp2/3-dependent actin assembly and revealed key signaling pathways regulating actin assembly in cells. Future analyses of microbe-host interactions are likely to continue uncovering new mechanisms regulating actin assembly and dynamics, as well as unexpected cellular functions for actin. Further, studies with known and newly emerging pathogens will also undoubtedly continue to enhance our understanding of the role of the actin cytoskeleton during pathogenesis and potentially highlight future therapeutic approaches.

Introduction

The host cell actin cytoskeleton is a key target of microbial pathogens. Bacterial pathogens frequently inhibit cellular processes by disabling the cytoskeleton using secreted toxins that target actin or its regulators (Aktories, 2011; Aktories et al., 2011). Alternatively, many bacterial pathogens and most viruses use actin assembly to promote their invasion or uptake, enabling cellular colonization or replication (Carabeo, 2011; Taylor et al., 2011). Many pathogens have also evolved a capacity to hijack the force-generating capacity of actin polymerization to power intracellular or surface-associated motility. The frequent occurrence of pathogen exploitation of host cell actin has led to the proposal that perturbing actin may be a hallmark of infection or “pattern of pathogenesis” (Vance et al., 2009).

Pathogens such as *Listeria monocytogenes* and *Shigella flexneri* use actin while they are within the cytosol to induce motility and promote collision of the pathogen with the plasma membrane, formation of a membranous protrusion containing the pathogen, and engulfment of the protrusion by adjoining cells, enabling cell-cell spread (Figure 1A). Pathogens such as vaccinia induce actin polymerization when the pathogen is bound to the outside of the cell in order to induce their motility and promote spread to adjacent cells (Figure 1B). Hijacking actin from inside or outside the cell represents topologically and biochemically distinct challenges that involve interfacing with components of signaling pathways that regulate actin assembly. Because pathogens exploit distinct layers of the actin regulatory machinery of their hosts, studies examining the mechanisms by which pathogens impact actin have illuminated key pathways of actin regulation.

Mobilizing the host actin cytoskeleton requires that pathogens deploy proteins that interact with actin or mimic regulators that mediate or control its polymerization. Actin assembly is regulated by a variety of signaling molecules, including tyrosine kinases, adaptor proteins such as Nck and Grb2, and Rho family GTPases including Rho, Rac, and Cdc42. These factors act

upstream of three major classes of proteins that nucleate actin filaments (F-actin) from actin monomers (G-actin) (Figure 2). One class, the formins, nucleate actin and processively associate with the fast-growing barbed end of the filament (Chesarone et al., 2010). A second class, the tandem-monomer-binding family, nucleate actin but do not associate with growing filament ends (Qualmann and Kessels, 2009). Although both of these classes can be exploited by pathogens, in this review we will focus on the third class, the Arp2/3 complex, and its activators, the nucleation promoting factors (NPFs) (Rotty et al., 2013), as this is the primary class used by pathogens to promote actin-based motility during infection. The Arp2/3 complex is a weak actin nucleator, but when activated by NPFs it binds to an actin filament and robustly nucleates a new filament that emerges to form a Y-branch (Campellone and Welch, 2010; Rotty et al., 2013). Mammalian class I NPFs include the WASP/N-WASP, WAVE/Scar, WHAMM, WASH, and JMY proteins, each of which exhibits a specific intracellular localization and activates actin assembly during distinct cellular processes including lamellipodia protrusion (WAVE/Scar), endocytosis and endosome remodeling (WASP/N-WASP, WASH), and anterograde transport (WHAMM) (Campellone and Welch, 2010; Rotty et al., 2013). Once nucleated, actin filament elongation provides the driving force for cellular movements.

Many unrelated bacterial and viral pathogens mobilize the Arp2/3 complex to nucleate actin by mimicking or exploiting molecules ranging from tyrosine kinase substrates to NPFs, proving the flexibility of evolutionary strategies to hijack the cytoskeleton (Haglund and Welch, 2011) (Figure 3). As it is unfortunately impossible to cover every pathogen that takes advantage of the Arp2/3 complex in the space available, we have focused our attention on *Listeria monocytogenes*, *Shigella flexneri*, vaccinia virus, and baculoviruses, each of which has developed the capacity to use Arp2/3-mediated actin polymerization to enhance their spread (Figure 3). We include early studies for historical perspective and contemporary studies for current

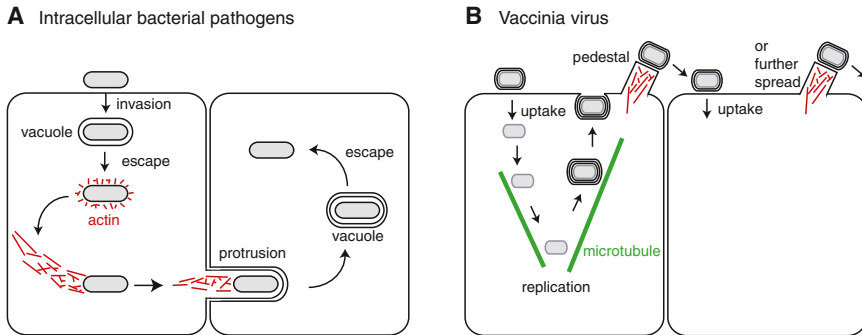


Figure 1. Schematic of Arp2/3-Induced Actin Polymerization in Pathogen Spread from Within or on the Surface of a Cell

(A) Following invasion and escape from the vacuole, actin-based bacterial motility promotes collision with the plasma membrane, protrusion formation, and protrusion engulfment by an adjacent cell.

(B) After being transported to the cell periphery on microtubules, vaccinia fuses with the plasma membrane and induces an outside-in signaling cascade to stimulate actin polymerization, which propels the virus onto neighboring cells. If the adjacent cell is already infected, the virus does not enter but again induces actin polymerization, which propels the virion across the cell surface enhancing its chances of reaching a noninfected cell.

information and future directions. Developments in the field over the past 25 years have highlighted that studying how pathogens exploit actin has enhanced our understanding of pathogenesis and revolutionized our knowledge of the host pathways that regulate actin assembly in uninfected cells.

The Discovery of Bacterial Actin-Based Motility

Intracellular bacterial movement was first observed in the 1950s by time-lapse microscopy of cells infected with *Rickettsia rickettsii* (Schaechter et al., 1957). Motility resulted in the interaction of bacteria with the host cell plasma membrane, the formation of bacteria-containing protrusions, and the release of bacteria from the cell. A similar phenomenon was subsequently described for *Shigella* in the 1960s, suggesting a role for bacterial movement in cell-to-cell spread (Ogawa et al., 1968). The direct association of protrusions with cell-to-cell spread was confirmed using ultrastructural analysis of epithelial cells infected with *Listeria*. Bacteria-containing membrane protrusions were seen to extend into invaginations in adjoining cells, where they became internalized (Rácz et al., 1970, 1972). Nevertheless, the molecular basis of intracellular motility and its functional connection with spread remained mysterious for nearly two decades.

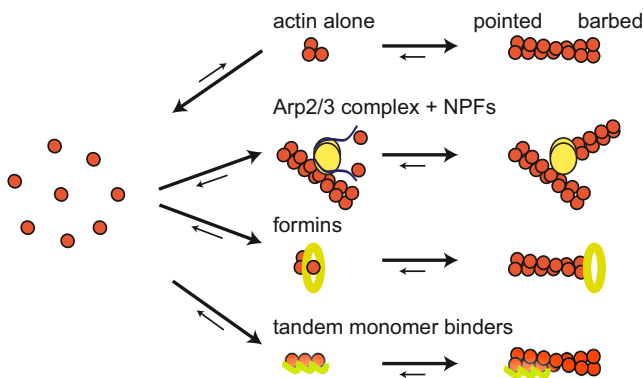


Figure 2. The Three Different Types of Actin Nucleation Mechanisms Spontaneous actin nucleation is the rate-limiting step in actin filament assembly. There are three major classes of host actin-nucleating factors that accelerate nucleation. The Arp2/3 complex is activated by NPFs, and nucleates a new filament from the side of an existing filament, linking the two filaments into a Y-branch. Formins nucleate a filament and then remain processively associated with the fast-growing barbed end as it elongates. Tandem-monomer-binding proteins nucleate a filament and can remain attached to the slow-growing pointed end.

Insights into the mechanism of motility came in the late 1980s from seminal studies on *Listeria*, *Shigella*, and *Rickettsia rickettsii*, which documented the association of cytoplasmic bacteria with the host actin cytoskeleton (Bernardini et al., 1989; Heinzen et al., 1993; Mounier et al., 1990; Tilney and Portnoy, 1989) (Figure 3). The first comprehensive study focused on *Listeria*, which was shown to associate with actin in stages. The bacteria were initially surrounded by an actin cloud, and then trailed by an actin comet tail (Tilney and Portnoy, 1989). Bacteria with actin tails often extended into protrusions of the host cell plasma membrane, with the bacterium at the tip. Some protrusions were internalized by neighboring cells, resulting in a double-membrane intracellular vacuole from which the bacterium eventually escaped. Subsequent studies confirmed this same pathway for *Shigella* and *Rickettsia conorii* (Gouin et al., 1999). In support of a function for actin in pathogen spread, treatment of infected cells with cytochalasin D, an inhibitor of actin assembly, prevented protrusion formation and spread of *Listeria* and *Shigella* (Bernardini et al., 1989; Tilney and Portnoy, 1989), as well as release of *Rickettsia rickettsii* from host cells (Heinzen et al., 1993). Together, these data supported a model in which the actin cytoskeleton promotes intracellular bacterial movement, protrusion formation, and penetration into neighboring cells.

Time-lapse imaging confirmed this model and revealed for the first time the kinetics of bacterial movement and spread. *Listeria*, *Shigella*, and *Rickettsia rickettsii* were shown to move at rates ranging from 2 to 60 $\mu\text{m}/\text{min}$, with variations between individual bacteria in a single cell and between bacteria in different cell types (Dabiri et al., 1990; Goldberg and Theriot, 1995; Sanger et al., 1992). The relationship between movement and spread was later directly observed for *Listeria* and *Shigella*. Moving bacteria collide with the plasma membrane and either ricochet back into the cytosol or enter into protrusions, with relative frequencies that depend on the strain and age of the cell monolayer (Monack and Theriot, 2001; Robbins et al., 1999). Protrusions that extend into the neighboring cell can be internalized, resolved into a vacuole, and then disrupted as bacteria escape into the cytosol. It is likely that this pathway of cell-to-cell spread is common to most intracellular bacterial pathogens that undergo actin-based motility. An exception are *Burkholderia* species, which have the intriguing ability to induce host cell-cell fusion to enable direct access between cells, bypassing reliance on actin-based protrusion formation and uptake (Stevens and Galayov, 2004).

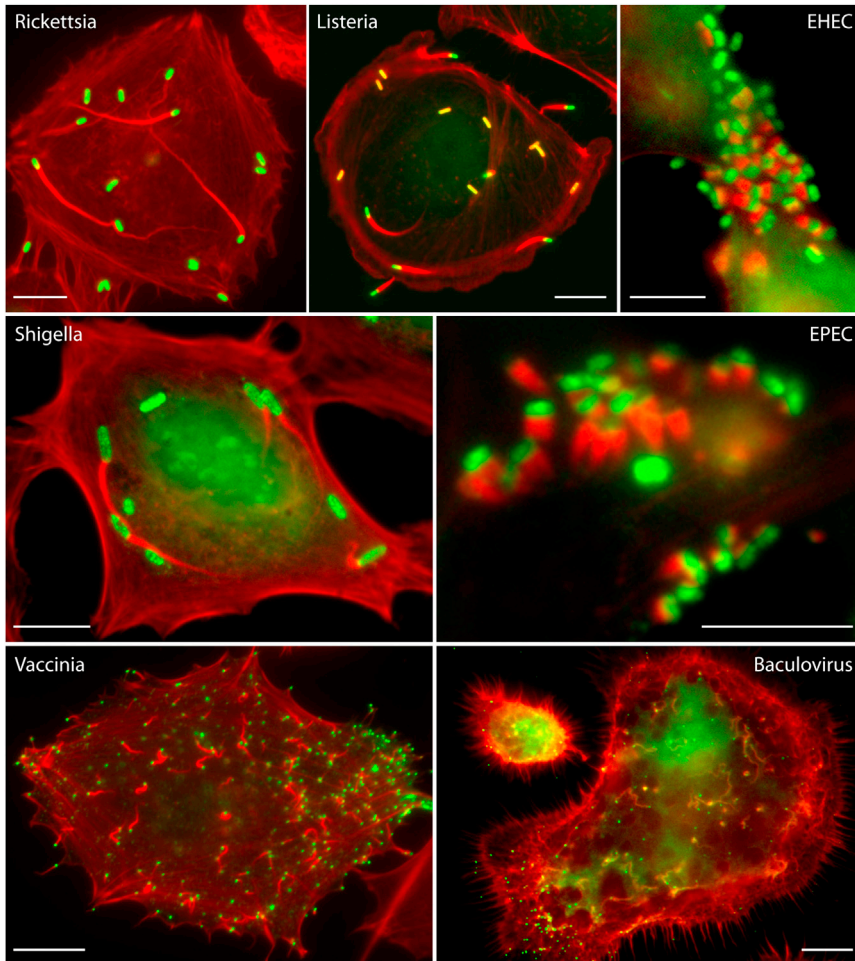


Figure 3. Immunofluorescence Images of Actin Tails or EPEC and EHEC Pedestals Polymerized by the Indicated Pathogen
F-actin, red; pathogens, green. All scale bars = 10 μ m.

bacterial proteins that regulate actin dynamics in the context of pathogen motility, providing insight into the underlying mechanisms of infectivity.

Bacterial Proteins Important for Actin Assembly

The identification of bacterial proteins required for actin assembly was first accomplished for *Listeria* and *Shigella*, where genes encoding these proteins were identified in screens for transposon mutants deficient in cell-to-cell spread and plaque formation. The discovery of these proteins initiated a revolution in our understanding of the molecular mechanisms of actin assembly by pathogens and by the host cell in the absence of infection.

IcsA of *Shigella* (also called VirG), which is encoded by the *icsA* locus on the virulence plasmid pWR100, was the first protein identified (Bernardini et al., 1989; Lett et al., 1989; Makino et al., 1986). IcsA is a member of the autotransporter (AT) family (or Type Va secretion system) and features an N-terminal signal sequence, central passenger domain,

The organization and dynamics of actin enable the role of actin polymerization in driving pathogen motility. Actin filaments are orientated with their fast-growing barbed ends facing the bacterium surface (Gouin et al., 1999; Tilney et al., 1992a, 1992b), and actin assembly at the surface is coupled to bacterial movement, resulting in the formation of the characteristic comet tails (Sanger et al., 1992; Theriot et al., 1992). Actin filaments in *Listeria* actin tails are organized into a dendritic network of Y-branches (Cameron et al., 2001), similar to the organization of actin in cellular lamellipodia (Svitkina and Borisy, 1999). Filaments in the comet tail remain fixed in place and are depolymerized with a half-life of 30 s for *Listeria* actin tails (Theriot et al., 1992) or 100 s for *Rickettsia rickettsii* tails (Heinzen et al., 1993), remarkably similar to actin dynamics in motile eukaryotic cells (Theriot and Mitchison, 1991; Theriot et al., 1992). Actin depolymerization is also crucial for bacterial movement as it replenishes the G-actin pool to fuel further actin assembly (Carlier et al., 1997; Rosenblatt et al., 1997). Based on the similarities in actin organization and dynamics in bacterial comet tails and cellular structures including lamellipodia, *Listeria* and *Shigella* motility have been used as a model to study the molecular mechanisms that control actin dynamics in cells. The use of such model systems has also led to the identification of specific

and C-terminal translocation domain that mediates insertion into the outer membrane of the Gram-negative bacterium (Figure 4). Although passenger domain sequences are essential for actin assembly (Suzuki et al., 1998), other than a series of glycine-rich repeats, the passenger domain shows minimal sequence similarity with other proteins known to be involved in cellular actin dynamics. For this reason, although early studies suggested that IcsA could be involved in pathogen motility and bacterial actin assembly, the molecular mechanism of IcsA function remained unclear for years.

Listeria ActA was identified later than IcsA and is encoded by *actA* on the bacterial chromosome (Kocks et al., 1992). ActA has an N-terminal signal sequence, a surface exposed region, and a single C-terminal transmembrane domain that inserts into the cytoplasmic membrane of the Gram-positive bacterium. The surface exposed region consists of sequence motifs including acidic (A), central (C), and actin-binding (AB) motifs that are crucial for actin assembly and exhibit some similarity to sequences in Wiskott-Aldrich Syndrome family proteins (Gouin et al., 2005) (Figure 4). Moreover, ActA contains 3–4 proline-rich repeat motifs that are not essential for actin assembly but are important for enhancing its efficiency (Lasa et al., 1995; Niebuhr et al., 1997; Skoble et al., 2000; Smith et al., 1996). As with IcsA, a lack of extensive sequence similarity with other

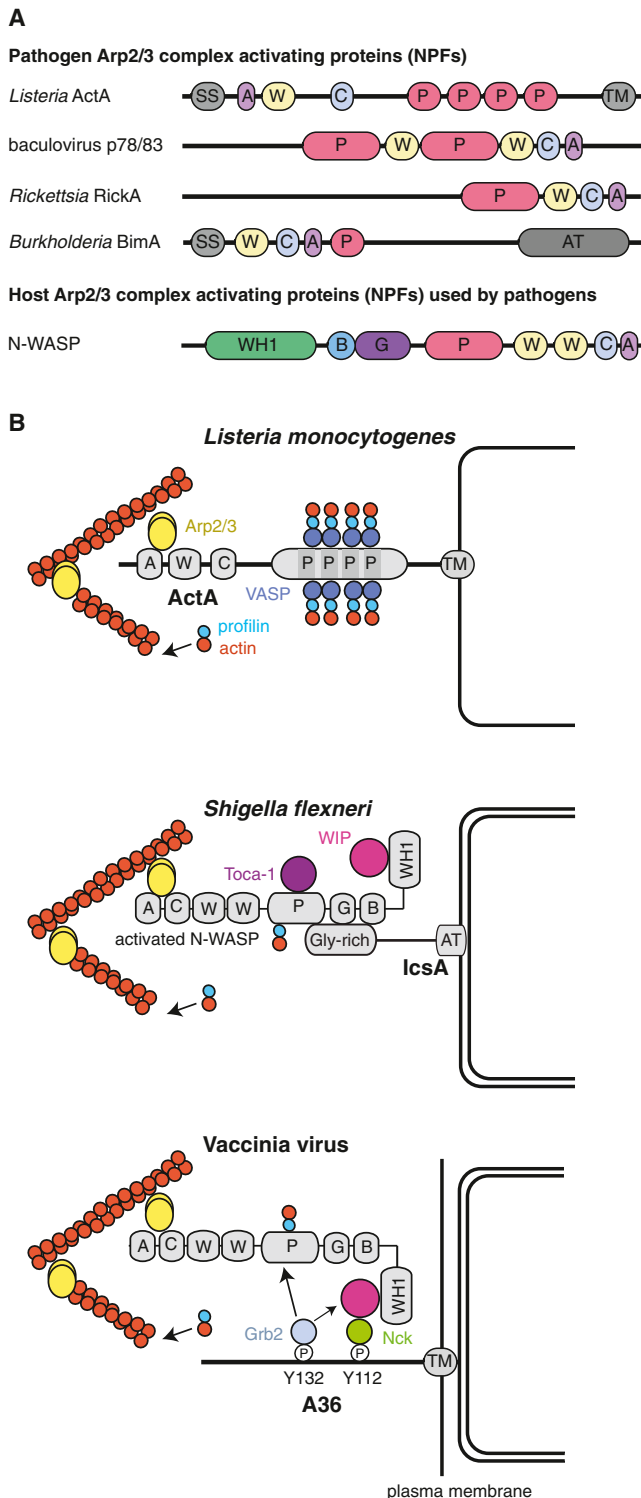


Figure 4. NPFs and Their Role in Pathogen Actin Assembly
(A) Schematic representation of the domains and motifs in pathogen NPFs and N-WASP, which is shown in its open conformation. SS = signal sequence; A = acidic; W = WASP homology 2 (WH2); C = central; p = proline rich; TM = transmembrane; AT = autotransporter; WH1 = WASP homology 1; B = basic; G = GTPase-binding.
(B) Schematic representation of the components that *Listeria*, *Shigella*, and vaccinia use to stimulate Arp2/3-mediated actin polymerization. ActA binds

proteins slowed progress in identifying its molecular mechanism of action.

Both IcsA and ActA are sufficient for actin assembly and motility, as expression of these proteins on the surface of bacteria that are unable to polymerize actin, for example *E. coli* for IcsA (Goldberg and Theriot, 1995) or *Listeria innocua* or *Streptococcus pneumoniae* for ActA (Kocks et al., 1995; Smith et al., 1995), enables motility. ActA is also sufficient to direct motility in the absence of other bacterial proteins (Cameron et al., 1999). Demonstrating this relied on techniques to reconstitute motility in cell cytoplasmic extracts, which was an approach used by cell biologists to identify key proteins in the actin dynamics of cellular motility (Theriot et al., 1994). Subsequently, within cytoplasmic extracts, ActA coated on the surface of plastic beads was sufficient to support their motility (Cameron et al., 1999). Thus, motility only requires display of ActA or IcsA on the bacterial surface, and the bacteria are otherwise passive participants, expending little of their own energy for movement and spread.

Although IcsA and ActA were the first pathogen actin assembly proteins to be identified, later studies revealed many such proteins that are expressed by numerous bacterial pathogens, including NPFs from *Rickettsia* and *Burkholderia* (Figure 4), and other actin polymerizing proteins from *Rickettsia*, *Chlamydia*, *Mycobacterium*, and *Vibrio* species. These proteins promote actin assembly by mimicking or hijacking representatives of all three major classes of actin assembly proteins in host cells (Haglund and Welch, 2011) (Figure 2).

***Listeria monocytogenes* and the Rise of the Host Arp2/3 Complex**

Bacteria expressing ActA or IcsA polymerize actin in cell extracts (Goldberg and Theriot, 1995; Marchand et al., 1995). However, ActA and IcsA themselves were unable to directly induce actin filament assembly, suggesting additional host proteins are required for actin nucleation (Loisel et al., 1999; Welch et al., 1997). Identification of the host actin assembly factor was made possible by the reconstitution of motility in cytoplasmic extracts, which could be fractionated and assayed for an activity that promotes actin assembly on the *Listeria* surface (Theriot et al., 1994; Welch et al., 1997). A host factor sufficient for actin assembly was purified and identified as the Arp2/3 complex. Arp2/3 had previously been identified in amoebae and was proposed to function in actin nucleation (Machesky et al., 1994), although no activity was detected in in vitro actin assembly assays (Kelleher et al., 1995). The Arp2/3 complex has since been shown to be necessary for *Listeria* actin assembly (Loisel et al., 1999; May et al., 1999; Yazar et al., 1999). Notably, although Arp2/3 can promote the assembly of actin by bacteria,

and activates the Arp2/3 complex while recruitment of VASP;profilin:actin complexes by its proline-rich repeats enhances actin filament elongation and *Listeria* actin-based motility. *Shigella* and vaccinia use different strategies to recruit host N-WASP to stimulate Arp2/3-induced actin polymerization. In the case of *Shigella*, the glycine-rich repeats of IcsA interact directly with the N-WASP:WIP complex, which also requires Toca-1 for N-WASP activation and actin polymerization. In contrast, vaccinia recruits N-WASP via a signaling network involving Nck and WIP, downstream of Src- and Abl-mediated phosphorylation of tyrosine 112 of the integral viral membrane protein A36. Grb2 recruitment is not essential, but its interaction with the proline-rich regions of WIP and N-WASP enhances actin tail formation.

it is not sufficient to enable motility, indicating that additional host components are required for full reconstitution of motility (Welch et al., 1997). Moreover, actin polymerization by Arp2/3 complex at the *Listeria* surface requires ActA (Welch et al., 1997), consistent with the essential nature of ActA in actin assembly during infection.

The fact that actin assembly by *Listeria* requires both ActA and Arp2/3 complex suggested that these factors act together to nucleate actin assembly. Subsequent experiments using purified ActA and Arp2/3 complex demonstrated that, although neither factor alone was sufficient, together the proteins formed an efficient nucleator (Welch et al., 1998). Based on the subunit composition of Arp2/3 complex and the presence of actin related proteins Arp2 and Arp3, it was proposed that ActA is an activator or NPF for Arp2/3. Subsequent work showed that ActA was indeed the first identified member of a broad class of NPF proteins, which are characterized by the presence of actin-binding WH2 domains (W), along with Arp2/3-binding C and A motifs (collectively called WCA) (Campellone and Welch, 2010). The WCA domain is the minimal region of NPF proteins that stimulates Arp2/3-dependent actin nucleation. The NPF family also includes other pathogen proteins such as baculovirus p78/83 (see later), *Rickettsia* spp. RickA (Gouin et al., 2004; Jeng et al., 2004) and *Burkholderia thailandensis* BimA (Sitthidit et al., 2010) (Figure 4). Thus, expressing proteins that mimic NPFs is a conserved mechanism of pathogenesis, and studying how pathogens deploy their NPFs will shed light on both pathogenic strategies as well as the function and regulation of actin assembly in uninfected cells.

It is noteworthy that *Listeria* actin-based motility appears to occur largely independently of regulation by host signaling pathways (tyrosine kinases and GTPases) that control actin assembly (Ebel et al., 1999; Marchand et al., 1995). However, the serine-threonine kinase CK2 phosphorylates ActA, enhancing Arp2/3 binding and *Listeria* motility, similar to the function for CK2 in phosphorylating host NPFs WASP and WAVE (Chong et al., 2009). Notably, even without the activity of CK2, bacterially expressed and purified ActA is active (Skoble et al., 2000; Welch et al., 1998). Thus, *Listeria* has evolved the ability to bypass the requirement for many host cell actin regulatory pathways, which differs from the behavior of other pathogens, including *Shigella* and vaccinia virus.

Shigella and the Discovery of Host NPFs

The discovery that ActA is an NPF for Arp2/3 complex suggested that *Shigella* IcsA might possess a similar activity. However, biochemical studies indicated that this is not the case (Egile et al., 1999). The mechanism that IcsA employs to promote actin nucleation emerged from a seminal study that implicated the cellular NPF N-WASP in *Shigella* motility (Suzuki et al., 1998). This study reported that N-WASP localizes at the site on *Shigella* from which the actin tail emerges (Figure 4). In contrast, N-WASP is not recruited by *Listeria*. Interestingly, *Shigella* specifically engages N-WASP but cannot recruit other NPFs, including the closely related WASP that is expressed in hematopoietic cell lineages (Suzuki et al., 2002). Consistent with this, *Shigella* cannot undergo actin-based motility in macrophages, which express WASP but not N-WASP (Suzuki et al., 2002). Moreover, *Shigella* do not move in cells expressing dominant-negative

variants of N-WASP or in N-WASP^{-/-} cells (Lommel et al., 2001; Snapper et al., 2001; Suzuki et al., 1998). Notably, N-WASP was implicated in *Shigella* motility before it was demonstrated to be an NPF for Arp2/3, and thus studies with *Shigella* were among the first to implicate N-WASP in actin nucleation.

The mechanism of N-WASP recruitment to *Shigella* involves direct binding to the IcsA protein. In particular, the glycine-rich repeats of IcsA, which are implicated in actin assembly, bind N-WASP in vitro (Suzuki et al., 1998) (Figure 4). An influential study showed that IcsA binding causes N-WASP to shift from an inactive, autoinhibited conformation to an open state where the WCA domain of N-WASP activates Arp2/3 complex (Egile et al., 1999) (Figure 4). Consistent with this, Arp2/3 complex is required for *Shigella* motility. This finding was remarkable because it built on a contemporary report that the host signaling molecules Cdc42 and PIP₂ also bind to N-WASP and cause a change from an inactive to an active NPF (Rohatgi et al., 1999). Thus, it appears that *Shigella* IcsA evolved as a mimic of host signaling pathways that recruit and activate N-WASP, in contrast with *Listeria* ActA, which mimics activated N-WASP.

IcsA is not sufficient to activate N-WASP in host cells entirely independently of host signaling proteins or pathways. The activities of Abl kinase (Burton et al., 2005) and Bruton's tyrosine kinase (Btk) (Dragoi et al., 2013) are important for N-WASP phosphorylation and *Shigella* motility. N-WASP activation and actin assembly by *Shigella* also requires Toca-1 (Leung et al., 2008), a cellular cofactor needed for N-WASP activation by Cdc42 and PIP₂ (Ho et al., 2004) (Figure 4). However, N-WASP activation is independent of other cellular factors needed for N-WASP activity, including Cdc42 or the WASP-interacting protein (WIP) (Moreau et al., 2000). Thus, IcsA bypasses the need for Cdc42 and PIP₂ in N-WASP activation, but not the requirement for other N-WASP activating inputs.

Reconstitution of Actin-Based Motility

The study of ActA and IcsA was instrumental in defining the involvement of the NPF-Arp2/3 pathway in actin nucleation by *Listeria* and *Shigella*. However, it was clear that additional factors beyond Arp2/3 and NPFs were required for motility, as purified Arp2/3 only promoted actin assembly but not movement (Welch et al., 1997). Just ten years after the initial discovery of bacterial actin-based motility, a crowning achievement in the field was the reconstitution of this process using purified proteins (Loisel et al., 1999). Surprisingly, the minimal reconstitution mix required for bacterial motility consisted of the NPF tethered to the bacterial surface and four other components: actin, Arp2/3 complex, capping protein, and ADF/cofilin. In the presence of these core factors, slow motility occurred (0.5 μm/min). The role of actin and Arp2/3 in motility was already discussed above. Two mechanisms have been proposed for the function of capping protein in the reconstituted motility mix. The funneling hypothesis proposes that capping older filaments and inhibiting their growth increases the concentration of available actin monomers, leading to more rapid elongation of new uncapped filaments at the bacterial surface (Carlier et al., 1997). The monomer-gating hypothesis proposes that capping acts as a switch that gates actin monomers to the Arp2/3 complex, enhancing the rate of actin nucleation (Akin and Mullins, 2008). Resolving these hypotheses will await further experimentation. The remaining essential factor,

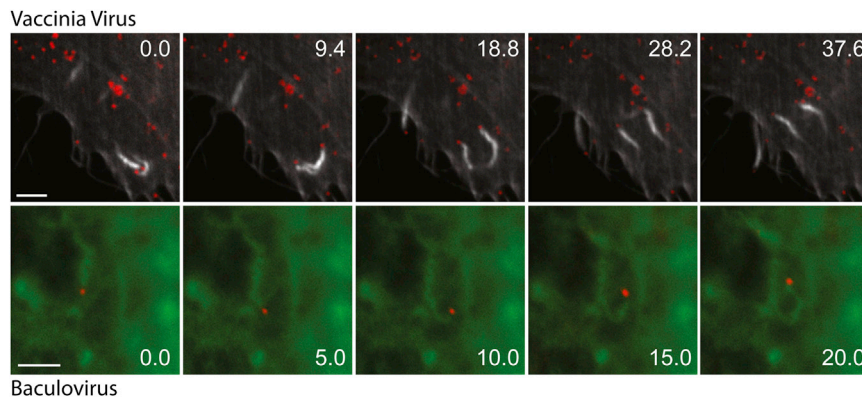


Figure 5. Viral-Induced Actin-Based Motility

The panels taken from [Movies S1](#) and [S2](#) show the actin-based motility of vaccinia virus (top, virus in red, actin in white) and baculovirus (bottom, virus in red, actin in green) at 8 and 1 hr postinfection, respectively. The scale bars represent 3 μm , and the time is indicated in seconds.

ADF/cofilin, severs and depolymerizes actin filaments, enabling monomer recycling for further polymerization ([Carlier et al., 1997](#); [Rosenblatt et al., 1997](#)). Thus, motility requires factors that enhance both actin assembly and disassembly.

Although motility can be reconstituted with only four components, further addition of the actin monomer-binding protein profilin and the adaptor protein Ena/VASP (for *Listeria* only) increases the speed of movement (to $\sim 3 \mu\text{m}/\text{min}$). Profilin is thought to promote filament elongation at barbed ends through several mechanisms, including enhancing ADP/ATP exchange, displacing monomers from sequestering proteins, and enhancing the local concentration of actin monomers available for assembly. Moreover, it is important for enhancing actin elongation during *Listeria* motility ([Grenklo et al., 2003](#)). Ena/VASP proteins, which specifically bind to the proline-rich repeat regions of ActA (but not IcsA or N-WASP), enhance motility by recruiting profilin ([Auerbuch et al., 2003](#); [Geese et al., 2002](#)) ([Figure 4](#)) and by enabling processive barbed end elongation while antagonizing the activity of capping proteins ([Breitsprecher et al., 2011](#); [Hansen and Mullins, 2010](#)). These factors synergize with Arp2/3 to enable rapid filament elongation and actin-based motility.

The reconstitution of actin-based bacterial motility demonstrates that the process is driven by a core set of proteins that regulate actin assembly dynamics. It also provides insights into the basic biochemical mechanisms that underlie host processes like lamellipodia protrusion during cell migration, which are driven by the same set of factors ([Campellone and Welch, 2010](#)). The reconstitution approach also highlights the fact that *Listeria* and *Shigella* motility rely on a short list of components, whereas the corresponding processes in host cells are more complex. Because they represent a stripped-down system, these pathogens have served as very useful models for cell biologists and biophysicists to study the basic mechanisms that control actin-based movement. Future studies will continue to make use of pathogens to study the biochemical basis of actin-based movement and how the biochemical properties of the system enable force generation to drive motility. In recent years, studies have also highlighted the fact that actin assembly by pathogens can play a role in processes outside of motility and spread.

Pathogen Actin Assembly and Autophagy

It was presumed for many years that the central function of actin assembly by *Listeria* and *Shigella* was to enable cell-to-

cell spread. More recently, a new role for actin polymerization has emerged. *Listeria actA* mutants that fail to recruit the host Arp2/3 complex or Ena/VASP proteins are more readily modified by ubiquitination, resulting in recruitment of the autophagy machinery ([Yoshikawa et al., 2009](#)). These results suggest that recruitment of the actin assembly machinery inhibits bacterial destruction by autophagy. Interestingly, actin recruitment appears to play the opposite role during *Shigella* infection, as inhibition of actin assembly by cytochalasin D or N-WASP depletion reduces targeting of *Shigella* by the autophagy machinery ([Mostowy et al., 2010, 2011](#)). Thus, the recruitment of actin nucleation factors and actin itself modulates autophagy, but the precise effect of actin differs between pathogens. This is an emerging area of research with many questions still to be answered, and future studies aimed at understanding how pathogens exploit actin should focus on the potential role for actin in autophagy. Such work also may reveal unexpected connections between autophagy and the cytoskeleton in uninfected host cells.

Vaccinia: A Virus Stimulates Actin Polymerization

Like intracellular bacteria, most viruses manipulate or use the actin cytoskeleton at some stage during their entry, replication, and spread ([Taylor et al., 2011](#)). One of the most striking examples is vaccinia virus, a large double-stranded DNA virus that is the most-studied member of the Orthopoxviridae. Vaccinia was used as the vaccine in the WHO global vaccination program against smallpox, a disease induced by its close relative, variola virus. Vaccinia promotes its entry into cells by stimulating actin-dependent macropinocytosis ([Mercer and Helenius, 2008](#)). Once inside, the virus quickly establishes a complex replication and assembly program in viral factories located near the microtubule-organizing center of the cell ([Roberts and Smith, 2008](#); [Smith et al., 2002](#)) ([Figure 1B](#)).

The first suggestion that vaccinia might use the actin cytoskeleton came from electron microscopy studies showing virions on the tips of large microvilli projecting from infected cells ([Stokes, 1976](#)). These viral-tipped projections, which appear late in infection, contained actin, α -actinin, fimbrin, and filamin, but not tropomyosin or myosin ([Hiller et al., 1979, 1981](#); [Krempien et al., 1981](#)). These initial studies were, however, largely forgotten until [Cudmore et al. \(1995\)](#) reported that, like *Listeria* and *Shigella*, vaccinia is moved by the power of actin polymerization on the tips of actin tails before extending out into adjacent noninfected cells ([Cudmore et al., 1995, 1996](#)) ([Figures 3 and 5](#) and [Movie S1](#)). As with *Listeria* and *Shigella*, actin tail assembly involved the polarized nucleation of actin on the virus, and the

filaments were orientated with their fast-growing ends toward the virion (Cudmore et al., 1996).

From the start it was clear that not all viruses in an infected cell induce actin tails (Cudmore et al., 1995). In an initial study using wide-field fluorescent imaging, it was thought that cytoplasmic intracellular enveloped virions (IEV) induced actin tails, as the latter were absent when IEV assembly was inhibited (Cudmore et al., 1995). The first suggestion that this might not be the case came when it was found that cell-associated enveloped virions (CEV) attached to the outside of the cell also induce actin tails (Cudmore et al., 1996) (Figure 1B). Subsequent studies demonstrated that actin tails are only induced by CEV when the IEV fuse with the plasma membrane after being transported from their perinuclear site of assembly on microtubules by kinesin-1 (Dodding and Way, 2011).

A Viral Protein that Induces Actin Assembly

The correlation between actin tail assembly and the presence of IEV, which are required for CEV formation, suggested that an IEV protein initiated actin tail formation. In 1996 only six viral proteins (A33, A34, A36, A56, B5, and F13) were known to be associated with the IEV membrane (Smith et al., 2002). Deletion of the genes encoding A33, A34, A36, B5, or F13 resulted in a loss of actin tails and a small plaque phenotype, which is indicative of defects in cell-to-cell spread (Roper et al., 1998; Röttger et al., 1999; Sanderson et al., 1998; Wolffe et al., 1997, 1998). However, only A36, an integral membrane protein, was required for actin tail formation and not IEV assembly (Röttger et al., 1999; Sanderson et al., 1998; Wolffe et al., 1998). Nevertheless, the proposed type II membrane topology of A36 (Parkinson and Smith, 1994) meant that it was not exposed on the surface of IEV, raising the question of how it could recruit the host proteins to stimulate actin polymerization. This conundrum was resolved when it was demonstrated that A36 has a type Ib membrane topology with an exposed cytoplasmic domain of ~195 residues on the IEV surface (Röttger et al., 1999; van Eijl et al., 2000) (Figure 4). Moreover, when IEV fuse with the plasma membrane A36 becomes localized in the membrane beneath CEV (Smith et al., 2002; van Eijl et al., 2000).

A36 was in the right place for the job, but in the absence of any obvious domains or sequence homologies to any other protein, it was not clear how it stimulated actin polymerization. Immunofluorescence analysis of infected cells suggested that, like *Listeria* and *Shigella*, vaccinia-induced actin polymerization was likely to involve the Arp2/3 complex (Frischknecht et al., 1999a). This study also revealed the presence of a phosphotyrosine signal at the site of vaccinia, but not bacterial actin tail assembly. Furthermore, microinjection of anti-phosphotyrosine antibodies into infected cells inhibited vaccinia but not *Listeria* actin tail formation (Frischknecht et al., 1999a). During vaccinia infection, three proteins at 200 (EGF-receptor), 80/85 (cortactin), and 50 kDa consistently become tyrosine phosphorylated (Frischknecht et al., 1999b). Furthermore, pTyr50 was absent in cells infected with the Δ A36R virus, suggesting that A36 was the unknown phosphorylated protein required for actin tail formation. Expression of A36 tyrosine (Y) to phenylalanine (F) mutants in cells infected with the Δ A36R virus revealed that only changing Y112 dramatically reduced actin tail formation (Frischknecht et al., 1999b). Inhibition of Src family kinases

also abrogated actin tail formation, consistent with the sequence surrounding Y112 conforming to a consensus for Src phosphorylation. The sequence also suggested that, when phosphorylated, Y112 was likely to bind the SH2 domain of Nck. Immunofluorescence analysis confirmed Nck was recruited to the virus, while in vitro peptide pull-down assays demonstrated that it bound phosphorylated Y112 (Frischknecht et al., 1999b) (Figure 4). Furthermore, expression of the SH2 domain of Nck largely inhibited actin tail formation. As seen with *Shigella* (Suzuki et al., 1998), N-WASP was also recruited to vaccinia, and expression of N-WASP lacking its C-terminal Arp2/3 binding site inhibited actin tail formation (Frischknecht et al., 1999b).

Curiously, the complete loss of actin tails and A36 phosphorylation was only observed when Y112 and Y132 were both mutated, even though mutation of Y132 alone had no impact on actin tail formation (Frischknecht et al., 1999b). However, the sequences surrounding Y132 matched the consensus-binding motif for the SH2 domain of Grb2 (Scaplehorn et al., 2002). Consistent with their predicted binding, Grb2 and Nck interacted with phosphorylated Y132 and Y112 of A36, respectively (Scaplehorn et al., 2002). The use of recombinant viruses encoding A36 mutants showed that phosphorylation of Y112 is essential for actin tail formation. In contrast, Y132 only enhances the number of actin tails (Scaplehorn et al., 2002). Vaccinia-induced actin polymerization had strong parallels with receptor tyrosine kinase signaling cascades, which also frequently involve multiple phosphorylation sites and adaptor proteins.

The ability to quantify actin tail formation meant that vaccinia was an excellent model to investigate how N-WASP couples Nck to actin polymerization. Unexpectedly, the WH1 domain and not the SH3 adaptor-binding proline-rich region of N-WASP was recruited to the virus (Moreau et al., 2000). This result was striking, as the majority of mutations leading to Wiskott-Aldrich syndrome are found in the WH1 domain of WASP (Jin et al., 2004). This observation led to the realization that vaccinia also recruits WIP, which interacts with the WH1 domain of WASP and Nck (Antón et al., 1998; Moreau et al., 2000; Ramesh et al., 1997; Zettl and Way, 2002) (Figure 4). Overexpression of the WASP binding domain (WBD) of WIP inhibited actin tails by blocking viral recruitment of N-WASP (Moreau et al., 2000; Zettl and Way, 2002). In contrast, even though WIP is recruited to the bacterium, expression of WBD did not inhibit *Shigella* actin tail formation (Moreau et al., 2000). Moreover, the WBD also inhibited the recruitment of endogenous WIP to vaccinia, suggesting that WIP and N-WASP are recruited as a complex (Moreau et al., 2000). Subsequent studies using N-WASP^{-/-} fibroblasts confirmed that WIP requires N-WASP for its recruitment to vaccinia (Snapper et al., 2001; Weisswange et al., 2009). There is also no recruitment of Grb2 in the absence of N-WASP (Weisswange et al., 2009). In contrast, Nck is still recruited to the virus, suggesting its recruitment is independent of WIP and N-WASP. Furthermore, the absence of WIP and its homolog WIRE results in the failure to recruit N-WASP but not Nck (Donnelly et al., 2013). Thus, Nck recruits a complex of WIP:N-WASP to the virus, which then associates with Grb2 interacting with phosphorylated Y132 of A36. This explains why Nck, but not Grb2, is essential for vaccinia to induce actin tails.

Molecular Dissection of the Vaccinia Signaling Network

Unraveling how a signaling cascade stimulates actin polymerization requires detailed knowledge of the interactions, dynamics, and stoichiometry of the proteins in the network. Unfortunately, many signaling networks controlling actin polymerization are not amenable to such quantitative analyses, as their components and/or activation are often transient and dispersed. In contrast, the signaling pathway used by vaccinia to induce actin polymerization is localized and sustained. Taking advantage of this, fluorescence recovery after photobleaching (FRAP) was used to analyze the dynamics of GFP-tagged Nck, Grb2, WIP, and N-WASP during vaccinia actin-based motility (Weisswange et al., 2009). All four proteins undergo rapid exchange, with a half-time of recovery of 0.14, 0.8, 0.8, and 2.7 s for Grb2, Nck, WIP, and N-WASP, respectively. The turnover of Nck, WIP, and N-WASP increases significantly in the absence of Grb2, demonstrating that Grb2 enhances vaccinia actin tail formation by stabilizing the signaling complex. Surprisingly, although Nck and WIP are responsible for N-WASP recruitment, N-WASP exchanges ~3.5 times slower than either of these proteins. N-WASP turnover also did not occur in the absence of Arp2/3 recruitment, suggesting that active actin polymerization promotes exchange of the vaccinia signaling complex. Consistent with this, the turnover rate of N-WASP depends on its interaction with both Grb2 and the growing plus ends of actin filaments. Loss of either of these interactions leads to a faster rate of N-WASP exchange and virus movement. This suggests that N-WASP not only activates the Arp2/3 complex, but also modulates the rate of actin-based motility by regulating the extent of actin polymerization, possibly by antagonizing filament capping.

Nck and N-WASP play a key role in connecting phosphotyrosine-based signaling to Arp2/3-mediated actin polymerization during a wide variety of cellular processes, in addition to driving actin-based motility of intracellular pathogens (Campellone and Welch, 2010; Dodding and Way, 2009; Lommel et al., 2001; Rotty et al., 2013; Snapper et al., 2001; Weisswange et al., 2009). What is less clear is the precise role played by WIP within Nck and N-WASP signaling networks. WIP inhibits the ability of N-WASP to activate the Arp2/3 complex until N-WASP receives the right signaling input (Ho et al., 2004; Martinez-Quiles et al., 2001; Takano et al., 2008). Recent analysis using MEFs lacking WIP, which have also been treated with RNAi against the WIP homolog WIRE, demonstrates that an interaction of WIP with the second SH3 domain of Nck is essential for vaccinia actin tail formation, as this interaction is required to recruit the WIP:N-WASP complex (Donnelly et al., 2013). Furthermore, the recruitment of N-WASP depends on its interaction with WIP rather than Nck. Finally, the first and third SH3 domains of Nck are not involved in recruiting the WIP:N-WASP complex but are essential to stimulate actin assembly. Vaccinia has thus provided essential insights into the connectivity within this important signaling network (Donnelly et al., 2013). WBD overexpression studies and infection of WIP^{-/-} cells suggest that WIP is not required for *Shigella* actin tail formation (Garber et al., 2012; Moreau et al., 2000). However, it remains to be determined whether *Shigella* still can recruit N-WASP and induce actin polymerization in the absence of both WIP and WIRE.

Role of Src and Abl Kinases in Vaccinia Actin Polymerization and Spread

A36 only promotes actin tail formation beneath CEV, although it is exposed on the surface of IEV. The reason is that Src activation and phosphorylation of A36 only occurs after the virus fuses with the plasma membrane (Newsome et al., 2004). This suggested that one or more of the four integral viral membrane proteins (A33, A34, A56, and B5) on the surface of CEV induces an outside-in signal to activate Src. The molecular mechanism for this still remains to be established, but the SCR4 domain of B5 is required to activate Src, phosphorylate A36, and induce actin tail formation (Newsome et al., 2004). Phosphorylation of A36 also promotes the release of kinesin-1 after the virus fuses with the plasma membrane.

Src is the prototypic member of a family of nonreceptor tyrosine kinases that play redundant roles in regulating a wide variety of cellular processes. It was not surprising, then, that vaccinia recruits multiple Src family kinases (Src, Fyn, and Yes) as well as the related Abl family kinases (Abl and Arg) to promote actin tail formation (Newsome et al., 2006; Reeves et al., 2005). While additional tyrosine kinases may also phosphorylate A36, inhibition of both Src and Abl family kinases is sufficient to inhibit vaccinia actin tail formation (Reeves et al., 2005). Consistent with this, in vitro kinase assays demonstrate that Abl, Arg, Fyn, Src, and Yes can phosphorylate Y112 of A36 (Newsome et al., 2006). Interestingly, Abl and Arg but not Src family kinases are also required to promote the release of CEV from infected cells (Reeves et al., 2005). Moreover, treatment of infected mice with Gleevec/STI-571/Imatinib, an Abl family kinase inhibitor used to treat chronic myelogenous leukemia, reduces viral spread and promotes survival from an otherwise lethal infection (Reeves et al., 2005, 2011).

In addition to the roles for Src and Abl kinases described above, recent work has begun to uncover additional potential effects of these key kinases in actin-based motility and infection. More than 30 years ago, Payne and Kristensson (1982) demonstrated that inhibition of actin polymerization blocked release of vaccinia from infected cells (Payne and Kristensson, 1982). Recently, studies have provided some molecular insights into this old observation (Horsington et al., 2013). The ability of Abl to promote virus release is independent of its ability to phosphorylate A36. Nevertheless, A36 phosphorylation and actin polymerization are required to drive CEV out of plasma membrane invaginations. In the absence of actin polymerization, CEV remain trapped within these invaginations and are not released unless the functionality of A34 or B5 is compromised. Consistent with this, structured illumination microscopy reveals that induction of actin polymerization polarizes A36 on the virus (Horsington et al., 2013). Actin is not the only cellular factor contributing to A36 polarization. Following their fusion with the plasma membrane, but before actin tail formation, vaccinia recruits clathrin in an AP-2-dependent fashion (Humphries et al., 2012). The clathrin is, however, left behind when CEV stimulate actin polymerization. Nevertheless, in the absence of clathrin recruitment, it takes longer for the virus to induce actin polymerization, and fewer actin tails are formed. Clathrin appears to have an organizational role, promoting clustering of A36, which in turn helps polarize and stabilize N-WASP, making initiation of actin-based motility easier (Humphries et al., 2012).

The activity of CK2 also enhances vaccinia actin tail formation and cell-to-cell spread (Alvarez and Agaisse, 2012). Loss of CK2 does not affect CEV formation, but does reduce the ability to recruit and activate Src. While the localization of CK2 during infection and the molecular basis for these observations remain to be established, it is curious that A36, which is heavily serine phosphorylated, contains several predicted CK2 phosphorylation sites (Alvarez and Agaisse, 2012; Wolffe et al., 2001). It is not just kinases that impact viral spread, however. The phosphoinositide 5-phosphatase SHIP2 acts as a negative regulator of viral release, although it is not required for actin tail formation (McNulty et al., 2011). The basis of this regulation remains to be established. Interestingly, SHIP2 recruitment to actin tails is dependent on its SH2 domain and N-WASP. Future analysis will confirm whether the activity of SHIP2 is related to the recent observations of Horsington et al. (2013).

Actin tail formation enhances the cell-to-cell spread of vaccinia. It had been unclear, however, how the virus can spread faster in a cell monolayer than its replication cycle would allow. Live-cell imaging of viral spread during plaque formation has now resolved this mystery (Doceul et al., 2010). If a virus (CEV or EEV) lands on a recently infected neighboring cell that lacks a virus factory, it forms a new actin tail without being internalized (Figure 1B). Motility then drives the virus across the surface of the infected cell and onto adjacent noninfected cells, where uptake occurs. In this way, vaccinia ignores already-infected cells, enhancing the rate of spread through the cell monolayer. This “super repulsion” is mediated by A33 and A36 on the surface of recently infected cells. The SCR4 domain of B5 is also required (Doceul et al., 2012). It remains to be established whether “super repulsion” depends on Src- and Abl-mediated phosphorylation of A36 and its downstream signaling network. The striking similarities between these two actin-dependent events, however, would suggest this is almost certainly the case.

A Common Mechanism to Promote the Spread of Poxvirus Infection

Actin-driven cell-to-cell spread of orthopoxviruses is likely to be common, as A36 is highly conserved (<http://www.poxvirus.org>). Consistent with this notion, variola and monkeypox viruses induce Abl and Src family kinase-dependent actin tails, while the A36 homolog of ectromelia virus, the causative agent of mousepox, is required for viral spread and actin tail formation (Lynn et al., 2012; Reeves et al., 2011). Actin tail formation is, however, not restricted to orthopoxviruses, as Yaba-like disease virus (YLDV; yatapoxvirus) and myxoma (leporipoxvirus) also induce actin tails (Duteyrat et al., 2006; Law et al., 2004), even though they lack an obvious A36 ortholog. Using a complementation approach, YL126 of YLDV was found to promote Nck- and N-WASP-dependent actin polymerization despite having less than 15% sequence identity with vaccinia A36 (Dodding and Way, 2009). Five phosphorylated tyrosines in YL126 can recruit Nck to promote actin polymerization. However, YL126-mediated actin tail formation, like that of A36, is also enhanced by the recruitment of Grb2 by a single phosphorylated tyrosine. Highly divergent YL126 orthologs in other vertebrate poxviruses, with as little as 6% homology to each other, can also induce Nck- and N-WASP-dependent actin polymerization. Actin-based motility thus appears to be a common mechanism used by verte-

brate poxviruses (*Chordopoxviridae*) to enhance cell-to-cell spread (Dodding and Way, 2009).

Baculoviruses Use the Arp2/3 Pathway for Motility and Nuclear Actin Assembly

Poxviruses are not the only viruses to hijack the Arp2/3 complex to promote actin polymerization. More than 20 years ago, it was observed that the baculovirus *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) induces thick actin cables in the cytoplasm of its host (Charlton and Volkman, 1991). These actin structures appear from 30 min postinfection and are tipped by a single nucleocapsid, suggesting the virus induces actin polymerization (Charlton and Volkman, 1993). Intriguingly, AcMNPV also stimulates the assembly of actin filaments in the nucleus (Charlton and Volkman, 1991, 1993; Volkman et al., 1992), a phenomenon that is essential for nucleocapsid assembly and progeny virus production (Hess et al., 1989; Ohkawa and Volkman, 1999; Volkman, 1988; Volkman et al., 1987, 1992). Subsequent *in vitro* assays demonstrated that purified AcMNPV nucleocapsids are capable of weakly stimulating actin polymerization (Lanier and Volkman, 1998). Furthermore, two viral capsid proteins, p39 and p78/83, were found to bind directly to actin. Examining the sequence of p78/83 suggested that it might represent a viral WASP-like protein, as it contained a proline-rich region, as well as a WCA motif that is indicative of G-actin and Arp2/3 binding (Machesky et al., 2001) (Figure 4).

Fifteen years after the discovery of baculovirus interactions with actin, p78/83 was shown to stimulate Arp2/3-dependent actin polymerization *in vitro*. Recent live-cell imaging shows that p78/83-mediated Arp2/3-induced actin polymerization propels AcMNPV nucleocapsids throughout the cytoplasm at rates of 7–22 $\mu\text{m}/\text{min}$ as early as 5 min postinfection (Ohkawa et al., 2010). Actin-based motility promotes nuclear collisions, a process that enables rapid nucleocapsid transit into the nucleus to speed the onset of early gene expression. Interestingly, once early gene expression is established, but before new viral progeny are produced, remaining cytoplasmic nucleocapsids accumulate in actin-rich protrusions at the cell surface, presumably ready to bud and spread to neighboring cells. This behavior may allow the virus to spread to neighboring cells before the initially infected cell is removed by apoptosis and sloughing, allowing the virus to establish an infection and enhancing its rapid dissemination (Ohkawa et al., 2010).

Interestingly, p78/83 and Arp2/3-dependent actin polymerization is also required later in infection for nuclear actin assembly (Goley et al., 2006). The role of nuclear actin remains unclear, however, and may include virus assembly, nuclear egress, and nuclear envelope remodeling during viral envelopment. Actin also plays a role in the nucleus of uninfected cells, yet the precise form and function of nuclear actin remains unclear (Weston et al., 2012). Baculoviruses represent an emerging system for studying how pathogens hijack actin in the nucleus and for studying the normal nuclear roles of actin.

What Can the Study of Pathogen Actin Assembly Still Tell Us?

Three decades after the discovery that *Listeria* and *Shigella* use actin-based motility to promote their cell-to-cell spread, we have achieved an understanding of how this process works at the

molecular level. Key proteins involved in Arp2/3-dependent motility have been identified, and reconstitution of the process has been achieved *in vitro* using purified proteins. These studies have revolutionized our understanding of how pathogens exploit actin and have revealed essential molecular pathways involved in actin regulation in host cells.

Nevertheless, much still remains to be discovered. With regard to *Listeria* and *Shigella*, we do not yet know at a biophysical level how Arp2/3-dependent actin polymerization is coupled to force generation to drive motility. Reconstitution of bacterial motility represents an experimentally accessible system to address this question, and what is learned will also apply to force-generating mechanisms involved in host cellular and intracellular motility. We do not understand how host motility is coupled with cell-to-cell spread, what membrane trafficking factors might be important for this process, or how membrane-cytoskeleton linkers like Ezrin and CD44 promote cell-to-cell spread (Pust et al., 2005). Moreover, we do not know how the ability to recruit Arp2/3 and actin plays other roles during infection, for example, in avoidance of autophagy. The information gained from studying *Listeria* and *Shigella* motility and cell-to-cell spread will continue to be of major importance in uncovering basic cell biological principles related to cytoskeletal function and regulation. It is also important to note that other bacteria, for example, *Rickettsia* and *Burkholderia* spp., are likely to exploit distinct host actin polymerization pathways involving formins or tandem-monomer-binding nucleators (Haglund and Welch, 2011). Thus, the study of how evolutionarily diverse bacterial pathogens usurp actin will undoubtedly shed light on the function and regulation of all three major host actin assembly pathways.

Vaccinia virus has also been a powerful model, in particular for understanding how a signaling network activated by Src and Abl family kinases functions to stimulate actin polymerization. However, as with bacterial pathogens, many outstanding questions remain. Vaccinia-induced actin polymerization is dependent on the activation of Src and Abl family kinases (Frischknecht et al., 1999b; Newsome et al., 2004, 2006; Reeves et al., 2005), but the molecular basis of how CEV activate Src and Abl family kinases remains to be determined. The temporal aspects of vaccinia-induced kinase activation as the virus fuses with the plasma membrane (or lands on another cell during “super repulsion”) and its relationship to and/or role in the release of kinesin-1 and recruitment of clathrin also need to be established. The virus is amenable to live imaging, including FRAP and FRET-based approaches, to address these important questions and also provides a great system for the development of new sensors to monitor the activity and interactions of these proteins together with components in the vaccinia-signaling cascade.

We also still lack a detailed understanding of how the initial level of A36 tyrosine phosphorylation determines the final output of the vaccinia-signaling network. Addressing this question is not easy for most phosphorylation-based signaling networks. However, the ability to manipulate the number of phosphorylation-competent A36 molecules beneath CEV (Humphries et al., 2012), combined with quantitative live imaging (Weisswange et al., 2009), provides a unique opportunity to determine how actin-based motility and dynamics of a signaling network change in response to the level of tyrosine phosphorylation. Within the

signaling network itself, we lack such basic information as the stoichiometry of components and how many A36 molecules are actually recruiting Nck. Does the slow exchange rate of N-WASP compared to Nck and WIP mean there is an additional binding partner in the system? We need to understand the exact sequence of events in the Nck-mediated recruitment and activation of the WIP:N-WASP complex, ideally at the single-molecule level. This will allow us to address such fundamental questions as whether each N-WASP molecule activates single or multiple Arp2/3 complexes before it dissociates from the virus. We currently have no information on the number and organization of actin filaments in the tail and why loss of clathrin recruitment results in the slower disassembly of the tail.

Many of the imaging approaches and quantitative aspects that are possible with vaccinia and other intracellular pathogens would be difficult, if not impossible, in other systems. Furthermore, the quantitative data derived from pathogens can be used to develop mathematical models that will provide additional molecular insights into the emergent properties and cooperative nature of signaling networks and how they regulate Arp2/3-dependent actin polymerization. The study of pathogens also has the potential to reveal insights into new functions for actin. One uncharted frontier in actin relates to its function in the nucleus. Nuclear actin is thought to participate in RNA biology, chromatin remodeling, nuclear shape, and cell differentiation (Weston et al., 2012). However, the regulation, state, dynamics, and roles of nuclear actin are poorly understood. Because of their ability to polymerize and harness actin in the nucleus, baculoviruses represent an outstanding model for understanding the regulation and function of nuclear actin. Finally, it should not be forgotten that pathogens cause a wide range of diseases that can have serious social and economic consequences. Understanding exactly how pathogens subvert cell signaling and the host actin cytoskeleton thus offers the potential to identify new therapeutic drug targets to combat infection that in the case of bacteria are independent of any acquired antibiotic resistance.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two movies and can be found with this article online at <http://dx.doi.org/10.1016/j.chom.2013.08.011>.

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