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Interactions of *Listeria monocytogenes* with mammalian cells during entry and actin-based movement: bacterial factors, cellular ligands and signaling

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Although <50 kb of its 3.3 megabase genome is known, *Listeria monocytogenes* has received much attention and an impressive amount of data has contributed in raising this bacterium among the best understood intracellular pathogens. The mechanisms that *Listeria* uses to enter cells, escape from the phagocytic vacuole and spread from one cell to another using an actin-based motility process have been analysed in detail. Several bacterial proteins contributing to these events have been identified, including the invasion proteins internalin A (InlA) and B (InlB), the secreted pore-forming toxin listeriolysin O (LLO) which promotes the escape from the phagocytic vacuole, and the surface protein ActA which is required for actin polymerization and bacterial movement. While LLO and ActA are critical for the infectious process and are not redundant with other listerial proteins, the precise role of InlA and InlB *in vivo* remains unclear. How InlA, InlB, LLO or ActA interact with the mammalian cells is beginning to be deciphered. The picture that emerges is that this bacterium uses general strategies also used by other invasive bacteria but has evolved a panel of specific tools and tricks to exploit mammalian cell functions. Their study may lead to a better understanding of important questions in cell biology such as ligand receptor signalling and dynamics of actin polymerization in mammalian cells.

Keywords: actin-based movement/actin polymerization/internalin/*Listeria*/PI-3 kinase

Introduction

Listeria monocytogenes is a food-borne pathogen responsible for meningitis, meningoencephalitis, septicaemias, abortions and, in some cases, gastroenteritis. The overall mortality rate is >20% (Lorber, 1996), the clinical symptoms being due to the unique ability of this organism to cross three barriers during infection: the intestinal barrier, the blood–brain barrier, and/or the placental barrier.

Listeriosis

Most of our knowledge of listeriosis comes from the many studies carried out in the mouse model which has long been used as a tool to study a rapidly appearing CD8+

T cell-dependent immune response (Mackaness, 1962). The portal of entry has long been a matter of debate, recent evidence indicating that there does not seem to be a preferential site of translocation (enterocytes or M cells) in the intestine (Pron *et al.*, 1998) with, however, a preferential site of early multiplication in the phagocytic cells underlying the Peyer's patches. This result is in agreement with the early observation that bacteria survive and even replicate in resident phagocytes of the lamina propria (Mackaness, 1962). Following translocation, bacteria, via the lymph and the blood, reach the spleen and liver. In the latter organ, most of the bacteria are killed by the Küpffer cells, though a fraction reach the hepatocytes where they induce apoptosis with concomitant release of chemoattractants which leads to an influx of neutrophils (Conlan and North, 1991; Rogers *et al.*, 1996). These phagocytic cells ingest bacteria or apoptotic hepatocytes and contribute to the rapid clearing of the infection before a complete sterilization is achieved by the immune response. The step of bacterial multiplication in the liver appears critical for the establishment of a 'successful' infection.

Although it may be hazardous to extrapolate these data to human listeriosis, the scenario of the human infection is believed to begin by ingestion of contaminated food (Schlech *et al.*, 1983). Bacteria then reach the gastrointestinal tract and cross the intestinal barrier. In the cases of the immunocompromised host or the pregnant woman, bacteria multiply unrestrictedly in the hepatocytes from which they further disseminate haematogeneously to the brain and placenta.

The cell infection

In most infected tissues, *Listeria* has an intracellular location due to its capacity to induce its own phagocytosis into cells that are normally non-phagocytic. Detailed analysis of infected tissue-cultured cells reveals a complex series of interactions between the bacteria and the cell (Figure 1). Host cell infection begins with the internalization of the bacteria either by phagocytosis in the case of macrophages or induced phagocytosis (invasion) in the case of normally non-phagocytic cells. Bacterial invasion starts by the interaction with the plasma membrane which progressively enwraps the bacterium. This process is different from the membrane ruffling observed upon *Salmonella* or *Shigella* entry and which culminates in macropinocytosis. It is usually referred to as the 'zipper' mechanism in contrast to the 'trigger' mechanism used by *Salmonella* or *Shigella* (Finlay and Ruschkowski, 1991; Isberg and Tran Van Nhieu, 1994; Adam *et al.*, 1995; Swanson and Baer, 1995; Mengaud *et al.*, 1996a). Following internalization, bacteria reside within membrane-bound

Fig. 1. Thin sections of the different steps of the infectious process [the third and fifth panels are reproduced with permission from *Cell* (Kocks *et al.*, 1992) and *Molecular Microbiology* (Cossart and Kocks, 1994)].

Fig. 2. Schematic representation of the cell infectious process by *L. monocytogenes*. The bacterial factors involved are indicated in blue. Adapted from Tilney and Portnoy (1989).

vacuoles for ~30 min before lysing the membrane. When free in the cytosol, they replicate and concomitantly become covered with actin filaments. These filaments rearrange within 2 h into long comet tails left behind in the cytosol while the bacteria move ahead at a speed of ~0.3 µm/s (Tilney and Portnoy, 1989; Dabiri *et al.*, 1990; Theriot *et al.*, 1992). When moving bacteria contact the plasma membrane, they induce the formation of bacterium-containing protrusions. Contact between these protrusions and neighbouring cells results in the internalization of the protrusion. In the newly infected cell, the bacterium is surrounded by two plasma membranes which must be lysed to initiate a new cycle of multiplication and movement. Thus, once *Listeria* has entered the cytoplasm, it can disseminate directly from cell to cell, circumventing host defences such as circulating antibodies and complement. This ability to disseminate in tissues by cell-to-cell spreading provides an explanation for the early observation that antibody is not protective and that immunity to *Listeria* is T cell-mediated.

The virulence gene cluster of *L. monocytogenes*

The different phases of the infection are shown schematically in Figure 2. Entry into mammalian cells is mediated by at least two bacterial factors: internalin A (InlA) and B (InlB). Escape from the vacuole requires expression of listeriolysin O (LLO), a pore-forming toxin which in some cells can function synergistically with or be replaced by a phosphatidylinositol-specific phospholipase C (PI-PLC). Intracellular movement requires expression of ActA, and lysis of the two-membrane vacuole is performed by a lecithinase (PLC-B). Most of the genes coding for these virulence factors are clustered on a 10 kb region of the chromosome. This region is absent from the non-pathogenic species (Figure 3) (Gouin *et al.*, 1994). The *inlAB* operon is located in another region. In contrast to well-characterized pathogenicity islands, the *Listeria* virulence gene cluster is rather small and has the same GC content as the rest of the chromosome. All known virulence genes are under the either absolute or partial control of a pleiotropic activator protein PrfA (Mengaud *et al.*, 1991; Chakraborty *et al.*, 1992). This protein is a member of the CAP/FnR family of transcriptional

Fig. 3. The PrfA regulon.

activators and has a critical helix-turn-helix motif similar to that of CAP (Sheehan *et al.*, 1996). Intriguingly, in some culture conditions, PrfA can be present but inactive, revealing a requirement either for a cofactor or a post-translational modification necessary for activation (Renzoni *et al.*, 1997). Recent evidence indicates that such a PrfA-activating factor exists (Dickneite *et al.*, 1998).

This review focuses on entry into non-phagocytic mammalian cells and actin-based motility. For more extensive details or other aspects of infection such as uptake by macrophages, the reader should refer to other recent reviews (Campbell, 1994; Cossart, 1997; Ireton and Cossart, 1997, 1998; Dramsi and Cossart, 1998; Kuhn and Goebel, 1998; Lasa *et al.*, 1998).

Invasion of mammalian cells

The *inlAB* locus

Invasion genes were identified by screening a library of transposon mutants of *L.monocytogenes* for loss of invasiveness into the intestinal epithelial cell line Caco-2 (Gaillard *et al.*, 1991). Three non-invasive mutants were obtained in which the transposon had inserted upstream from and inactivated expression of two open reading frames, *inlA* and *inlB*.

The role of *inlA* was demonstrated by expression in the non-invasive bacterium *Listeria innocua*, which became able to invade Caco-2 cells and suggested that the InlA protein may be sufficient for entry (Gaillard *et al.*, 1991); hence the name 'internalin' given to this protein. The role of the second gene was elucidated by deleting each of these two genes and testing the corresponding mutants in various cell lines (Dramsi *et al.*, 1995, 1997; Ireton *et al.*, 1996). These experiments showed that InlB is also an invasion protein involved in entry into some hepatocyte-like cell lines, HeLa cells, Vero cells, CHO cells and fibroblasts, and suggested that the *inlAB* operon may be involved in cell tropism.

Internalin, a LRR protein sufficient for entry

Internalin A is an 800 amino acid protein which displays two regions of repeats, the first being a succession of 15, 22 amino acid-long leucine-rich repeats (LRRs) (Figure 4). It has the features of a protein which is targeted to and exposed on the bacterial surface, i.e. a signal peptide, and a C-terminal region containing a LPXTG peptide

Fig. 4. Schematic representation of InlA and InlB.

followed by a hydrophobic sequence and a few charged residues. This type of C-terminus is found in >50 Gram-positive bacterial surface proteins (Fischetti *et al.*, 1990) and allows covalent linkage of the protein to the peptidoglycan, after cleavage of the T-G link (Schneewind *et al.*, 1995). Several lines of evidence indicate that internalin is sufficient for entry. Indeed, expression of *inlA* not only in *L.innocua* but also in another Gram-positive bacterium such as *Enterococcus faecalis*, confers invasiveness (Lecuit *et al.*, 1997). Moreover, latex beads coated with internalin are invasive (Lecuit *et al.*, 1997).

E-cadherin is the epithelial cell receptor for internalin

The internalin receptor was identified by a chromatography affinity approach with an internalin column (Mengaud *et al.*, 1996a). Two polypeptides from Caco-2 cells were retained and their N-terminal sequence determined. Comparison with protein sequence data banks revealed that these two polypeptides were human E-cadherin and its proteolytic fragment normally produced in the conditions used to prepare the extracts (Mengaud *et al.*, 1996a).

E-cadherin is a transmembrane glycoprotein which mediates calcium-dependent cell-cell adhesion through homophilic interactions between extracellular domains. Cadherins are specifically expressed in different tissues, E-cadherin in epithelial cells, N-cadherin in neuronal cells, etc. (Geiger and Ayalon, 1992). These proteins play a critical role in cell sorting during development and in maintenance of tissue cohesion and architecture during adult life. In polarized epithelial cells, E-cadherin is mainly expressed at the adherens junctions and on the basolateral face. Integrity of the intracytoplasmic domain of cadherins is required for optimal intercellular adhesion (Nagafuchi

and Takeichi, 1988). This domain interacts with proteins named catenins, which in turn interact with the actin cytoskeleton, highlighting the importance of the cytoskeleton in maintaining adhesion of adjacent epithelial cells (Kemler, 1993).

A set of transfected cell lines was used to demonstrate that the internalin–E-cadherin interaction promotes not only specific binding but also entry of *L.monocytogenes* (Mengaud *et al.*, 1996a). Both *L.monocytogenes* or *L.innocua* expressing internalin enter efficiently into cells expressing the chicken E-cadherin (L-CAM), in contrast to cells expressing N-cadherin or no cadherin. Similar results have also recently been obtained with internalin-coated beads (Lecuit *et al.*, 1997), establishing definitively that E-cadherin acts as a receptor for internalin and promotes entry. We are currently investigating how internalin interacts with E-cadherin. Recent data suggest that the LRRs region interacts directly with E-cadherin (Mengaud *et al.*, 1996b), but the particular region in the extracellular domain of E-cadherin involved in this interaction has not yet been identified. Preliminary experiments indicate that the cytoplasmic domain of E-cadherin is required for entry of the bacteria but not adhesion (our unpublished results).

Given the basolateral localization of E-cadherin in polarized epithelial cells, our data suggest that, *in vivo*, *L.monocytogenes* does not penetrate the intestinal barrier by the apical pole of enterocytes. This hypothesis is in agreement with *in vitro* observations that *Listeria* preferentially invades Caco-2 cell islets by their basolateral face (Gaillard and Finlay, 1996). If entry into enterocytes occurs, this would represent a secondary step taking place at their basolateral face after translocation through M cells or between epithelial cells, as has been proposed for *Shigella* (Perdomo *et al.*, 1995).

The role *in vivo* of internalin is still unclear. Indeed, after oral inoculation of mice, an *inlA* mutant reaches the mesenteric lymph nodes and the liver as rapidly as in the wild-type strain (Drams *et al.*, 1995; Gaillard *et al.*, 1996; Pron *et al.*, 1998). *InlA* could play a role in a subsequent step of the infectious process such as dissemination into the liver or to its final targets, the brain and placenta.

***InlB*, an invasion protein with GW modules, a novel cell wall association motif**

InlB is found both at the bacterial surface and to some extent in bacterial culture supernatants (Drams *et al.*, 1995). It has a signal sequence and eight tandem leucine-rich repeats (LRRs) very similar to those of *InlA* (Figure 4). However, *InlB* does not contain the LPXTG motif or any hydrophobic region which would indicate a possible transmembrane region. Recent experiments indicate that the 231 C-terminal amino acids of *InlB* are necessary and sufficient to anchor *InlB* to the bacterial surface (Braun *et al.*, 1997). This region contains tandem repeats of ~80 amino acids beginning with the sequence GW. Interestingly, similar repeats are found in a newly identified surface protein of *L.monocytogenes*, named Ami, and to a lesser extent in lysostaphin. Lysostaphin is secreted by *Staphylococcus simulans* and associates with the cell wall of *Staphylococcus aureus*, even when added exogenously (Baba and Schneewind, 1996). *InlB* is also able, when externally added, to associate with *L.monocytogenes* and

several other Gram-positive bacteria. This external association leads to entry of a *ΔinlB* mutant and also promotes entry of the non-invasive species *Staphylococcus carnosus*. These results suggest not only that *InlB* may interact with the cell wall after secretion or release from the bacterial surface but also that this interaction could contribute to invasion (Braun *et al.*, 1997). We have recently shown that latex beads coated with *InlB* enter efficiently into Vero cells, demonstrating that *InlB* is sufficient for entry (Braun *et al.*, 1998). The *InlB* receptor remains elusive but it is clear that it is not E-cadherin. In the mouse model, *InlB* appears to play a role in the hepatic phase of the infection (Drams *et al.*, 1995; Gaillard *et al.*, 1996). *InlB* mutants replicate less efficiently in the liver, though whether *InlB* mediates entry in hepatocytes *in vivo* or plays a role in survival is unclear (Gaillard *et al.*, 1996; Gregory *et al.*, 1996, 1997).

***PI-3 kinase*, a signalling protein required for and activated upon entry**

Treatment of cells with either tyrosine kinase inhibitors (Tang *et al.*, 1994; Velge *et al.*, 1994) or cytochalasin D (Gaillard *et al.*, 1987) inhibit entry but not adhesion of *Listeria*, suggesting that bacterial invasion requires tyrosine kinase activity and an intact actin cytoskeleton. Recent evidence indicates that the PI-3 kinase P85/P110, a signalling protein implicated in actin polymerization in response to receptor stimulation and tyrosine phosphorylation, is involved in entry of *L.monocytogenes* in mammalian cells (Ireton *et al.*, 1996).

Upon receptor stimulation, PI-3 kinase translocates from the cytosol to a membrane-associated tyrosine-phosphorylated protein, i.e. an activated tyrosine kinase receptor or a tyrosine-phosphorylated adaptor protein. Migration to the plasma membrane stimulates activity of PI-3 kinase, by placing the enzyme in a compartment where its substrates are located and also by inducing conformational changes after protein–protein interactions. PI-3 kinase phosphorylates the D3 position of the inositol ring of PI, PIP and PIP₂ giving rise to PI₃P, PI₃,4P₂ and PI₃,4,5P₃ (Carpenter and Cantley, 1996). These last two phosphoinositides are virtually absent in resting cells and their levels increase dramatically upon stimulation. PI₃,4P₂ and PI₃,4,5P₃ are not substrates for known phospholipases and appear to act as second messengers by interacting with various kinases such as the serine/threonine kinase Akt (Franke *et al.*, 1997).

Pretreatment of various mammalian cells with wortmannin, a fungal metabolite which specifically inhibits PI-3 kinase, inhibits *Listeria* entry (Ireton *et al.*, 1996). In addition, expression of a dominant-negative form of P85 (ΔP85) also inhibits *Listeria* entry, establishing that the P85/P110 PI-3 kinase is required for entry (Ireton *et al.*, 1996). Measurements of the levels of cellular phosphoinositides reveal that entry of *Listeria* stimulates synthesis of both PI₃,4P₂ and PI₃,4,5P₃ in Vero cells. This synthesis is inhibited by pretreating cells with wortmannin, but not by pretreatment with cytochalasin D, suggesting that cytoskeleton rearrangements may occur downstream from the PI-3 kinase stimulation during bacterial invasion. Interestingly, PI-3 kinase stimulation is inhibited by genistein, an inhibitor of tyrosine kinases. Accordingly, shortly after infection, PI-3 kinase associates with at least

one tyrosine-phosphorylated protein and it is possible that this association stimulates PI-3 kinase activation.

A Δ *inlB* mutant still adheres to Vero cells but does not efficiently stimulate PI-3 kinase and does not stimulate association of PI-3 kinase with tyrosine-phosphorylated proteins. Thus, InlB seems to play a critical role in the stimulation of P85/P110.

How stimulation of PI-3 kinase affects bacterial invasion is not known, but one possible mechanism is by controlling actin polymerization or reorganization of the actin cytoskeleton. PI3,4P2 and PI3,4,5P3 are able to uncap barbed ends of actin filaments in permeabilized platelets, suggesting a simple means by which stimulation of PI-3 kinase activity by adherent bacteria could drive local cytoskeletal changes needed for entry (Hartwig *et al.*, 1995). It is also possible that a high concentration of phosphoinositides may affect the local curvature of the lipid bilayer, facilitating bacterial internalization. The generation of phospholipids in the membrane may also attract proteins which bind specifically to these phospholipids that are normally absent, e.g. proteins with PH domains (Toker and Cantley, 1997). PI-3 kinase itself can interact with other signalling proteins that regulate organization of the actin cytoskeleton, such as pp125FAK (Chen and Guan, 1994) or Rho-GTPases (Tolias *et al.*, 1995), and such interactions may play a role in entry. PI-3 kinase has also been implicated in endocytic processes (Araki *et al.*, 1996) and it is possible that some of the components used for endocytosis are also employed by bacterial pathogens to gain entry into host cells. Intriguingly, the internalin–E-cadherin-mediated entry in Caco-2 cells, which is also affected by wortmannin, does not stimulate PI-3 kinase activity. In these cells, the basal levels of PI3,4P2 and PI3,4,5P3 do not increase significantly upon entry. In fact, these lipids are already present at high concentration in uninfected Caco-2 cells. Thus in Caco-2 cells, the internalin–E-cadherin pathway may exploit a pre-activated PI-3 kinase. The future challenges are now to identify the upstream tyrosine-phosphorylated proteins and the downstream targets of PI3,4,5P3. Preliminary experiments indicate that one of the phosphorylated proteins is c-Cbl (Ireton and Cossart, 1997). A very recent study in HeLa cells in which entry is InlB-dependant demonstrates a role for MEK1 mitogen activated protein (MAP) kinase kinase/ERK2 in entry. Treatment with wortmannin does not affect ERK2 activation. How the PI-3 kinase pathway and MEK1/ERK2 pathway contribute to entry in HeLa cells deserve investigation (Tang *et al.*, 1998).

The two pathways of entry

Listeria can enter into mammalian cells by at least two pathways (Figure 5). One of them, the internalin–E-cadherin pathway, can be compared with that of *Yersinia*, in which the bacterial factor Invasin interacts with a β 1-integrin, a cell adhesion molecule known to be connected to the cytoskeleton (Tran Van Nhieu and Isberg, 1993). Although the molecules involved are different, some functional similarity exists between the two systems, such as the sensitivity to tyrosine kinase inhibitors and to cytochalasin D. The other strategy for entry is InlB-dependent. This pathway seems to share similarities with growth factor-mediated signalling pathways. As evidenced

Fig. 5. The InlA and InlB pathways of entry in mammalian cells.

by the residual level of entry of a Δ *inlAB* mutant, other mechanisms of entry exist. InlA and InlB are two members of the internalin multigene family which contains five other members—InlC, InlD, InlE and InlF—and it was anticipated that these genes could also play a role in entry, but this does not seem to be the case (Engelbrecht *et al.*, 1996; Domann *et al.*, 1997; Dramsi *et al.*, 1997). A recent report indicates that ActA, the protein involved in actin polymerization, could also participate in invasion (Alvarez-Dominguez *et al.*, 1997).

Escape from the phagosome

Once internalized, *Listeria* lyses the vacuolar membrane. This event is achieved by a potent pore-forming toxin, listeriolysin O (LLO) (Gaillard *et al.*, 1987; Bielecki *et al.*, 1990). Mutants not expressing LLO are avirulent in the mouse, demonstrating that escape of the bacteria to the cytosol is critical for the establishment of the infection (Gaillard *et al.*, 1986; Portnoy *et al.*, 1988; Bielecki *et al.*, 1990). The bacterial phosphatidyl inositol phospholipase C (PI-PLC), is also involved in the lysis of the vacuole, albeit to a lesser extent (Camilli *et al.*, 1991). Another bacterial factor, namely ClpC, a member of the Clp-ATPase family of stress proteins (Rouquette *et al.*, 1996), also contributes to the escape from the phagosome, at least in macrophages (Rouquette *et al.*, 1998).

Apart from its crucial role in the escape from the vacuole, LLO is also able to induce apoptosis (Guzman *et al.*, 1996), stimulate MAP kinases (Tang *et al.*, 1996), and contribute to induction in expression of cell-adhesion molecules in infected endothelial cells (Drevets, 1997; Krull *et al.*, 1997). The function of LLO is thus not restricted to vacuolar lysis and listeriolysin appears to act as a multifunctional factor.

Actin-based motility

Listeria has evolved an efficient mechanism that couples actin polymerization to intracellular movement. This actin-based motility has recently received a great deal of attention since it is reminiscent of cellular events that occur

Fig. 6. ActA, a composite protein.

at the leading edge of moving cells such as neutrophils migrating to a site of infection, or metastatic cancer cells and which remain elusive. Hence the enthusiasm for a system which, by its relative simplicity, can be manipulated and studied more easily. Similar mechanisms of actin-based motility are used by *Shigella flexneri*, *Rickettsia* and Vaccinia virus (for reviews, see Cossart, 1995, 1997; Theriot, 1995; Finlay and Cossart, 1997; Ireton and Cossart, 1997; Smith and Portnoy, 1997; Dramsi and Cossart, 1998; Lasa *et al.*, 1998).

The relationship between actin tail formation and movement

The early observations of thin sections of *Listeria*-infected cells decorated with the S1 fragment of myosin revealed that the actin tails are made of cross-linked short filaments with barbed (fast-polymerizing) ends oriented towards the bacterium, and suggested that actin polymerization takes place at the rear of the bacterium (Tilney and Portnoy, 1989). Videomicroscopy of infected cells microinjected with fluorescent actin monomers then demonstrated that the actin polymerization takes place at one pole of the bacterium and that the bacterium moves away while the actin tail remains stationary in the cytosol (Dabiri *et al.*, 1990; Sanger *et al.*, 1992; Theriot *et al.*, 1992). The rate of tail formation and bacterial speed are strictly correlated, suggesting that the force for propulsion is provided by actin polymerization itself (Theriot *et al.*, 1992).

The ActA protein

ActA was discovered by analysing mutants which were completely unable to polymerize actin and move inside cells (Domann *et al.*, 1992; Kocks *et al.*, 1992). These mutants form intracellular microcolonies, are unable to spread from cell to cell, and are avirulent when injected into mice. The gene inactivated in these mutants, *actA*, encodes a 610 amino acid protein.

ActA has the features of a protein targeted to the bacterial surface (Figure 6), including a signal sequence and a C-terminal hydrophobic region which may anchor this protein in the bacterial membrane. ActA has a polar distribution on the bacterial surface, with a higher distribution at one pole of the bacterium (Kocks *et al.*, 1993). In infected cells, ActA is not released in the tail (Kocks *et al.*, 1993; Niebuhr *et al.*, 1993) but is located at the base of the actin tail, suggesting that its polar distribution predetermines the site of actin assembly and the direction of movement (Kocks *et al.*, 1993).

ActA is sufficient to induce actin polymerization and movement

To determine whether ActA is sufficient to induce actin polymerization, the *actA* gene was transfected into mam-

malian cells where it was able to induce the polymerization of globular actin (G-actin) into filamentous actin (F-actin) (Pistor *et al.*, 1994; Friederich *et al.*, 1995). Two approaches were used to demonstrate that ActA is not only sufficient to induce actin polymerization, but also produces movement: (i) expression of ActA in the non-pathogenic species *L. innocua* (Kocks *et al.*, 1995) and conversion of this normally non-motile bacterium into an organism capable of actin polymerization and movement; and (ii) incubation of *Streptococcus pneumoniae* with a recombinant ActA-LytA hybrid protein (Smith *et al.*, 1995) which, when adsorbed onto the pneumococcal surface, allowed the bacteria to polymerize actin and move. These experiments were done using *Xenopus* cytoplasmic extracts, a very instrumental *in vitro* system now commonly used to observe *Listeria* movement (Theriot *et al.*, 1994; Marchand *et al.*, 1995).

Homologies between ActA and other proteins

The ActA protein can be artificially divided into three parts, an N-terminal domain (aa 1–233) which is highly charged, a central proline-rich repeat region (aa 234–395) and a C-terminal region (aa 396–610). Recent amino acid sequence comparisons have revealed that ActA is a composite protein. The proline-rich repeats and the C-terminal domain share significant sequence similarity with zyxin, a protein associated with focal contacts and actin stress fibres (Golsteyn *et al.*, 1997). The N-terminal domain of ActA is similar (25% identity) to the C-terminal region (aa 879–1066) of vinculin, which is a protein recently shown to be able to bind actin precisely through its C-terminal region (Gilmore and Burridge, 1996; Lasa *et al.*, 1998). Thus, ActA seems to have domains similar to eukaryotic proteins involved in cytoskeleton organization (Figure 6).

Cellular factors involved in actin-based motility

Early attempts to demonstrate interactions between ActA and actin have failed, suggesting that this protein does not interact directly with actin and that at least another cellular factor is involved in the actin polymerization process.

To identify such factors, the main approach has been immunofluorescence of fixed infected cells using antibodies to known cytoskeletal proteins (for reviews, see Ireton and Cossart, 1997; Dramsi and Cossart, 1998; Lasa *et al.*, 1998). Proteins identified by this technique include α -actinin, tropomyosin, vinculin, villin, talin, ezrin/radixin, fimbrin, cofilin, profilin and VASP. Only profilin and VASP co-localize with the beginning of the actin tail. The other proteins are present all along the tail. VASP was shown to bind purified ActA *in vitro*, establishing the first direct link between ActA and the cytoskeleton (Chakraborty *et al.*, 1995). Since VASP binds profilin, co-localization of these two proteins at the beginning of the actin tail is probably due to VASP binding to ActA (Chakraborty *et al.*, 1995); however, this has not yet been demonstrated.

The role of some of the other proteins was assessed by various approaches. In the case of profilin, depletion of cytoplasmic extracts with polyproline beads was used. Bacterial movement was then tested in the depleted extracts. Clearly, most profilin can be depleted without

Fig. 7. Current model of actin assembly.

affecting bacterial motility (Theriot *et al.*, 1994; Marchand *et al.*, 1995). The role of α -actinin was assessed by microinjection of a fragment which acted as a dominant-negative mutant and inhibited movement. The results demonstrated that this cross-linking protein is required for efficient actin tail formation and movement (Dold *et al.*, 1994).

A different approach has led to the identification of other cellular factors involved in actin-based motility (Welch *et al.*, 1997a,b). Cytoplasmic extracts isolated from human platelets were fractionated and a seven-polypeptide complex was purified which was sufficient to initiate ActA-dependent actin polymerization at the surface of *L. monocytogenes*. This complex is similar to that originally isolated from *Acanthamoeba* by affinity on a profilin column (Machesky *et al.*, 1994). Two subunits of this protein complex are actin-related proteins (Arps) belonging to the Arp2 and Arp3 subfamilies. The Arp3 subunit, as well as p34-Arc and p21-Arc, localize to the surface of stationary bacteria and the tails of motile bacteria in *L. monocytogenes*-infected tissue culture cells, consistent with a role for the complex in promoting actin assembly *in vivo*. Whether this complex is responsible for actin nucleation in intact host cells and whether it binds to the amino-terminal part of ActA are not yet known.

Cofilin/ADF, which belongs to a group of small (15–22 kDa) actin-binding proteins, was also identified as being involved in the *Listeria* actin-based motility. Cofilin/ADF is a protein which has affinity for ADP-actin, can increase the off rate at the pointed ends of actin filaments, and enhance filament turnover. Both depletion and addition experiments in extracts indicate that cofilin is critical for the dynamics of the process (Carlier *et al.*, 1997; Rosenblatt *et al.*, 1997).

Genetic analysis of ActA

In order to identify the regions of ActA critical for its function, deletions in *actA* were generated which demonstrated that the N-terminal (ActAN) is absolutely essential and that the central region acts as a stimulator of movement (Lasa *et al.*, 1995, 1997). These results were confirmed by expressing in *Listeria* an ActAN–LacZ fusion which was functional for movement (Lasa *et al.*, 1997). It was also shown that in infected cells, VASP co-localizes with ActA-expressing bacteria only if the central region of ActA is present, suggesting that VASP binds to the proline-rich region of ActA, a result recently confirmed by Niebuhr *et al.* (1997).

A recent analysis of the N-terminus of ActA has demonstrated that this region contains two critical regions involved in actin polymerization (Lasa *et al.*, 1997). These

Fig. 8. Common strategies/specific features.

two regions (region T, aa 116–122; region C, aa 21–97) are not involved in the early steps of the process because their deletion does not abrogate actin assembly. Deletion of region T prevents tail formation. Deletion of region C leads to discontinuous actin tails (see below).

Current hypothesis

Our current view of actin-based motility is that in wild-type bacteria, the apparently continuous movement occurs in three steps: (i) the generation of free barbed ends, by either nucleation, or severing or uncapping of pre-existing filaments; (ii) monomer addition and movement; and (iii) continuous filament release/capping/cross-linking and generation of new free barbed ends. It is probable that the balance between these last two events allows continuity of the process. We think that in the $\Delta 21-97$ mutant (Lasa *et al.*, 1997), free barbed ends are generated and polymerization takes place, but capping occurs more rapidly than in the wild-type, so that bacteria are stalling until a critical number of free barbed ends is reached. These observations have led us to propose that ActA could play a role in protection of filament ends from capping proteins. This hypothesis implies that ActA could interact with actin and would, as vinculin, contain a cryptic actin binding site which has to be activated to be detected. In fact, as we have recently shown, a peptide spanning aa 30–50 can bind actin (Lasa *et al.*, 1997).

All these considerations led to a model, presented in Figure 7 (see also Dramsi and Cossart, 1998; Lasa *et al.*, 1998), in which we have incorporated the recent information that ActA is a dimer (Mourrain *et al.*, 1997). In the model, the central part of the ActA dimer binds VASP, which is a tetramer. VASP binds profilin, which binds actin monomers that can be used by the N-terminal part to elongate free barbed ends. These filament ends are protected from capping proteins by the N-terminal part of ActA. Whether Arp2 and Arp3 are recruited by the N-terminal domain of ActA or whether there is another factor involved remains to be established. It is thus possible that ActA has several functions, one of them being to protect filaments from capping proteins.

Concluding remarks

Listeria monocytogenes is an invasive bacterium which has some features in common with other invasive pathogens (Figure 8). It enters cells by a 'zipper' mechanism similar

to the Invasin-mediated type of entry of *Yersinia*. This 'zipper' mechanism is different from the 'trigger' mechanism used by *Shigella* or *Salmonella*. The proteins used for entry by *Listeria* (InlA and InlB) and *Yersinia* (Invasin) differ, while those used by *Shigella* (IpaB) and *Salmonella* (Sips) are similar. Later stages of the *Listeria* infection are similar to those of *Shigella*, which is also internalized in a vacuole, escapes from this compartment and spreads from cell to cell using an actin-based motility process (Figure 8). In *Listeria*, these different steps are mediated by LLO, ActA and a lecithinase (PLC-B), while in *Shigella*, they are mediated by the unrelated proteins IpaB, IcsA and IcsB. These findings illustrate that bacteria have evolved a variety of factors to exploit mammalian cell functions. These factors may provide useful tools to address basic questions in cell biology (Cossart *et al.*, 1996; Finlay and Cossart, 1997; Finlay and Falkow, 1997; Dramsi and Cossart, 1998). For example, the three main proteins discussed in this review, internalin A, internalin B and ActA can be used and manipulated to address cadherin function, PI-3 kinase activation and signalling, and actin-based motility.

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