InlB, a surface protein of *Listeria monocytogenes* that behaves as an invasin and a growth factor

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Summary

Molecules from some pathogenic bacteria mimic natural host cell ligands and trigger engulfment of the bacterium after specifically interacting with cell-surface receptors. The leucine-rich repeat (LRR)-containing protein InlB of *Listeria monocytogenes* is one such molecule. It triggers bacterial entry by interacting with the hepatocyte growth factor receptor (HGF-R or Met) and two other cellular components: gC1q-R and proteoglycans. Recent studies point to significant similarities between the molecular mechanisms underlying InlB-mediated entry into cells and classic phagocytosis. In addition, InlB, in common with HGF, activates signaling cascades that are not involved in bacterial entry. Therefore, studies of InlB may help us to analyze the previously noticed similarities between growth factor receptor activation and phagocytosis.

Key words: HGF, Met, gC1q-R, PI 3-kinase, Cytoskeleton, Phagocytosis

Introduction

Some bacterial pathogens have evolved sophisticated strategies to enter and 'invade' cells that are non-professional phagocytes, such as epithelial cells underlying mucosal surfaces or endothelial cells inside blood vessels (Finlay and Falkow, 1997; Finlay and Cossart, 1997). These strategies allow them to cross tight tissue barriers and to proliferate in protected niches, escaping the first host immune defenses such as circulating antibodies and complement. Two general mechanisms of bacterium-induced-phagocytosis have been described (Dramsi and Cossart, 1998). Both require cytoskeletal rearrangements and remodeling of the plasma membrane. In the 'trigger' mechanism, a bacterium in contact with the cell delivers directly into the host cytoplasm virulence factors that activate signal transduction pathways, leading to the capture of the bacterium into large membrane ruffles (Cornelis and Van Gijsegem, 2000; Tran Van Nhieu et al., 2000; Galan, 2001). In the other pathway, the 'zipper-like' mechanism, a bacterial ligand interacts with and activates a mammalian receptor, which results in the tight envelopment of the bacterial body by the cell membrane (Ireton and Cossart, 1997), a phenomenon reminiscent of phagocytosis in macrophages (Swanson and Baer, 1995).

Listeria monocytogenes, a Gram-positive bacterium responsible for serious infections in immunocompromised people and pregnant women, promotes its own internalization into various cell types by the zipper mechanism (Cossart and Bierne, 2001; Vazquez-Boland et al., 2001; Cossart, 2002). A genetic screen for *Listeria* mutants unable to enter mammalian cells led to the discovery of two related leucine-rich repeat (LRR)-containing proteins involved in that process: InlA (also called internalin) and InlB (Gaillard et al., 1991; Dramsi et al., 1995). Both proteins induce particle uptake when coated on latex beads, which suggests that they are sufficient for internalization (Lecuit et al., 1997; Braun et al., 1998). InlA-mediated entry is restricted to a few epithelial cells, whereas InlB promotes entry into various cell types, such as hepatocytes, epithelial cells and endothelial cells (Dramsi et al., 1995; Greiffenberg et al., 1998; Lingnau et al., 1995; Parida et al., 1998). This tropism is determined by specific host cell receptors. InlA is a ligand for E-cadherin, a cell adhesion molecule present in epithelial tissues and involved in the formation of intercellular junctions (Mengaud et al., 1996). InlB is an agonist of the hepatocyte growth factor receptor (HGF-R/Met), a widely expressed receptor tyrosine kinase involved in complex cellular processes, such as cell proliferation, dissociation, migration and differentiation (Shen et al., 2000). InlB also interacts with gC1q-R, a ubiquitous glycoprotein (Braun et al., 2000), and with proteoglycans (Jonquieres et al., 2001) that might potentiate interactions with Met.

Our knowledge of InlA- and InlB-mediated entry pathways has recently improved. Studies of InlA point to an essential role of this protein in the crossing of the human intestinal barrier (Lecuit et al., 1999; Lecuit et al., 2001). At the cellular level, our knowledge of the signals transduced downstream of the InlA-E-cadherin interaction is still fragmentary (Lecuit et al., 2000). By contrast, signaling pathways activated by InlB have been dissected in more detail, revealing the strikingly potent signaling properties of InlB.

Phagocytosis and signaling via Fc receptors in macrophages share many characteristic features with those of growth factor receptors activation (Castellano et al., 2001; Cox and Greenberg, 2001). Here, we highlight how InlB, the first-identified bacterial agonist of a receptor tyrosine kinase, bridges these two biological processes, bringing them closer than ever. InlB thus might prove as instrumental as ActA, IcsA and other bacterial factors (Finlay and Cossart, 1997; Cossart, 2000; Stebbins and Galan, 2001) in addressing key issues in cell biology.

A modular protein with two functional domains

InlB is a 67 kDa protein whose primary structure is mainly characterized by two regions of repeats located at the N- and C-terminus (Fig. 1A).

The LRRs-IR 'internalin' domain

InlB is a member of the internalin-related protein family, which contains 24 members in *L. monocytogenes* (Glaser et al., 2001), including the other invasion protein InlA. Most internalin-like proteins have a short N-terminal conserved cap region, followed by several leucine-rich tandem 22-residue repeats (LRR) and an inter-repeat (IR) region (Dramsi et al., 1997; Schubert et al., 2001). In some cases, a second repeat region of up to three repeats of ~70 residues, the B-repeats, is present. InlB possesses eight LRRs and one B repeat. Although all of this domain is necessary for efficient internalization by InlB, the N-terminal 213-residue region (the cap and LRR) is sufficient to induce entry of bacteria or InlB-coated beads into cells and to activate signal transduction pathways (Braun et al., 1999; Shen et al., 2000).

The crystal structure of this domain reveals that it is a long and slightly curved tube made of successive β loop-3_{10}helix-loop motifs (Fig. 1B) (Marino et al., 1999; Marino et al., 2000). This structure shares similarities with those of previously described LRR-containing proteins, such as the porcine and human ribonuclease inhibitors (Kobe and Deisenhofer, 1993; Papageorgiou et al., 1997) and the U2LRR fragment of the U2 snRNP (Price et al., 1998). Recently, the structure of the whole cap-LRR-IR domain was also solved, confirming the curved and elongated shape of the LRR region but also revealing some interesting properties of the cap and IR regions (Schubert et al., 2001). The cap region is a truncated EF-hand-like domain, comparable to one of the tandem EF-hand calcium-binding domains identified in calmodulin and related proteins (Babu et al., 1988; Flaherty et al., 1993). However, the presence of potential calcium-binding sites in this region is controversial (Marino et al., 1999; Schubert et al., 2001). The IR region is structurally related to the immunoglobulin (Ig)-like domain, several copies of which are present in antibodies and numerous eukaryotic cell-surface proteins (Harpaz and Chothia, 1994). It is not yet known whether this Ig-like domain makes specific contacts with eukaryotic cell-surface proteins or whether it has only a structural role in stabilizing the LRR region. The curvature of the internalin domain makes it ideally shaped to embrace globular protein domains. Interestingly, LRR and Ig-like domains in other bacterial proteins are mostly found in virulence factors (Kajava, 1998; Schubert et al., 2001). The fusion of these two domains in InlB and in other internalins may represent an optimal adaptation to its eukaryotic host during evolution.

The bacterial-surface-anchoring domain

The C-terminal region of InlB contains three tandem ~80-residues repeats, which are highly basic and start with the dipeptide GW (Braun et al., 1997). These repeats mediate a loose association of the protein with the bacterial surface, mainly through non-covalent interactions with lipoteichoic acid, a membrane-anchored polymer present on the surface of Gram-positive bacteria (Jonquieres et al., 1999). Strikingly, they also confer on InlB the unusual property of adhering to bacteria when added from the extracellular medium. Association/re-association of InlB with bacteria after secretion or release from the bacterial surface might play an important role during invasion of cells. Indeed, InlB is buried in the bacterial cell wall, and this puzzling localization suggested that external factors regulate its accessibility, possibly by acting on GW repeats. This hypothesis is supported by the demonstration that GW repeats bind to cellular proteoglycans and that these interactions are required for efficient entry (see below) (Jonquieres et al., 2001).

Host cell receptors

HGF-R/Met

InlB, when present at the bacterial surface, mediates entry into the host cell by zipper-type phagocytosis. Strikingly, soluble

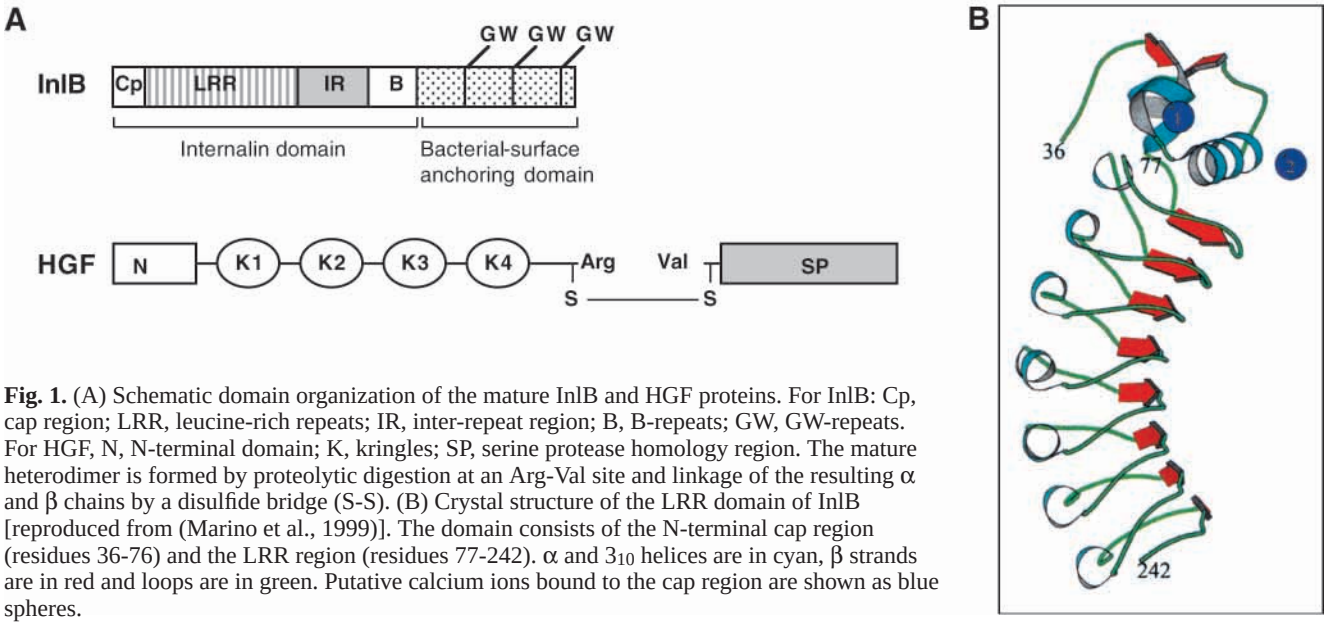

Fig. 1. (A) Schematic domain organization of the mature InlB and HGF proteins. For InlB: Cp, cap region; LRR, leucine-rich repeats; IR, inter-repeat region; B, B-repeats; GW, GW-repeats. For HGF, N, N-terminal domain; K, kringles; SP, serine protease homology region. The mature heterodimer is formed by proteolytic digestion at an Arg-Val site and linkage of the resulting α and β chains by a disulfide bridge (S-S). (B) Crystal structure of the LRR domain of InlB [reproduced from (Marino et al., 1999)]. The domain consists of the N-terminal cap region (residues 36-76) and the LRR region (residues 77-242). α and 3_{10} helices are in cyan, β strands are in red and loops are in green. Putative calcium ions bound to the cap region are shown as blue spheres.

InlB induces membrane ruffling, which is well known to be induced by growth factors. Both phenomena coincide with tyrosine phosphorylation of several proteins, including the adapter proteins Gab1, Cbl and Shc, and with phosphoinositide (PI) 3-kinase activation (Ireton et al., 1999). Furthermore, they can both be inhibited by pretreatment of cells with tyrosine kinase inhibitors and/or PI 3-kinase inhibitors. These observations suggested that InlB activates a growth factor receptor, which was recently identified as Met (Shen et al., 2000). Met signaling plays important roles in the regulation of several processes, such as development and tissue regeneration (Jiang and Hiscox, 1997; Birchmeier and Gherardi, 1998; Stella and Comoglio, 1999), as well as tumor invasiveness (Trusolino and Comoglio, 2002). It is now also implicated in *Listeria* invasion.

Met is a disulfide-linked heterodimer composed of a 45 kDa extracellular α-subunit and a 145 kDa transmembrane β-subunit, which contains the tyrosine kinase catalytic domain (Furge et al., 2000). Receptor activation is mediated in part by autophosphorylation of specific tyrosine residues within the intracellular region. Phosphorylation of two tyrosine residues (Y1234 and Y1235) within the tyrosine kinase domain activates the intrinsic kinase activity of the receptor, whereas the two phosphorylated tyrosine residues in the C-terminus (Y1349 and Y1356) form a specific docking site for multiple signal transducers and adapters. In common with the natural ligand HGF, purified InlB stimulates the sequential tyrosine phosphorylation of Met, recruitment and phosphorylation of Gab1, Cbl and Shc, and formation of complexes containing these adapters and the p85 subunit of PI 3-kinase.

InlB interacts with the extracellular domain of Met through its LRR domain, but the full-length protein is required for maximal activation (Shen et al., 2000). Interestingly, several pieces of evidence indicate that InlB does not strictly mimic HGF. First, HGF and InlB do not share sequence similarity. HGF is a disulfide-linked αβ heterodimer that shows structural similarity to enzymes of the blood coagulation cascade (Stella and Comoglio, 1999). The 69 kDa α subunit contains an N-terminal hairpin loop and four kringle domains, and the 34 kDa β subunit contains a catalytically inactive serine proteinase domain (Fig. 1A). The N and first kringle domain (NK1) in the α chain are sufficient to bind to Met, but the crystal structures of the LRR in InlB and the NK1 in HGF seem structurally unrelated (Chirgadze et al., 1999). Second, InlB and HGF do not seem to interact with Met at the same site, because an excess of HGF does not inhibit binding of InlB to Met (Shen et al., 2000). Third, InlB-induced phosphorylation of Met is more transient (peaking after 10-20 minutes and undetectable at 60 minutes) than that produced by HGF (which remains unchanged after two hours). Whether the difference in binding-site location explains this difference in the duration remains to be established. Interestingly, differences in the kinetics of Met activation seem to induce divergent biological responses triggered by this receptor (Boccaccio et al., 2002). In common with HGF, InlB stimulates scattering of MDCK cells; however, whether InlB can elicit all of the complex responses induced by HGF, such as mitogenesis and morphogenesis, is not known. These responses might also depend on whether Met is activated by bacterial-surface-bound InlB or by a soluble form released in the extracellular medium.

Proteoglycans

Activation of Met by HGF is enhanced by glycosaminoglycans (GAGs), such as heparan sulfates, which are negatively charged polysaccharides present at the surface of all cell types (Trusolino et al., 1998). GAGs can be secreted into the extracellular medium but usually decorate a protein moiety in proteoglycans. Proteoglycans are required for optimal activity of HGF and many other growth factors (Rusnati and Presta, 1996; Kresse and Schonherr, 2001; Rubin et al., 2001), possibly immobilizing them at the cell surface, protecting them from degradation, transferring them to the high-affinity receptors and facilitating their oligomerization. Interestingly, InlB also binds to GAGs through its GW repeats, and the presence of GAGs on the cell surface significantly increases InlB-dependent invasion (Jonquieres et al., 2001). In addition, the internalin domain of InlB is less efficient in activating Met and in inducing cell scattering than the full-length InlB protein (Shen et al., 2000). Thus, binding of GW repeats to cellular GAGs could enhance interaction of the LRR domain with Met.

Soluble heparin detaches InlB from the bacterial surface and induces InlB clustering, as it does with HGF. This suggests that GAGs compete with lipoteichoic acid for binding to GW repeats. Although GAGs and lipoteichoic acid are structurally different, they each have a highly negative charge density, whereas GW repeats are highly basic. GAGs might therefore stimulate the release of InlB as a soluble factor, which could act as a growth factor independently of invasion. As in the case of HGF, cellular responses to InlB might depend on the GAG composition of the target cell surface.

gC1q-R

InlB interacts with another cellular protein, gC1q-R, identified through affinity chromatography (Braun et al., 2000). gC1q-R is a highly acidic multiligand-binding glycoprotein of 33 kDa that is predominantly associated with the mitochondria and the nucleus but also found at the cell surface and in body fluids. Originally identified as the receptor for the globular head of C1q, the first component of the complement cascade, gC1q-R is in fact a multifunctional protein that has affinity for diverse ligands, including plasma, cellular and microbial proteins (Ghebrehiwet et al., 2001). It interacts with several viral proteins, such as HIV-1 Rev and Tat (Luo et al., 1994), protein V of adenovirus (Matthews and Russell, 1998), Epstein-Barr virus nuclear antigen-1 (EBNA-1) (Wang et al., 1997) and hepatite C virus core protein (Kittlesen et al., 2000), and at least two bacterial proteins, InlB and protein A from *Staphylococcus aureus* (Nguyen et al., 2000). This molecule could thus be involved in several aspects of host-pathogen interactions, although its physiological roles are not yet clear.

The crystal structure of human gC1q-R reveals a donut-shaped ternary complex (Jiang et al., 1999) but provides little clues to its mode of attachment at the cellular surface. It is not a GPI-anchored protein; instead it could bind to the cell surface by ionic interactions. Nevertheless, interaction of InlB with a surface-associated gC1q-R form appears to be critical for InlB-mediated entry, since gC1q-R antibodies and C1q compete with InlB for binding to gC1q-R and are able to inhibit specifically InlB-dependent entry of *L. monocytogenes* into cells (Braun et al., 2000). However, antibodies against gC1q-

R only partially inhibit InlB-mediated signaling. Therefore, an attractive hypothesis is that gC1q-R facilitates interaction of InlB with Met. This bridging effect could be cell-type dependent, relying on the presence of surface-bound gC1q-R. Interestingly recent data indicate that the highly acidic protein gC1q-R binds to the basic GW repeats of InlB (M. Marino, M. Banerjkee, T. Chapman et al., unpublished). Therefore, it may modulate InlB accessibility in a similar way to proteoglycans. Another intriguing question is whether gC1q-R interacts with InlB intracellularly, after the escape of bacteria from the phagocytic vacuole. Further studies are therefore required to clarify the role of gC1q-R in the cellular infectious process.

InlB-mediated internalization

A phagocytic process?

Phagocytosis is a highly sophisticated mechanism for ingestion and destruction of microbial pathogens as well as of apoptotic cells and debris. It occurs primarily in specialized phagocytic cells, such as macrophages and neutrophils, by distinct pathways that differ with respect to morphology, signaling and functional consequences (Swanson and Baer, 1995; Aderem and Underhill, 1999; Greenberg, 2001). However, the initial engulfment process involves a succession of common events: particle binding, receptor clustering, actin assembly, membrane extension, phagosome closure and actin disassembly around the phagosome. The InlB-mediated internalization of *L. monocytogenes* into cells that are not professional phagocytes requires all these steps and therefore can be compared to phagocytosis. From a morphological point of view, InlB-induced phagocytosis is related to the zipper mechanism induced by the macrophage Fc and complement receptors [FcR and CR3 (Kaplan, 1977; Swanson and Baer, 1995)]. It involves extension of tightly adherent membranous structures around the particle and assembly of a continuous F-actin cup (Fig. 2A) (Braun et al., 1998; Parida et al., 1998; Bierne et al., 2001). From a signaling point of view, InlB-induced phagocytosis is more specifically related to that triggered by FcR than by CR3 (see below) (Cox and Greenberg, 2001; May and Machesky, 2001).

Actin rearrangements

Since InlB-Met signaling leads to both phagocytosis (Fig. 2A) and membrane ruffling (Fig. 2B), these two types of actin-based process might share some downstream effectors. Both require actin polymerization, tyrosine phosphorylation and PI 3-kinase activation (Ireton et al., 1996; Braun et al., 1998; Ireton et al., 1999), as does FcR-mediated phagocytosis (Cox and Greenberg, 2001). Recently, the Arp2/3 complex, cofilin, LIM-kinase and the GTPase Rac, all well known regulators of transient actin polymerization/depolymerization, were shown to be involved in InlB-mediated actin reorganization (Bierne et al., 2001). The Arp2/3 complex is an assembly of seven proteins that together promote nucleation of actin filaments on the side of older filaments and therefore participate in the formation of branched actin networks (Machesky and Gould, 1999; Welch, 1999; Robinson, 2001). It is recruited to InlB-induced phagocytic cups and membrane ruffles. Moreover, formation of these actin-based structures is inhibited when Arp2/3 is sequestered by the C-terminus of Scar (Bierne et al., 2001). These results suggest that the Arp2/3 complex regulates actin dynamics during InlB-induced phagocytosis, as it does during FcR and CR3-mediated phagocytosis (May et al., 2000) and at the leading edge of motile mammalian cells (Bailly et al., 1999; Svitkina and Borisy, 1999).

Actin polymerization is thought to provide the driving force that propels membranes around the bacterium. However, the shaping of the phagocytic cup also requires actin depolymerization events, particularly beneath the particle, to facilitate retraction of the cup. Proteins of the ADF/cofilin family (Bamburg, 1999; Chen et al., 2000), which increase actin depolymerization at free pointed ends are candidates for mediators of this process, although they have been characterized mainly as enhancers of actin dynamics rather than actin disrupters. These proteins increase the rate of actin turnover and the number of free actin ends available for polymerization (Carlier et al., 1997; Rosenblatt et al., 1997; Chan et al., 2000). They are inactivated through LIM-kinase-induced phosphorylation (Arber et al., 1998; Yang et al., 1998) and reactivated through dephosphorylation.

Interestingly, analysis of the role of cofilin during InlB-induced phagocytosis has shown that InlB participates in both formation and disruption of the actin phagocytic cup (Bierne et al., 2001). First, not only is cofilin recruited at InlB-induced F-actin cups, but, strikingly, it seems to accumulate progressively and transiently around the phagosome (Fig. 3,1). Second, InlB-induced phagocytosis is inhibited in cells deregulated for the cofilin phosphocycle. Inactivating cofilin by LIM-kinase induces F-actin overaccumulation at the entry site of InlB particles and inhibits closure of the phagocytic cup

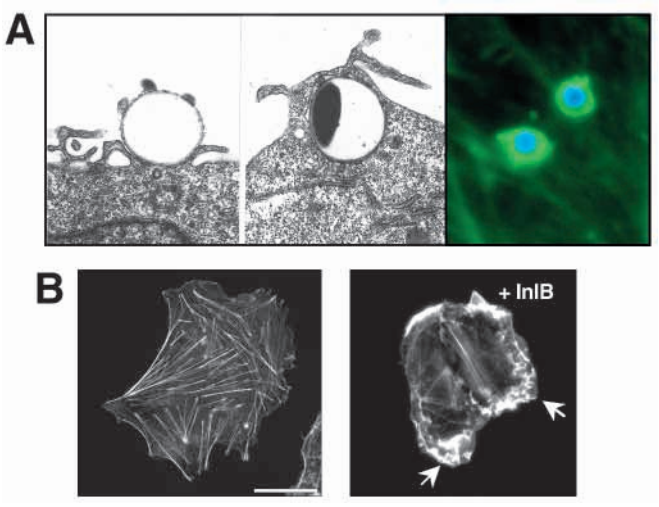

Fig. 2. InlB-induced actin-based processes. (A) InlB-mediated internalization by the 'zipper-like' mechanism. On the left is a transmission electron microscopy section of an InlB-coated bead being internalized into an epithelial cell [reproduced from (Braun et al., 1998)]. The particle is engulfed within tightly adherent membranous structures before lying in a phagocytic vacuole. Note the presence of small membrane projections at the entry site of the particle. On the right, the F-actin phagocytic cup is stained with FITC-phalloidin (in green) at the entry site of two InlB-coated beads (in blue). (B) InlB-induced membrane ruffling in Vero cells. A cell untreated (left) or incubated with 4.5 nM purified InlB for 5 minutes (right) and stained with FITC-phalloidin to detect F-actin.

(Fig. 3,3). Conversely, increasing cofilin activity by overexpressing a constitutively active cofilin mutant (S3A cofilin), or a dominant-negative LIMK1 mutant, induces loss of F-actin at phagocytic cups and also inhibits phagocytosis (Bierne et al., 2001) (Fig. 3,2). Together, these data fit with a two-step model (Fig. 3). At low activity, controlled by LIM-kinase, cofilin could be involved in the phagocytic cup extension by stimulating actin dynamics. Then, dephosphorylation of cofilin and its progressive accumulation on filaments would ultimately favor the disassembly of the actin network during the retraction of the phagocytic cup and around the newly formed phagosome. It will now be important to address the role of Slingshot (SSH), the newly identified cofilin phosphatase (Niwa et al., 2002) in this process.

From Met to the cytoskeleton

What links Met activation and recruitment of Arp2/3, cofilin and LIM-kinase? Likely candidates are the Rho-GTPases Rac and Cdc42, which regulate actin cytoskeleton rearrangements both during lamellipodia formation and phagocytosis (Cox et al., 1997; Caron and Hall, 1998; Ridley, 2001). Indeed, HGF activates Cdc42, Rac and PAK (Royal et al., 2000), which is an upstream activator of LIM-kinase (Edwards et al., 1999). Recruitment of Rac to the Met receptor seems to require the adapters CrkII and Dock180 (Furge et al., 2000). It is still unknown whether Rac and Cdc42 are activated by guanine-nucleotide-exchange factors (GEFs) downstream of Met. InlB-induced membrane ruffling in Vero cells is impaired by Rac1-N17 and Cdc42-N17 dominant-negative mutants, which suggests that both Rac and Cdc42 are involved in InlB-mediated cytoskeletal rearrangements. However, only Rac1-N17 blocks InlB-induced phagocytosis in Vero cells, whereas Cdc42-N17 has no effect on entry in this cell line (Bierne et al., 2001). This observation suggests that the formation of the F-actin cup is controlled mainly by Rac in this system. InlB-Met interactions probably elicit a Rac/PAK/LIM-kinase/cofilin cascade. In addition, Rac has recently been shown in other systems to activate the Arp2/3 complex by a cascade of events involving the adapters IRSp53 and WAVE, a member of the Wiskott-Aldrich Syndrome protein (WASP) family (Miki et al., 2000). The current model is that activated Rac recruits IRSP53, which binds to the proline-rich region of WAVE. As a result, the C-terminal region of WAVE is exposed and activates the Arp2/3 complex (Takenawa and Miki, 2001). Preliminary results indicate that these molecules play a role in InlB-Met-induced cytoskeletal rearrangements (H.B. and P.C., unpublished).

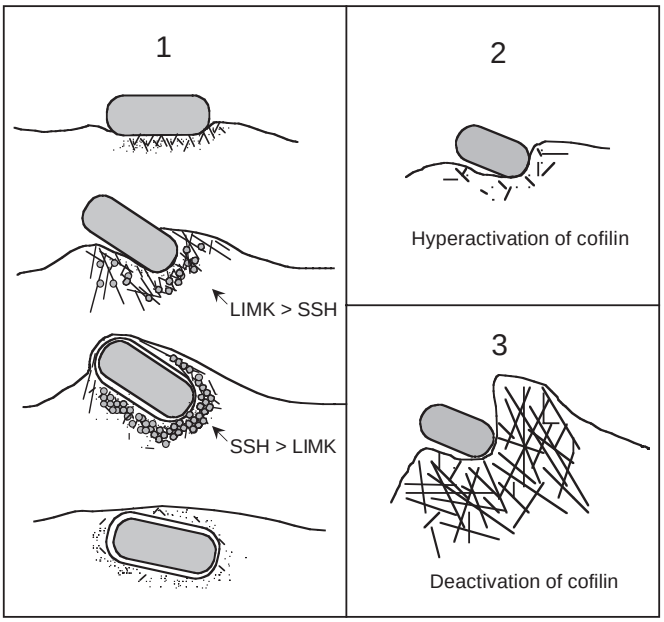

Fig. 3. Role of cofilin during InlB-induced phagocytosis. Adapted from (Bierne et al., 2001). (1) Interaction of InlB with its receptors induces recruitment of the Arp2/3 complex (not shown) and of cofilin (represented by grey circles), which stimulate actin polymerization (represented by lines). LIM-kinase and the phosphatase SSH could be recruited to the phagocytic cup to regulate cofilin's activity. In a first step, LIM-kinase prevents excessive depolymerization of actin filaments by partly inactivating cofilin. Then, SSH would reactivate cofilin, which finally accumulates on the filaments and favors the disruption of the actin network during the retraction of the phagocytic cup and around the newly formed phagosome. Cofilin would thus be involved in both assembly and disassembly of the InlB-induced phagocytic cup. (2) Increasing the pool of active cofilin by expressing the constitutively active S3A cofilin or by inhibiting endogenous LIM-kinase blocks phagocytic cup formation, presumably because of an excess of depolymerizing activity. (3) Partial inactivation of cofilin by overexpressing LIMK1 induces an intense and disorganized accumulation of actin filaments at the phagocytic cup, preventing the engulfment of the particle into the cell.

Putative roles for PI 3-kinase

PI 3-kinase is an essential component of InlB-mediated phagocytic signaling, but its downstream effectors are not yet identified. In FcR-mediated phagocytosis, PI 3-kinase appears to function in pseudopod extension and closure of the phagocytic cup by regulating exocytosis of endomembranes and membrane fusion events (Araki et al., 1996; Booth et al., 2001; Cox and Greenberg, 2001). Inhibition of PI 3-kinase does not prevent accumulation of the subcortical actin at the sites of particle attachment, which suggests that it does not regulate actin polymerization. However, reorganization of the cortical actin cytoskeleton required for lamellipodia and membrane ruffle formation in response to various stimuli (Wymann and Arcaro, 1994; Kotani et al., 1994; Reif et al., 1996; Arrieumerlou et al., 1998; Hill et al., 2000), including soluble InlB (Ireton et al., 1999), does require PI 3-kinase activity, highlighting a direct connection between PI 3-kinase activation and the cytoskeleton in InlB-Met signaling. To reconcile these findings, we propose that PI 3-kinase plays multiple roles in InlB-mediated internalization, including recruitment of both membrane vesicles and actin regulatory proteins. Proteins of the Vav family (Bustelo, 2001), which act as GEFs for Rac and are downstream effectors of PI 3-kinase signalling (Han et al., 1998), are involved in both growth factor and phagocytic signalling (Moores et al., 2000; Patel et al., 2002). They might have a role in Rac activation downstream of Met. One possible scenario is that Met clustering recruits and activates Rac, leading to the initiation of actin polymerization. Then, PI 3-kinase activity leads to a Vav-induced sustained activation of Rac, which drives actin

rearrangements at the phagocytic cup. In line with this idea, although recruitment of activated Rac at the plasma membrane is sufficient to trigger phagocytosis, it is not sufficient to promote a detectable accumulation of F-actin at the phagocytic cup (Castellano et al., 2000).

New effectors

A powerful way of identifying new components of the phagocytic machinery is to isolate phagocytic vacuoles and proceed to a proteomic approach (Duclos and Desjardins, 2000; Garin et al., 2001). In recent work, analysis of vacuoles produced during the uptake of InlB-coated beads identified MSF as a putative new effector of the InlB-dependent pathway (Pizarro-Cerda et al., 2002). MSF is not only present in phagosomes but also recruited to the entry site of InlB-coated beads, where it colocalizes with actin. It is a member of the septin family of GTPases (Osaka et al., 1999), which form filaments that can interact with actin-based structures (Field et al., 1996; Kinoshita et al., 1997). Septins regulate vesicle transport in exocytosis through interaction with SNARE proteins (Beites et al., 1999). However, the precise function of MSF in bacterial uptake is unknown.

Other InlB-mediated cellular responses

Not only does InlB promote phagocytic events, but it also activates signaling pathways that are not directly linked to phagocytosis (Fig. 4). In common with HGF, InlB, especially as a soluble factor, might therefore be able to regulate many

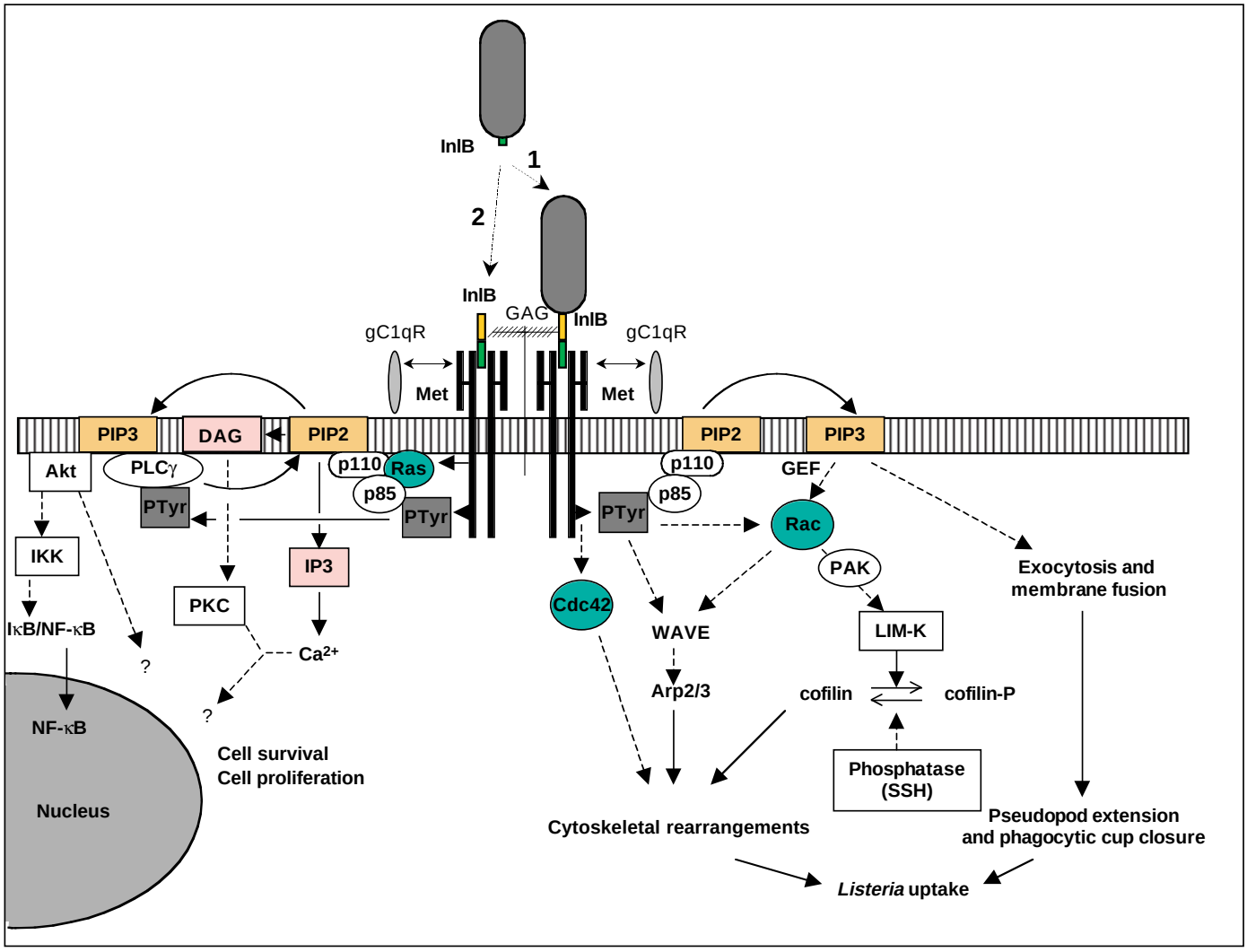

Fig. 4. The InlB-mediated signaling pathways. In bacteria present in the environment, InlB is buried into the bacterial cell wall, somehow protected from proteolytic degradation and external aggressive agents. Upon infection, in proximity with the targeted host cell, it may dissociate from the bacterial surface, by interacting with GAGs and gC1q-R and become accessible to the Met receptor. (1) The surface-exposed protein will trigger entry of the bacteria. (2) A pool of the protein may be released in the medium and induce signals as a prelude to or independently from entry. InlB-Met interactions induce recruitment of adapters proteins, some of which become tyrosine-phosphorylated, and recruitment and activation of the p85-p110 PI 3-kinase. Some downstream events induce actin cytoskeleton rearrangements involving Rho GTPases and cytoskeletal regulatory proteins, and membrane reorganization, leading to bacteria uptake. Other downstream events, involving activation of other enzymes, such as PLCγ or Akt, might affect the bacterial fate into the cell and/or the host cell behavior. Hashed arrows indicate hypothetical steps.

cellular processes. These events may be critical for cell survival after internalization of the bacterium and release of it into the cytosolic compartment.

PLC-γ1 and calcium

Phospholipases C play critical roles in receptor-mediated signal transduction via the generation of inositol 1,4,5-trisphosphate [InsPtd(1,4,5)P_3] and diacylglycerol (DAG) after hydrolysis of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2]. InsPtd(1,4,5)P_3 induces calcium release from internal stores, and DAG activates a large family of calcium/phospholipid-dependent protein kinase C (PKC) isoenzymes. The PLC-γ1 isoform is widely expressed and activated by a variety of receptor and non-receptor tyrosine kinases by tyrosine phosphorylation (Carpenter and Ji, 1999). In addition, the activity of PLC-γ1 is enhanced through binding of the PH and SH2 domains of the enzyme to the PI 3-kinase product PtdIns(3,4,5)P_3 (Falasca et al., 1998; Rameh et al., 1998).

InlB activates PLC-γ1 in HEp-2 cells and induces intracellular calcium rises (Bierne et al., 2000). Activation of PLC-γ1 results from its PI 3-kinase dependent association with tyrosine-phosphorylated proteins but does not require tyrosine phosphorylation of PLC-γ1. It is possible that the adapter Gab1, which becomes tyrosine phosphorylated in response to InlB stimulation, recruits PLC-γ1 as it does in HGF-Met signaling (Gual et al., 2000). InlB stimulation induces very transient increases in intracellular InsPtd(1,4,5)P_3 and calcium levels and does not provoke a sustained response. Therefore the intracellular Ca^{2+} released is likely to activate highly localized cellular processes, which would be able to respond to slight changes in the concentration of this potent signaling ion (Bootman et al., 2001).

What then is the function of PLC-γ1 in InlB-mediated signaling? PLC-γ has been proposed to be involved in actin rearrangements, because PtdIns(4,5)P_2 and calcium are well known regulators of actin-binding proteins (Lee and Rhee, 1995). However, PLC-γ1 is not required for the reorganization of the actin cytoskeleton that occurs during InlB-induced phagocytosis (Bierne et al., 2000). Indeed, entry of InlB-coated beads or InlB-expressing bacteria is not affected by the PLC inhibitor U73122 or the calcium chelator BAPTA/AM. In addition, *L. monocytogenes* internalization is not decreased in *Plcg1*-knockout cells. Therefore, InlB-mediated PLC-γ1 activation and intracellular calcium rises are apparently not involved in the internalization process. Similarly, the PLC-γ/calcium signaling cascade that occurs upon ingestion of particles by professional phagocytes is not a prerequisite for uptake (Di Virgilio et al., 1988). Interestingly, recruitment of PLC-γ1 is not required for HGF-mediated cell scattering, and the inhibitor U73122 does not block this process, which suggests that PLC-γ signaling is also not involved in HGF-mediated cytoskeletal reorganization. By contrast, PLC-γ appears to be critical for HGF-mediated tubular morphogenesis, a phenomenon similar to differentiation (Machide et al., 1998). PLC-γ1 also mediates an intracellular signal for the HGF-enhanced mitogenesis in rat primary hepatocytes (Okano et al., 1993). Taken together, these data suggest that InlB-induced PLC-γ1 activation and calcium mobilization are probably involved in post-internalization steps, such as the control of cell growth and/or of gene expression.

Akt and NF-κB

The eukaryotic transcription factor NF-κB is an important regulator of many genes involved in inflammation, immunity, stress responses and the inhibition of apoptosis. In unstimulated cells, NF-κB is sequestered in the cytoplasm by the inhibitory protein IκB (May and Ghosh, 1997; Baeuerle, 1998; May and Ghosh, 1999). Signal-induced phosphorylation and consequent proteolytic degradation of IκB allows NF-κB to enter the nucleus and induce transcription. Several bacterial surface components, such as lipopolysaccaride (LPS) in Gram-negative bacteria and lipoteichoic acids (LTA) in Gram-positive bacteria, are potent activators of NF-κB. InlB also activates NF-κB in some macrophages and epithelial cell lines and induces NF-κB-dependent expression of the cytokines TNF-α and IL6 (Mansell et al., 2000). The effect is rapid and sustained and involves the degradation of both IκBα and IκBβ.

Mansell et al. have recently examined the InlB-NF-κB signaling cascade in murine J774 macrophages (Mansell et al., 2001), in which Met is expressed (N. Khelef and P.C., unpublished). First, in common with HGF, InlB induces the sequential activation of the small G-protein Ras and of PI 3-kinase. Then, PI 3-kinase activates the Akt/protein kinase B (PKB), which activates NF-κB by an uncharacterized pathway. Several other studies indicate that Akt is involved in NF-κB activation (Kane et al., 1999; Ozes et al., 1999; Burow et al., 2000). One proposed mechanism is that Akt activates the IκB kinase complex (IKK) that phosphorylates IκB.

Interestingly, Akt is thought to play an important role in protecting cells from apoptosis and in promoting cell survival (Downward, 1998; Burow et al., 2000). In particular, it was recently shown to be part of anti-apoptotic HGF and PDGF signaling (Romashkova and Makarov, 1999; Xiao et al., 2001). Therefore, one attractive hypothesis is that InlB-mediated Akt activation plays a role in the survival of the infected host cell. Since *L. monocytogenes* is an intracellular pathogen, host cell survival could facilitate the dissemination of the bacteria in tissues. The role of NF-κB activation upon InlB stimulation may not be linked to anti-apoptosis, since it has been recently shown that HGF-induced NF-κB activation is dispensable for the anti-apoptotic function of HGF (Muller et al., 2002). Further studies are require to understand the precise role of this regulator in the InlB pathway.

Concluding remarks

Many InlB-induced signals are dependent on the cell type. For instance, InlB mediates entry in many, but not all, cell lines. It will thus be important to determine the respective roles of the three known receptors, Met, gC1q-R and proteoglycans, in cell tropism and to search for other critical factors. In addition, some cell lines can be permissive for InlB-induced phagocytosis but not for other InlB-induced cellular events. For instance, InlB activates NF-κB in J774 and P388D1 macrophages, as well as in the epithelial cell line HEp-2, but not in Vero cells (Mansell et al., 2000). Similarly, InlB activates PLC-γ in HEp-2 cells but not in Vero cells (H.B. and P.C., unpublished). The same is true for HGF, which also selectively

activates PLC-γ in some cell types and not in others, by a 'switch on-off' mechanism that probably contributes to specific biological responses (Machide et al., 2000). In addition, when apparently similar processes take place, subtle differences occur. For example, InlB activates a p85-p110 class IA PI 3-kinase in many cell lines, but the nature of the p85 isoform recruited varies: although both isoforms are present, it is p85α that is recruited in Vero cells and p85β in HEp-2 cells (Ireton et al., 1999; Bierne et al., 2000). These isoforms might have different functions (Gout et al., 1992; Hartley et al., 1995; Shepherd et al., 1997). Thus, if InlB has different functions depending on the cell type, it will be very informative to determine where and when each of these functions operates during infection.

InlB acts as an invasin in vitro and promotes entry into cells that are not (or poorly) permissive for the other invasion protein InlA entry pathway, such as hepatocytes (Dramsi et al., 1995) and endothelial cells (Greiffenberg et al., 1998; Parida et al., 1998). Is InlB really an invasin in vivo? In the murine model, a Δ*inlB* mutant of *L. monocytogenes* produces fewer bacterial counts in the liver (Dramsi et al., 1995; Gaillard et al., 1996). However, it is not yet clear whether InlB promotes invasion of hepatocytes per se or whether another process is involved, such as intracellular bacterial multiplication (Gregory et al., 1997). The role of the InlB pathway in other tissues, and its interplay with the InlA pathway, deserves more investigation.

InlB shares properties with HGF in vitro. Does InlB act as a growth factor in vivo? If it were the case, it would probably have to be released from the bacterial surface as a soluble signaling molecule. This phenomenon remains to be demonstrated. Activation of cell growth and cell survival by InlB, especially that of hepatocytes, endothelial cells and macrophages, could be of primary importance for bacterial dissemination in tissues. Moreover, in common with HGF, soluble InlB triggers scattering of some epithelial cells (Shen et al., 2000). Does InlB open cellular junctions in epithelia and facilitate interaction between InlA and its receptor E-cadherin, as recently proposed (Cossart, 2001)? Does InlB share the deleterious effects of HGF, which plays a role in metastasis? Much effort is still required to discover all of the properties of this fascinating protein.

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