Host/pathogen interactions. Subversion of the mammalian cell cytoskeleton by invasive bacteria.

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Perspective

Perspectives Series: Host/Pathogen Interactions

Subversion of the Mammalian Cell Cytoskeleton by Invasive Bacteria

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The high plasticity of the actin cytoskeleton and its dynamics are often exploited by bacterial pathogens, during entry, and in some cases movement and dissemination of these organisms in mammalian cells. Indeed, some bacterial pathogens, including the gram-negative bacteria *Salmonella*, *Shigella*, and *Yersinia* and the gram-positive bacterium *Listeria monocytogenes*, have the capacity to induce their own uptake into mammalian cells which are normally nonphagocytic. Entry of all of these bacteria requires rearrangement of the host cell actin cytoskeleton, since uptake is impaired by inhibitors of actin polymerization such as cytochalasin D. Exploitation of the cytoskeleton by pathogenic bacteria during entry can be divided, although artificially, into two general strategies, according to the type of morphological changes that occur in the host cell. Entry of *Yersinia* or *Listeria* has been described as occurring through a "zipper" type event, while entry of *Salmonella* or *Shigella* is often referred to as a "trigger" phenotype. However, even pathogens that share a common strategy appear to target different host proteins to induce uptake. After internalization, invasive bacteria either reside in membrane-bound vacuoles (*Yersinia* and *Salmonella*), or rapidly lyse such vacuoles and move within the cytoplasm by a process involving continuous polymerization of host actin at the posterior end of the bacterium (*Listeria* and *Shigella*). This intracytoplasmic movement is a prerequisite for direct cell to cell spreading. While the actin-based motilities of *Listeria* and *Shigella* are similar in many aspects, different bacterial proteins are involved, again suggesting that a similar strategy can be accomplished by nonidentical molecular mechanisms.

Internalization of bacterial pathogens by nonphagocytic cells

Yersinia species. After ingestion of contaminated food, the two enteropathogenic *Yersinia*, *Y. pseudotuberculosis* and *Y. enterocolitica* translocate across the intestinal barrier, primarily via M cells in Peyer's patches of the epithelium. Bacterial uptake can be reproduced in vitro; most normally nonphagocytic cell lines internalize *Yersinia* in a membrane-bound compartment in which bacteria survive but do not multiply (1, 2). Internalization of *Y. pseudotuberculosis* requires expression of a 986–amino acid outer membrane protein called invasin, the 192 carboxy-terminal amino acids of which are sufficient to bind mammalian cells. *Escherichia coli*–expressing invasin or latex beads covered with invasin are internalized, demonstrating that invasin is sufficient for entry. Invasin mediates uptake by binding tightly to a subset of β1 integrins on the surface of host cells. Each integrin is a heterodimer of α and β chains, and several different α chains can associate with the β1 chain to form complexes capable of interacting with invasin or natural ligands such as the extracellular matrix proteins fibronectin, collagen, and laminin. Interestingly, invasin binds to its receptor with an ~ 100-fold higher affinity than do the natural ligands, and binding of ligands like fibronectin does not lead to uptake of small particles. Therefore, high-affinity binding is needed for internalization. At the microscopic level, invasin-mediated entry closely resembles Fcγ or complement receptor–mediated phagocytosis, and is morphologically characterized by zippering of a closely apposed host membrane around the bacterium. Host F-actin and signal transduction via tyrosine phosphorylation are needed for entry, but the specific kinases involved are not known. It was originally thought that direct association of integrins with the cytoskeleton might be required for bacterial internalization, since the intracytoplasmic domain of the β1 subunit normally interacts with the cytoskeleton by binding to the actin-binding proteins talin and α-actinin. However, mutations in β1 that reduce interaction with the cytoskeleton increase bacterial uptake, while mutations that impair uptake are in the sequence NPIY (3). This sequence is also found in the LDL receptor, and appears to mediate clustering of this protein into clathrin-coated pits by binding to the heterotetrameric adaptor protein AP2. Intriguingly, large lattices of clathrin and AP2 adaptor complexes are formed beneath bacteria in the early stages of internalization, suggesting that integrin-mediated uptake may involve clathrin and share similarities with clathrin-mediated endocytosis.

Salmonella and Shigella species. Like enteropathogenic *Yersinia*, *Salmonella* and *Shigella* species also initiate infection in the small bowel primarily by entering and transcytosing across M cells. Experiments with infected mice indicate that for *Salmonella* a proportion of transcytosed bacteria disseminate to regional lymph nodes, establishing a systemic infection in susceptible hosts that may result in large foci of infection in the liver and spleen. Some bacteria may infect enterocytes (1, 2). In the case of *Shigella*, bacteria that have traversed M cells are then engulfed by underlying macrophages, where they induce apoptosis. Apoptotic macrophages release IL-1β, contributing to a severe inflammatory response. Bacteria released from these dying cells can invade enterocytes through the basolateral surface (2, 4).

Entry of *Salmonella* and *Shigella* into cultured mammalian cells shares a similar morphological phenotype that is strikingly different from that of *Yersinia* species. Upon initial contact with the host cell, these bacteria trigger dramatic rearrangements of cellular F-actin, characterized by large membrane

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projections similar to membrane ruffles induced by some growth factors or oncogenes. This process is evident within 1 min after contact between pathogen and host, and the affected membrane region then rapidly enlarges over the next 3 min. Bacterial-induced ruffling may lead to macropinocytosis and passive entry of other bacteria or particles which are trapped by the highly dynamic membranes. In contrast to the zipper entry by *Yersinia*, the host membrane is not closely apposed to the bacterium, but instead engulfs the microorganism in a large vacuole formed by fusion of membrane projections.

The bacterial factors that mediate entry of *Salmonella* and *Shigella* are not significantly secreted during growth in broth, but instead are released upon contact with mammalian cells. This contact-mediated secretion is one of the properties specific to so-called "type III secretion systems" which are found in a growing number of bacterial pathogens. These systems comprise $\sim$ 20 genes encoding secreted proteins and proteins involved in the secretion machinery. The secreted proteins do not have a classical signal sequence, and appear to be translocated across the two bacterial membranes by a protein complex located in the bacterial envelope. Secretion occurs in response to a signal which may be a contact between a key bacterial protein and the mammalian cell, but it can also be induced by soluble factors present in serum. This key bacterial protein seems to act as a plug, which when released allows secretion and eventually translocation of effector proteins into the mammalian cell. In *Salmonella*, this secretion system is encoded by a "pathogenicity island" or cluster of chromosomal genes known as the *inv/spa* complex. In *Shigella*, the *mxi/spa* complex located on the virulence plasmid encodes a homologous secretion system with secreted proteins involved in entry (IpaA, B, C, and D) encoded by *ipa* genes. The *Shigella*-secreted proteins IpaB and IpaC associate into a soluble complex that can be immunopurified on latex beads. These beads are sufficient to cause membrane ruffling and can be internalized in HeLa cells. In the case of *Salmonella*, interestingly, upon contact with mammalian cells, bacteria become rapidly covered with appendages comprised of unidentified proteins, and assembly of these structures apparently requires the *inv/spa* type III secretion system (5).

Entry of *Salmonella* into cells correlates with increases in intracellular Ca^{2+} levels and IP3 production. It seems likely that the bacterium stimulates host phospholipase C inducing IP3 production which in turn mobilizes Ca^{2+} from intracellular stores. Calcium fluxes could affect uptake by controlling activity of various actin-binding proteins such as α-actinin, talin, and ezrin which are recruited to the site of entry.

Internalization of *Shigella* seems to involve tyrosine phosphorylation of several key host proteins, including cortactin, pp125FAK, and paxillin. Cortactin is an actin-associated protein and a substrate for the nonreceptor tyrosine kinase c-Src. Tyrosine phosphorylation of c-Src increases during *Shigella* invasion, and this kinase is recruited to the site of entry. Transient overexpression of c-Src in transfected cells induces membrane ruffling and stimulates entry of normally noninvasive *Shigella* mutants, suggesting that this kinase plays a role in entry. T-plastin is an actin-bundling protein that appears to have a function in bacterial uptake, possibly by bundling newly formed actin filaments in membrane extensions. Vinculin, another actin-binding protein, also colocalizes to the site of entry of *Shigella*, and this protein can be coimmunoprecipitated with IpaA (6). Bacteria that do not express IpaA still recruit vinculin to the site of entry, but are impaired in recruitment of α-actinin, a vinculin-binding protein. These results suggest that IpaA affects vinculin activity after the recruitment step.

In mammalian cells, agonist/receptor-mediated rearrangements of the actin cytoskeleton leading to membrane ruffling, actin stress fiber formation, or filopodia formation are controlled by small GTP-binding proteins of the Rho family (Rac, Rho, and CDC42). Therefore, it was anticipated that invasive bacteria might regulate Rho GTPases to induce cytoskeletal changes needed for uptake. Recently, it was demonstrated that invasion by *Salmonella* requires CDC42 function, but not Rac or Rho (7). In contrast, invasion by *Shigella* requires Rho activity, indicating that the similar trigger phenotypes observed with *Salmonella* and *Shigella* occur by at least partly different molecular mechanisms (4).

Interestingly, an α5β1 integrin in Chinese hamster ovary cells has been shown to be a receptor for *Shigella*, probably by interaction with IpaB, IpaC, and IpaD (8). The fact that this integrin is also one of the receptors for the invasin protein of *Yersinia* raises the possibility that the same host cell receptor may be exploited in different ways to cause remarkably different mechanisms (zipper or trigger) of entry.

Listeria. Like *Yersinia*, *Salmonella*, and *Shigella*, *L. monocytogenes* is a food-borne pathogen which can cross the epithelial barrier, possibly via M cells and/or enterocytes (9). After translocation, bacteria are engulfed by macrophages and disseminate to the spleen and liver. In the immunocompromised host, bacterial replication can occur in hepatocytes leading to a systemic infection with further spreading to the brain or the placenta. Thus, in the infected animal and in tissue culture models, *L. monocytogenes* invades a wide variety of nonphagocytic cells, in addition to being internalized by professional phagocytes.

The morphological events associated with entry have been studied mainly in epithelial cells, and uptake resembles the zipper entry of *Yersinia*, characterized by absence of membrane ruffling, and highly local apposition of the plasma membrane with the incoming microbe (10). Bacteria appear to progressively sink into the mammalian cell surface. Therefore, although actin cytoskeleton rearrangement is necessary for internalization, rearrangement is apparently highly local, and no striking accumulation of F-actin near the site of entry is observed.

Two bacterial factors allowing entry have been identified. Internalin (or InlA) is a surface protein that mediates entry into cultured intestinal epithelial cells. This protein confers invasiveness to the noninvasive species *Listeria innocua*, suggesting that, like invasin of *Yersinia*, this protein is sufficient to promote entry. Internalin contains multiple tandem copies of a 22–amino acid leucine-rich motif called a leucine-rich repeat (LRR).[1] LRRs are a feature of several proteins (mostly eukaryotic) that generally participate in protein–protein interaction. The mammalian receptor for internalin is E-cadherin, a transmembrane cell adhesion protein normally involved in homophilic cell–cell interactions (10). Apart from internalin, the only other heterophilic ligand known for E-cadherin is the integrin αEβ7 expressed in intraepithelial lymphocytes. The in-

1. *Abbreviations used in this paper:* LRR, leucine-rich repeat; PI, phosphoinositide.

tracytoplasmic region of E-cadherin is linked to the cytoskeleton through a complex of α and β catenins, and is needed for homophilic interactions and cell–cell adhesion. Interestingly, this region is not required for interaction between αEβ7 and E-cadherin (11). Whether the cytoplasmic domain of E-cadherin is involved in internalin-mediated entry is currently unknown.

Internalin is not required for the entry of *L. monocytogenes* into a number of other cultured cell lines, suggesting that this bacterium has additional strategies for invasion. InlB, a surface protein that contains LRRs similar to those in InlA, mediates entry in cultured hepatocytes and in several epithelial or fibroblast cell lines including HeLa, Chinese hamster ovary, Hep-2, and Vero (12, 13). The InlB receptor has not been identified yet.

Tyrosine kinases are presumed to participate in both internalin- and InlB-mediated entry, since inhibitors of such kinases block bacterial invasion. However, the kinases involved have not been identified. Another signaling protein needed for entry is the phosphoinositide (PI)-3 kinase, p85/p110 (13). Entry of *L. monocytogenes* is impaired by treatment of host cells with specific inhibitors of this kinase or by expression of dominant negative form of p85. In addition, in Vero cells PI-3 kinase activity is stimulated within minutes after infection, indicating that *L. monocytogenes* activates host cell p85/p110. This activation requires InlB, tyrosine phosphorylation in the host cell and association of p85 with at least one host tyrosine phosphorylated protein. How activation of PI-3 kinase mediates uptake is unknown, but it is possible that this kinase controls changes in the actin cytoskeleton during entry. Treatment of Vero cells with cytochalasin D inhibits entry, but does prevent bacterial adhesion or activation of PI-3 kinase, suggesting that stimulation of this kinase may precede cytoskeletal rearrangements. One attractive possibility, recently shown in platelets, is that the lipid products of PI-3 kinase (PI3,4P2 and PI3,4,5P3) may directly affect F-actin by uncapping barbed ends of actin filaments. Alternatively, PI-3 kinase has been shown to regulate the GTPase rac in fibroblasts, and it is possible that regulation of this G-protein by p85-p110 is involved in entry of *L. monocytogenes*.

Bacterial actin-based motility

Bacteria such as *L. monocytogenes*, *L. ivanovii*, *S. flexneri*, and also several *Rickettsiae* have the capacity to move intracellularly using polymerization of host cell actin as a driving force for movement. These organisms have provided new tools to tackle the unsolved questions of actin-based motility and cell shape changes (14). Actin polymerization and movement seem tightly coupled in both bacterial and mammalian systems, but there are important differences: in cells, actin polymerization is initiated at the plasma membrane where a signal is received (for example, a chemoattractant in the case of neutrophils or a growth factor) and transduced to the cytoskeletal machinery. In the case of bacteria, the process involves no membrane and no stimulus. The capacity to polymerize actin is roughly constitutive. In addition, bacteria are relatively easy to manipulate, i.e., they are easy to grow and to store, and more importantly they can be genetically engineered. Hence, a real enthusiasm for bacterial actin-based motility emerged almost 10 yr ago with work performed mostly on *L. monocytogenes* and to a lesser extent on *Shigella*. *Rickettsiae* are strict intracellular bacteria and are still difficult to manipulate.

The Listeria actin comet tail. After entry, *Listeria* are trapped within vacuoles which are then rapidly lysed, releasing the bacteria into the cytosol where they replicate and become covered with actin filaments (F-actin). This actin coat then rearranges into a long tail which remains stationary in the cytoplasm and trails behind the moving bacteria. Bacteria migrate around the cytoplasm at speeds ranging from 6 to 60 $\mu m\ min^{-1}$ with their tail lengths proportional to their speed, faster bacteria having longer tails (up to 40 μm). Decoration with S1 myosin heads revealed that the actin filaments in the *Listeria* tails are short (0.2 μm) and oriented with their barbed (fast polymerizing end) towards the bacterium, suggesting that actin polymerization takes place at the bacterial surface. Video microscopy of *Listeria*-infected cells after microinjection of fluorescent or photoactivatable monomeric actin (G-actin) and α-actinin demonstrated that continuous assembly of actin microfilaments, followed by their release and cross-linking, takes place at the posterior end of the bacterium, providing the propulsive force for movement. In contrast to actin polymerization that occurs only at the base of the actin tail, and at varying rates, actin filament depolymerization occurs at a constant rate throughout the tail and in all tails.

Bacterial movement can be analyzed in cell free systems such as cytoplasmic extracts of *Xenopus laevis* eggs or human platelets. The speed of bacterial movement and density profiles of actin tails indicate that these extracts provide a reasonable substitute for the cytoplasm of mammalian cells.

The bacterial factor ActA. The bacterial protein ActA is needed for polymerization of host actin and movement (9, 14). The gene encoding this 610–amino acid protein, *actA*, was identified by analysis of mutants that formed microcolonies inside cells, failed to polymerize actin, and did not spread from one cell to the other. ActA contains a highly charged amino-terminal domain, a central region made of four tandem proline-rich repeats and a carboxy-terminal region. Recent protein alignments highlight that ActA may be a composite protein with the central proline-rich region and the carboxy-terminal domain that can be aligned with human Zyxin, an 83-kD actin-associated protein (Goldsteyn, R., E. Friedrich, and D. Louvard, personal communication). The amino-terminal domain of ActA (amino acids 12 –233) has sequence homology (25% identity) with human vinculin (amino acids 878–1065), a protein associated with stress fibers and focal adhesions (Dehoux, P., and P. Cossart, manuscript in preparation). In addition, the proline-rich region of ActA has homology with the proline-rich domain of vinculin. These sequence similarities underscore the role of ActA in actin assembly.

One interesting property of ActA is its polar distribution on the bacterial surface, with highest concentration at one of the two poles. Establishment of ActA polarity is linked to bacterial replication. In infected cells, ActA colocalizes with the base of the actin tail and determines both the site of actin assembly and direction of movement.

Several approaches were used to demonstrate that ActA is sufficient to induce actin polymerization., including transfection of ActA in mammalian cells. However, this approach does not allow analysis of movement. Two other strategies were thus used. Expression of *actA* into the nonpathogenic bacterium *L. innocua* rendered this organism capable of actin based motility. In another approach, coating of *Streptococcus pneumoniae* with a recombinant ActA-LytA hybrid protein resulted in decorated bacteria that polymerized actin and moved

in a cell free system. However, movement only occurred after division had generated polar distribution of ActA. Thus, expression of ActA and its polar distribution are required for actin-based movement.

The cellular proteins. Many proteins have been detected in the actin tails. They include: α-actinin, tropomyosin, fimbrin, (plastin), profilin, vinculin, villin, ezrin (radixin), talin, gelsolin, VASP, and the Arp2/Arp3 complex. Only profilin and VASP colocalize with the base of the actin tails. Note that the actin concentration in the tail is so high compared with the rest of the cell that detection of a given actin-binding protein in the tail can only be considered relevant if a functional analysis has been performed. It is the case for α-actinin, profilin, VASP, and Arp2/Arp3.

Microinjection of infected cells with a 53-kD fragment of α-actinin, an actin cross-linking protein, causes the disappearance of bacteria containing filopodia and an arrest in movement suggesting that this fragment acts as a dominant negative protein and that cross-linking of actin filaments is important for movement. The role of profilin, a G-actin–binding protein which also has affinity for polyproline sequences, has been addressed by depletion experiments or microinjection of proline-rich peptides. The fact that actin assembly and movement can occur in the absence of most profilin raises the possibility that profilin may not be as critical as originally thought. VASP is the first and only identified ligand of ActA. It can bind purified ActA and also binds profilin, zyxin, and vinculin. Thus VASP could bring polymerization-competent profilin/actin complexes to bacteria by binding simultaneously to ActA and Profilin. Arp2 and Arp3, two actin-related proteins, are components of an eight polypeptide complex purified from human platelets that has the capacity to initiate ActA-dependent actin polymerization around bacteria (15). Arp2 and Arp3 localize at the surface of stationary bacteria and in the tails of locomoting bacteria. These unconventional actins which interact with actin might serve as a link between ActA and actin.

Structure function analysis of ActA. Genetic analysis of *actA* has shown that the amino-terminal domain of ActA is necessary and sufficient to promote actin-based motility (9, 14, 16). Yet, the central region is an active participant in the process and its presence stimulates by a factor of two the rate of movement. As revealed by immunofluorescence on cells infected with various mutants, VASP binds to the central proline-rich region, suggesting that VASP may not be absolutely essential for actin assembly.

Transfection experiments and analysis of bacteria expressing various *actA* mutations showed that the amino-terminal domain contains at least two critical regions (9, 14, 16). The first region (region T for tail formation) spans residues 116–122 and is necessary for tail formation. Deletion of region T does not prevent actin assembly. The second region (region C for continuity) spanning amino acids 21–97 appears to be involved in the continuity of the actin polymerization process. Deletion of this region leads to an intriguing phenotype of discontinuous actin tails which may be due to disruption of the balance between the number of new free barbed ends generated at the bacterial surface and capping of these barbed ends. In the wild-type situation, region C could act by protecting actin filaments from capping proteins. In its absence, premature capping would occur and movement will stop until a certain number of free barbed ends available for polymerization have been generated at the bacterial surface.

Current model for actA-mediated movement. Data available suggest that the amino-terminal domain of ActA generates free barbed ends either by recruiting or acting as a nucleator or by severing actin filaments or by uncapping actin filaments. Polymerization then ensues and depends on the maintenance of these free barbed ends. Filaments are then released, capped, and cross-linked, generating the tail. The central region of ActA binds VASP which can bind profilin or profilin/actin complexes. An attractive possibility is that profilin/actin complexes attracted by VASP in the vicinity of the bacteria can bind to free barbed ends with further release of the free profilin, thereby participating to the dynamic of the process. This hypothesis would explain why profilin and VASP are only found at the site of actin polymerization. The Arp2/Arp3 complex appears as a good candidate for interacting with both ActA and actin. It is tempting to speculate that the Arp2/Arp3 complex interacts with the amino-terminal domain of ActA and acts as a link between actin and ActA but this hypothesis awaits experimental evidence.

The recent observation that peptides encompassing the amino-terminal domain of ActA are able to bind actin reactivates the early hypothesis that ActA may interact directly with actin (16). The observation that bacteria grown in broth do not nucleate actin efficiently has led to the suggestion that ActA may not be a nucleator or may have to be modified to bind actin, or that it may recruit a nucleator. Another emerging possibility, however, is that ActA (as shown previously in the case of vinculin) could contain cryptic actin-binding sites which need to be unmasked to become accessible to actin. The recently discovered homology between the amino-terminal domain of ActA and the actin-binding domain of vinculin underscores this possibility. Finally, the discovery of a mutant moving discontinuously suggests that ActA may also play a role in protecting free barbed ends from uncapping (16).

The Shigella actin-based motility. Like *Listeria*, *Shigella* can move intracellularly. Intriguingly, the bacterial gene used, *icsA*, encodes a protein with no homology to ActA (14). This protein is also polarly distributed on the bacterial surface. IcsA was shown recently to bind vinculin (17). Whether ActA and IcsA activate the same signaling cascade is under investigation.

Conclusions

Bacterial pathogens have developed strategies to maximally exploit the exceptional properties of the actin cytoskeleton during entry and intracellular movement. Interactions between pathogens and the cytoskeleton also occur in other instances. For example, adhesive bacteria, such as EPEC, also require an active cytoskeleton at the site of bacterium cell contact to adhere tightly with the host cell. The signals underlying this adherence are currently being elucidated. All the interactions described above require cytoskeleton rearrangements stimulated by the pathogen, but there are examples where the pathogen inhibits activity of the cytoskeleton. It is the case for some toxins which modify actin directly, such as the C2 toxin of *Clostridium botulinum* or indirectly like the C3 toxin acting on Rho. One of the most sophisticated systems is the Yop system of *Yersinia.* Indeed, in addition to strategies to invade mammalian cells, these bacteria have evolved strategies to inhibit their phagocytosis by phagocytes. Upon contact with the macrophage, the *Yersinia* inject within the macrophage, via a type III secretion system, several proteins, in particular YopE which can paralyze the cytoskeleton and thus prevent phagocytosis

(2). Exploitation of the cytoskeleton is not limited to bacterial pathogens since its has been discovered recently that a vaccinia virus can move intracellularly (18). It is clear that the challenge for the future will be to determine and compare the stimuli that generate free actin barbed ends, an absolute requirement for cytoskeletal rearrangements.

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"Host/Pathogen Interactions: Understanding the Strategies of Microbial Virulence and Host Defense"

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