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InlB-Dependent Internalization of *Listeria* Is Mediated by the Met Receptor Tyrosine Kinase

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Summary

The *Listeria monocytogenes* surface protein InlB promotes bacterial entry into mammalian cells. Here, we identify a cellular surface receptor required for InlB-mediated entry. Treatment of mammalian cells with InlB protein or infection with *L. monocytogenes* induces rapid tyrosine phosphorylation of Met, a receptor tyrosine kinase (RTK) for which the only known ligand is Hepatocyte Growth Factor (HGF). Like HGF, InlB binds to the extracellular domain of Met and induces "scattering" of epithelial cells. Experiments with Met-positive and Met-deficient cell lines demonstrate that Met is required for InlB-dependent entry of *L. monocytogenes*. InlB is a novel Met agonist that induces bacterial entry through exploitation of a host RTK pathway.

Introduction

Listeria monocytogenes is a food-borne bacterium capable of causing meningitis or abortions in pregnant women (Gellin and Broome, 1989). Like many microbial pathogens, this intracellular bacterium induces uptake ("enters") into host cells that are normally non-phagocytic (Cossart and Lecuit, 1998). Entry of *L. monocytogenes* into epithelial cells, endothelial cells, and hepatocytes in vivo is likely to play an important role in traversing the intestinal, blood-brain, and placental barriers, and in colonization of the liver.

Entry is mediated by the *L. monocytogenes* surface proteins InlA (internalin) and InlB (Gaillard et al., 1991; Dramsi et al., 1995). Different mammalian cell lines have different susceptibilities to InlA- or InlB-mediated uptake. In the monkey kidney cell line Vero, mouse hepatocytes, or human endothelial cells, bacterial entry is mediated exclusively by InlB (Dramsi et al., 1995; Greiffenberg et al., 1998; Ireton et al., 1996; Parida et al., 1998). In the human intestinal epithelial cell line Caco-2 and the hepatocyte cell line HepG2 both InlA and InlB have functions in entry (Gaillard et al., 1991; Dramsi et al., 1995).

The amino-terminal domains of both InlA and InlB contain multiple, tandem 22 amino acid Leucine Rich Repeats (LRRs) that are essential for bacterial entry (Braun et al., 1999; Cossart and Lecuit, 1998). The number and exact amino acid sequence of the LRRs differs in InlA and InlB, and these differences allow interaction with distinct mammalian targets. The host receptor for InlA is the calcium-dependent cell surface adhesion molecule E-cadherin (Mengaud et al., 1996). InlB does not interact with E-cadherin, and is believed to mediate entry through a distinct mammalian receptor (Mengaud et al., 1996; Cossart and Lecuit, 1998).

Entry of *L. monocytogenes* is an active process requiring tyrosine phosphorylation and re-organization of the actin cytoskeleton in the host cell (Cossart and Lecuit, 1998). Essentially nothing is known about signaling that couples InlA/E-cadherin interaction to internalization of *L. monocytogenes*. In contrast, an InlB-dependent signaling pathway triggered in the host cell has been partly characterized. Infection of Vero cells with *L. monocytogenes* results in activation of the host Phosphoinositide (PI) 3-kinase p85-p110 (Ireton et al., 1996). P85-p110 is required for bacterial uptake, since entry is blocked by genetic or chemical inhibition of PI 3-kinase activity. Experiments with purified InlB demonstrate that this bacterial protein is sufficient to activate PI 3-kinase in cells (Ireton et al., 1999). Activation of p85-p110 coincides with tyrosine phosphorylation of the mammalian adaptor proteins Gab1 and Cbl, and the formation of Gab1.p85 complexes. How interaction of InlB with the host cell leads to these signaling events is not understood.

P85-p110 is a heterodimeric lipid kinase that regulates mammalian cell growth, apoptosis, and/or the actin cytoskeleton in response to oncogenic transformation or engagement of several surface receptors (Fruman et al., 1998). The Met Receptor Tyrosine Kinase (RTK) is one of the receptors known to be coupled to p85-p110 and the adapters Gab1 and Cbl (Birchmeier and Gherardi, 1998; Trusolino et al., 1998). Met is a high affinity receptor for Hepatocyte Growth Factor (HGF), also known as scatter factor. Met is expressed in epithelial and endothelial cells, whereas HGF is produced by fibroblasts. In vivo, Met and HGF are essential for development of the liver, placenta, and specific muscle groups (Birchmeier and Gherardi, 1998; Trusolino et al., 1998). In cultured cells, HGF/Met interaction stimulates movement or "scattering" of epithelial cells grown in monolayer, differentiation of cells grown in collagen gels into branching tubules, and proliferation of hepatocytes or endothelial cells. HGF/Met interaction leads to phosphorylation of Gab1 and Cbl, and overexpression studies indicate that Gab1 participates in scattering and tubule formation (Maroun et al., 1999; Nguyen et al., 1997; Weidner et al., 1996). Scattering also requires p85-p110 (Royal and Park, 1995).

Mature Met is a disulfide-linked heterodimer made of 45 kDa α- and 145 kDa β-subunits (Birchmeier and

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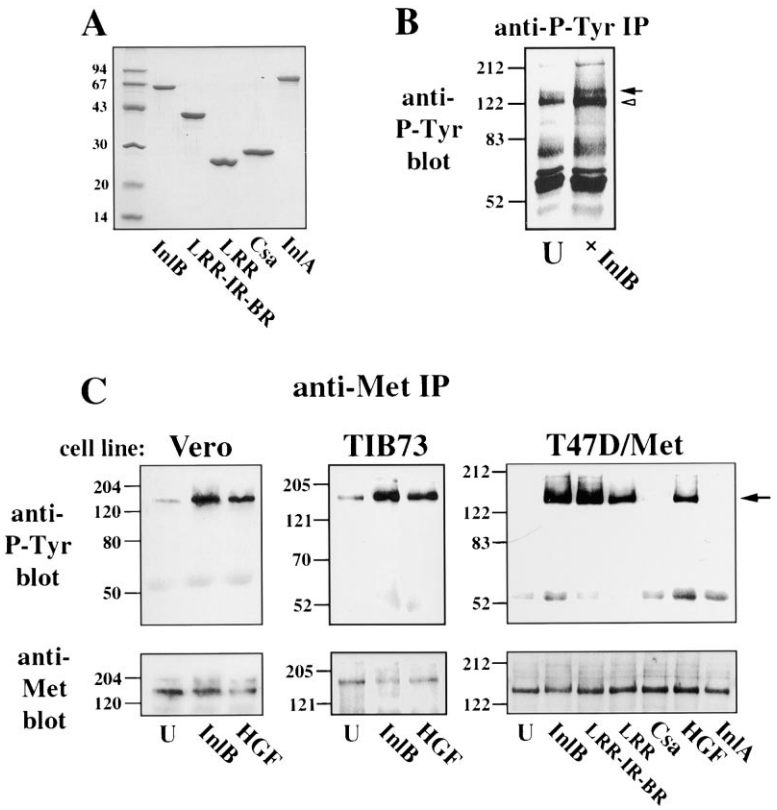

Figure 1. InlB Stimulates Tyrosine Phosphorylation of Met

(A) Purified proteins used. InlB: full-length InlB; LRR-IR-BR: a derivative composed of the Leucine-Rich Repeat (LRR), Inter-repeat (IR), and B repeat (BR) domains in InlB; LRR: a derivative consisting of only the LRR region in InlB; Csa: a derivative containing only the cell surface-anchoring region (Csa) in InlB; InlA: full-length InlA. After purification, three micrograms of each protein were loaded on a 12% SDS/PAGE gel, which was subsequently stained with Coomassie Blue.

(B) InlB stimulates tyrosine phosphorylation of a ~145 kDa protein in mammalian cells. Vero cells were left untreated (U) or treated with 3 nM InlB for 1 min, followed by solubilization in lysis buffer and immunoprecipitation with the anti-phosphotyrosine antibody 4G10. The immunoprecipitates were migrated on a 7.5% SDS/PAGE gel, transferred to nitrocellulose, and probed with 4G10 to detect total tyrosine phosphorylated proteins. The open arrowhead indicates the position of the ~110–120 kDa phosphorylated adaptor proteins Gab1 and Cbl, and the closed arrow indicates the ~145 kDa phosphorylated protein.

(C) InlB stimulates tyrosine phosphorylation of Met in several mammalian cell lines. Vero, TIB73, or T47D/Met cells were left untreated (U) or incubated for 1 min (Vero) or 5 min (TIB73 and T47D/Met) with 1.5 nM InlB, 2 nM LRR-IR-BR, 2 nM LRR, 2 nM Csa, 1.5 nM HGF, or 3.5 nM InlA, as indicated. Cells were then solubilized and Met was immunoprecipitated with antibodies DO-24 (Vero), 101 (TIB73), or C-12 (T47D/Met). Tyrosine-phosphorylated proteins were detected by Western blotting with 4G10 (upper panel). The membranes were then stripped and probed with anti-Met antibodies DL-21 (Vero and T47D/Met) or 101 (TIB73) to verify precipitation of Met. The arrow indicates the position of the ~145 kDa tyrosine-phosphorylated β subunit of Met in the three different cell lines. No phosphorylated proteins were present when control mouse or rabbit preimmune IgG were used to prepare precipitates from InlB-treated Vero, TIB73, or T47D/Met cells (data not shown).

Gherardi, 1998; Trusolino et al., 1998). The β subunit and the amino-terminal region of the α subunit form the extracellular domain. The remainder of the β chain spans the plasma membrane and contains a cytoplasmic region with tyrosine kinase activity. Interaction of Met with HGF results in autophosphorylation at multiple tyrosines in the receptor's cytoplasmic domain and in the recruitment of several signaling molecules, including Gab1, Cbl, and p85-p110. Autophosphorylation of Met is essential for all of its known biological functions.

In this report, we show that Met binds to and is activated by the *L. monocytogenes* protein InlB. Met is required for InlB-dependent bacterial entry into mammalian cells. InlB/Met interaction represents a novel mechanism of internalization of a microbial pathogen through engagement of a host RTK.

Results

InlB Stimulates Tyrosine Phosphorylation of Met

Treatment of Vero cells with InlB protein (Figure 1A) results in the appearance of a ~145 kDa tyrosine-phosphorylated protein that migrates above the 110–120 kDa proteins corresponding to phosphorylated Gab1 and Cbl (Ireton et al., 1999, Figure 1B). Since the β subunit of the Met RTK is ~145 kDa, we tested whether InlB activates Met. Vero cells were treated with 3 nM of InlB protein for 1 min (Figure 1C), conditions which cause optimal activation of p85-p110 and phosphorylation of Gab1 (Ireton et al., 1999). InlB stimulated tyrosine-phosphorylation of the ~145 kDa β subunit of Met (Figure 1C). In contrast, InlB does not induce tyrosine phosphorylation of the EGF receptor in Vero cells (Ireton et al., 1999), indicating that this bacterial protein does not cause promiscuous activation of RTKs.

InlB also stimulated Met phosphorylation in the mouse hepatocyte cell line TIB73, the human hepatocyte cell line HepG2, the human epithelial cell lines HeLa, and HEp-2, and the dog kidney cell line MDCK (Figure 1C and data not shown). Bacterial entry into all of these cell lines is dependent, either fully or partly, on InlB (Dramsi et al., 1995; Ireton et al., 1996). The human mammary epithelial cell line T47D expresses very low levels of Met. Met was not detected in anti-Met immunoprecipitates from these cells after brief exposure to film (30 min) (Figure 3A), but a weak signal corresponding to the Met β chain was detected after overnight exposure (data not shown). As expected, tyrosine-phosphorylated proteins were not readily observed in anti-Met precipitates prepared from T47D cells treated with InlB (Figure 3A). T47D cells were used to construct a cell line that stably expresses high levels of human Met (Experimental Procedures). Met phosphorylation was readily detected in the resulting T47D/Met cells (Figure 1C; 3A). In contrast to InlB, the *Listeria* protein InlA did not induce phosphorylation of Met (Figure 1C). These results indi-

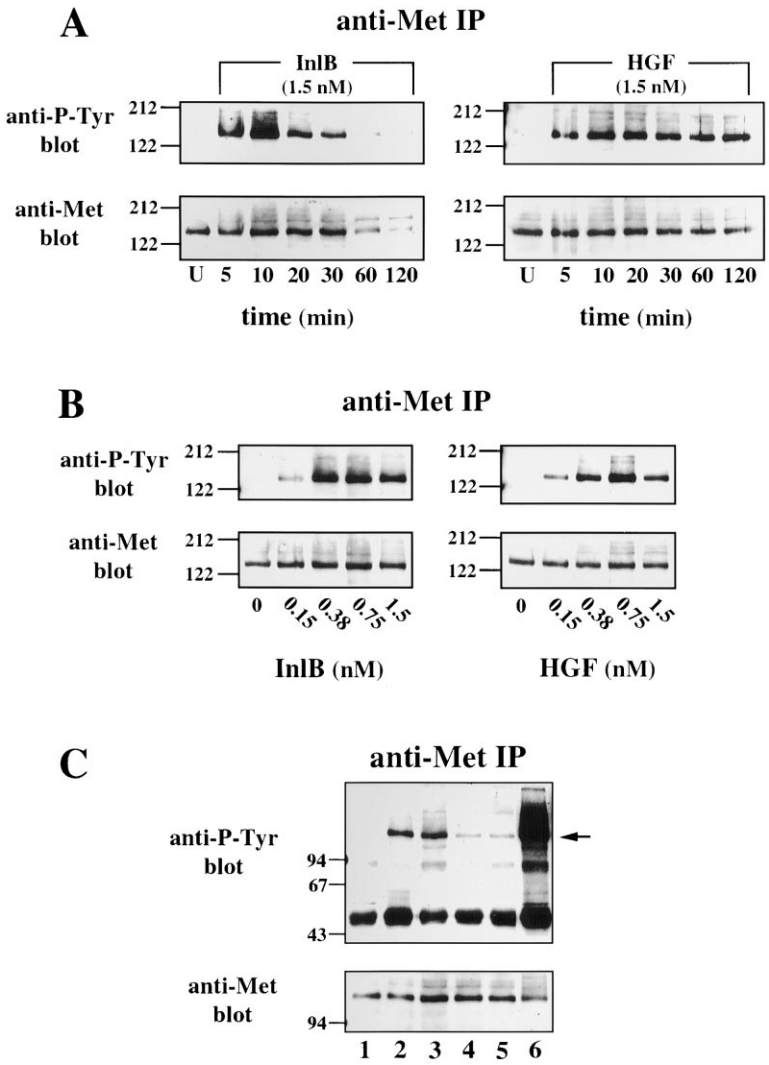

Figure 2. Kinetics and Dosage Dependence of InlB-Induced Phosphorylation of Met

(A) The kinetics of InlB- and HGF-induced phosphorylation of Met. T47D/Met cells were left untreated (U), or incubated with 1.5 nM InlB or 1.5 nM HGF for the indicated times.

(B) The effects of different concentrations of InlB or HGF on Met phosphorylation. T47D/Met cells were left untreated (U) or treated with increasing concentrations of InlB or HGF.

Met was immunoprecipitated from lysates with antibody C-12. Upper panel: tyrosine-phosphorylated Met, as detected with antibody 4G10. Lower panel: total precipitated Met, as detected by probing stripped membranes with antibody DL-21. The results are representative of three (A) or two (B) experiments. The decrease in the steady-state level of Met after 60 min incubation with InlB (Fig. 2A; lower panel) was reproducible. The cause of this decrease is not known, but may be related to proteolysis occurring after Met activation.

(C) Infection with *L. monocytogenes* induces Met phosphorylation. T47D/Met cells were left uninfected (lane 1) or infected with wt *L. monocytogenes* (lane 2), the Δ*inlA* mutant strain (lane 3), the Δ*inlB* mutant (lane 4), the Δ*inlAB* double mutant (lane 5), or treated with 3 nM InlB protein (lane 6) for 5 min. Upper panel: tyrosine-phosphorylated Met, as detected with antibody 4G10. Lower panel: total precipitated Met, as detected with antibody DL-21. The arrow indicates the position of the phosphorylated Met β chain. The apparent small increase in Met phosphorylation in cells infected with the Δ*inlB* and Δ*inlAB* mutant strains was not consistently observed in repeats of this experiment.

cate that InlB stimulates Met phosphorylation in several mammalian cell lines.

InlB-induced phosphorylation of Met in T47D/Met cells reached a maximum at 10 to 20 min, and then decreased to undetectable levels by 60 min (Figure 2A). Interestingly, Met phosphorylation in response to HGF was more sustained, appearing essentially undiminished 120 min after addition of the growth factor. InlB- and HGF- induced phosphorylation of Met occurred over a similar ligand concentration range (0.15 nM or greater concentrations) (Figure 2B).

Based on peptide sequence, InlB can be divided into an amino-terminal 213 amino acid Leucine Rich Repeat (LRR) domain, a 94 residue Inter-repeat (IR) region, a 57 residue "B repeat" (BR) region, and a carboxyl-terminal 232 amino acid cell surface-anchoring (Csa) domain (Cossart and Lecuit, 1998). The LRR domain alone is necessary and sufficient for entry into mammalian cells (Braun et al., 1999). When used at concentrations of 1.5 nM or greater, InlB derivatives consisting of the LRR, IR, and BR regions (LRR-IR-BR), or of the LRR region alone induced Met phosphorylation (Figure 1C). At concentrations below 1.5 nM, these two derivatives were less effective than full-length InlB in promoting phosphorylation (data not shown). The protein consisting of only the Csa region had no effect on Met phosphorylation when used at 1.5 nM (Figure 1C) to 12 nM (data not shown). These results indicate that the LRR domain of InlB is critical for stimulation of Met phosphorylation. Full activity requires both the Csa and the LRR domains.

Consistent with the above results, Met was phosphorylated upon infection with the wt *L. monocytogenes* strain EGD or with an isogenic mutant strain containing a deletion in the *inlA* gene (Δ*inlA*) (Figure 2C). In contrast, strains deleted only for the *inlB* gene (Δ*inlB*) or for both *inlA* and *inlB* (Δ*inlAB*) did not stimulate Met phosphorylation. These results demonstrate that endogenous InlB activates Met during infection. The lower magnitude of bacterially-induced Met phosphorylation compared to phosphorylation by saturating concentrations of InlB protein may be due to differences in amounts and/or presentation of InlB (i.e. in a particle-bound or soluble form).

Met Acts Upstream of Signaling Involving Gab1, Cbl, and p85

We used the T47D and T47D/Met cell lines expressing different levels of Met (Figure 3A) to determine if Met is

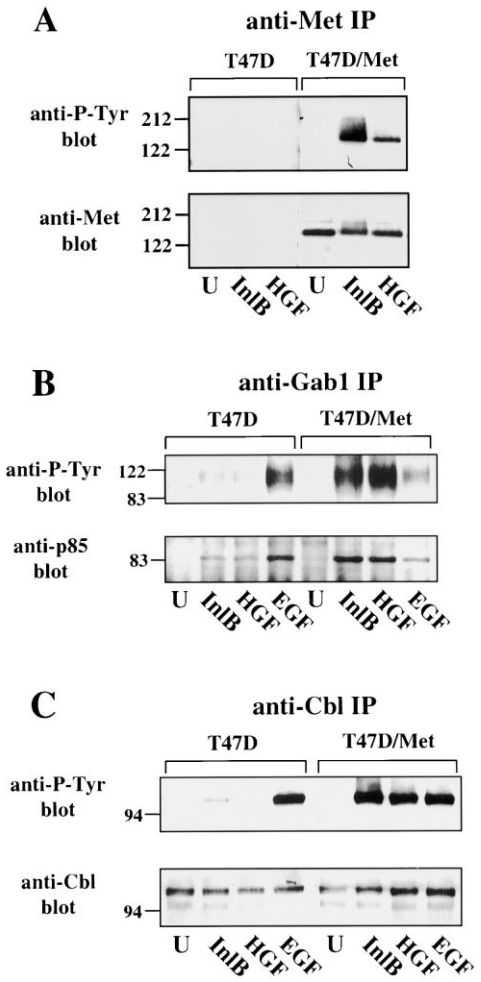

Figure 3. Met Is Required for InlB-Induced Phosphorylation of Gab1 and Cbl

T47D or T47D/Met cells were left untreated (U) or incubated for 5 min with 3 nM InlB, 3 nM HGF, or 17 nM EGF, as indicated. Cells were solubilized in lysis buffer and immunoprecipitations were performed.

(A) Tyrosine phosphorylation of Met. Met was immunoprecipitated with antibody C-12, and phosphorylation of the β chain of Met was detected with antibody 4G10 (upper panel). The membrane was then stripped and total precipitated Met was detected with antibody DL-21 (lower panel).

(B) Tyrosine phosphorylation of Gab1. Gab1was immunoprecipitated and detected by Western blotting with antibody 4G10 (upper panel). The membrane was stripped and probed with an antibody against the p85 subunit of PI 3-kinase (lower panel).

(C) Tyrosine phosphorylation of Cbl. Cbl was immunoprecipitated and detected with antibody 4G10 (upper panel). After stripping, total Cbl was detected with an anti-Cbl antibody (lower panel).

required for InlB-induced phosphorylation of Gab1 or Cbl. As previously observed with Vero cells (Ireton et al., 1999), incubation of T47D/Met cells with InlB caused tyrosine phosphorylation of Gab1 and Cbl and interaction (co-immunoprecipitation) of Gab1 with p85 (Figure 3B,C). Under these conditions, p85 itself is not detectably tyrosine phosphorylated (Ireton et al., 1999; data not shown). In contrast to the situation in T47D/Met cells, InlB-induced phosphorylation of Gab1 or Cbl in the parent T47D cell line was severely compromised. Western blotting of total cell lysates showed that Gab1, Cbl, and p85 were present at equivalent levels in both of these cell lines (data not shown). As controls, T47D/Met and T47D cells were treated with HGF or EGF, since both of these growth factors stimulate phosphorylation of both Gab1 and Cbl (Holgado-Madruga et al., 1996; Nguyen et al., 1997; Weidner et al., 1996). As expected, Gab1 and Cbl phosphorylation in response to HGF was detected in T47D/Met cells and was virtually absent in T47D cells. In contrast, phosphorylation of these adapters in response to EGF was not diminished in T47D cells compared to in T47D/Met cells. Taken together, these results demonstrate that Met is required for InlB- and HGF-induced phosphorylation of Gab1 and Cbl and for the formation of Gab1.p85 complexes. Thus Met acts upstream of InlB-induced cytosolic signaling involving Gab1, Cbl, and p85.

We note that although phosphorylation of Gab1 and Cbl was much more efficient in T47D/Met cells, low-level phosphorylation of these adapters and formation Gab1.p85 complexes was often observed in T47D cells treated with InlB or HGF (Figure 3B,C). It seems likely that these residual signaling events occur through the low levels of endogenous Met present in T47D cells.

InlB Stimulates Scattering of Epithelial Cells

HGF stimulates dispersal or "scattering" of Madin-Darby canine kidney (MDCK) cells (Birchmeier and Gherardi, 1998; Trusolino et al., 1998). InlB also induced cell scattering (Figure 4). The lowest concentration of InlB or HGF that effectively promoted scatter was 20 pM, demonstrating that these two proteins are active in a similar concentration range. In contrast, InlA did not induce cell scatter, even when used at 12 nM. Like scattering promoted by HGF (Royal and Park, 1995; Figure 4), InlB-induced scattering was sensitive to treatment of cells with a PI 3-kinase inhibitor (LY294002) (Figure 4). These results indicate that InlB is able to stimulate PI 3-kinase–dependent cell scatter in a manner similar to HGF.

Interestingly, the LRR-IR-BR and LRR derivatives of InlB did not efficiently induce scattering of MDCK cells (data not shown). The inability of these proteins to promote cell motility may be related to their lower activity in induction of Met phosphorylation and/or to differences in the cells used for assaying phosphorylation (T47D/Met) and scatter (MDCK).

InlB Binds to the Extracellular Domain of Met

InlB and Met were co-immunoprecipitated from lysates of Vero cells that were pre-incubated with purified InlB (Figure 5A). These results suggested that InlB and Met associate either directly or indirectly.

To test if InlB binds directly to Met, we used a fusion protein (Met-Fc) containing the extracellular domain of Met and the Fc region of human IgG1. This Met-Fc protein was previously shown to bind to HGF with high affinity (Mark et al., 1992). After incubation of soluble InlB with Met-Fc, InlB co-precipitated with the fusion protein (Figure 5B). In contrast, InlB did not co-precipitate with a fusion protein comprised of the extracellular region of the PDGF-R and Fc (PDGFR-Fc). The proteins containing the LRR, IR, and BR regions (LRR-IR-BR) or only the LRR region in InlB also bound to Met-Fc. In

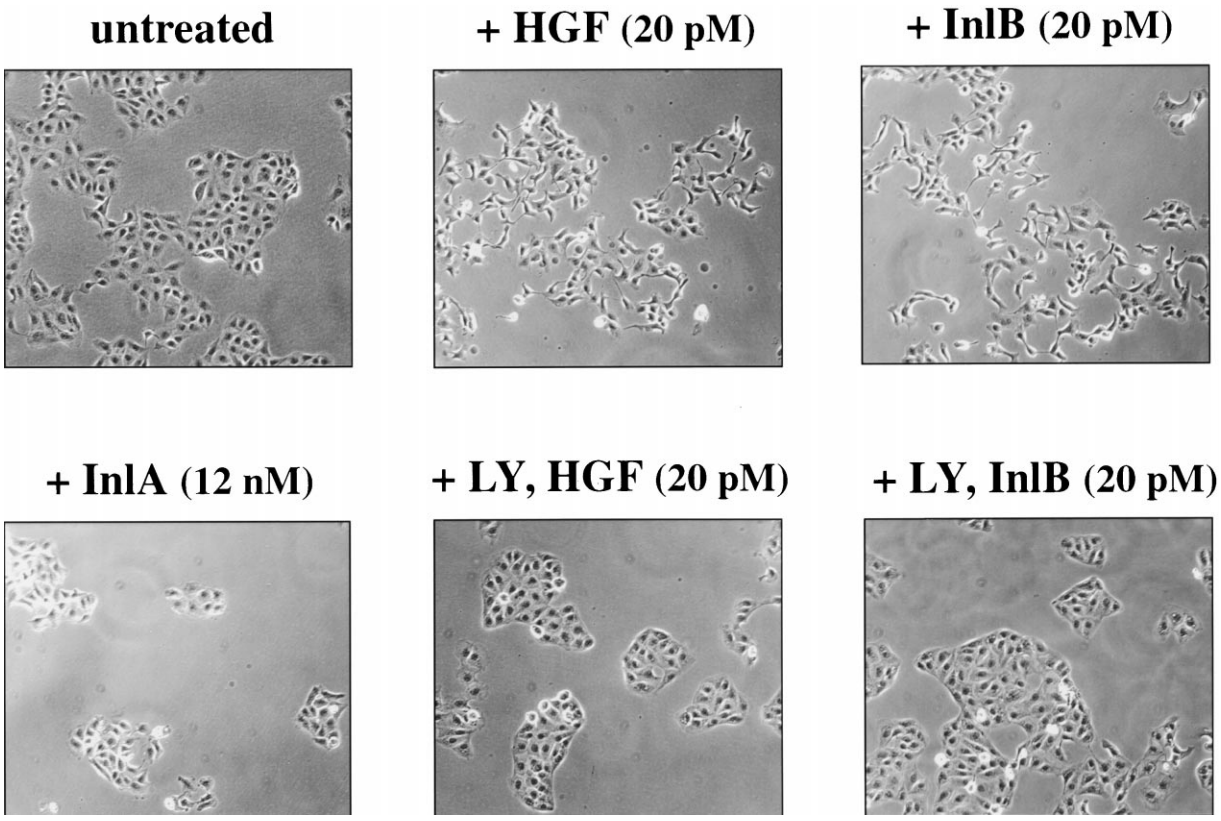

Figure 4. InlB Induces Scattering of Epithelial Cells

Serum-starved MDCK cells grown in monolayer were pre-incubated for 30 min with 50 μM LY294002 (LY) or with the solvent DMSO (all other panels). HGF (20 pM), full-length InlB (20 pM), or InlA (12 nM) were added as indicated. Photographs were taken ~8 hr after addition of proteins. The results are representative of two to three experiments, depending on the condition.

contrast, neither InlA nor the Csa derivative of InlB interacted with Met-Fc (Figure 5B and data not shown). Addition of a 10- and 50-fold excess of InlB inhibited the binding of 1.5 nM biotinylated InlB, whereas the same fold excess of InlA protein had no effect. Interestingly, a 50-fold molar excess of HGF also failed to inhibit binding of InlB to Met, suggesting that InlB and HGF may interact with different sites on this receptor. These results demonstrate that InlB binds to the extracellular domain of Met. The LRR domain is critical for binding.

Consistent with the above data, the Met-Fc protein bound to *L. monocytogenes* strains that express *inlB* (wt and Δ*inlA* mutant bacteria), but not to strains deleted for the *inlB* gene (Δ*inlB* and Δ*inlAB* mutants) (Figure 5C and data not shown). Thus InlB on intact bacteria interacts with the extracellular region of Met.

InlB-Mediated Entry of *L. monocytogenes* Requires Host Met

InlB mediates entry into non-phagocytic mammalian cells. To determine if Met is required for InlB-dependent entry, we evaluated uptake of various *L. monocytogenes* wt and mutant strains into T47D/Met and T47D cells (Table 1). These two cell lines are identical except for levels of Met (Figure 3A). In T47D cells, entry of the Δ*inlA* mutant was greatly reduced compared to entry of the wt bacterial strain. This result was expected, since T47D cells express the receptor for InlA, E-cadherin (H. Khoury and M. Park, personal communication). Deletion of *inlB* either alone (Δ*inlB* mutant) or in combination with *inlA* (Δ*inlAB* mutant) did not cause a further reduction in entry in T47D cells. Thus uptake into these cells depended on InlA, but not on InlB. In contrast, entry into T47D/Met cells depended on InlB as well as on InlA. Entry of the Δ*inlAB* mutant was only about 12% of that of the Δ*inlA* mutant indicating that, in the absence of InlA, InlB is required for uptake into T47D/Met cells. In addition, entry of Δ*inlA* (InlB+) bacteria into T47D/Met cells was ~9-fold more efficient than entry into parental T47D cells. In contrast, entry of Δ*inlB* (InlA+) or Δ*inlAB* bacteria was identical in the Met-positive and Met-deficient cell lines. These results provide genetic evidence that Met is needed for InlB-, but not InlA-, mediated entry.

Additional evidence was obtained by utilizing an antibody that recognizes an epitope in the Met extracellular domain (Figure 6A). Entry of *L. monocytogenes* into Vero cells depends on InlB but not on InlA (Ireton et al., 1996). Incubation of Vero cells with the anti-Met antibody DO-24 caused an ~95% inhibition in internalization of wt bacteria (Figure 6A). Incubation of T47D/Met cells with DO-24 caused a ~70% inhibition in entry of Δ*inlA* mutant bacteria, but did not affect entry of the Δ*inlB* or Δ*inlAB* mutant strains. Thus DO-24 inhibited InlB-dependent

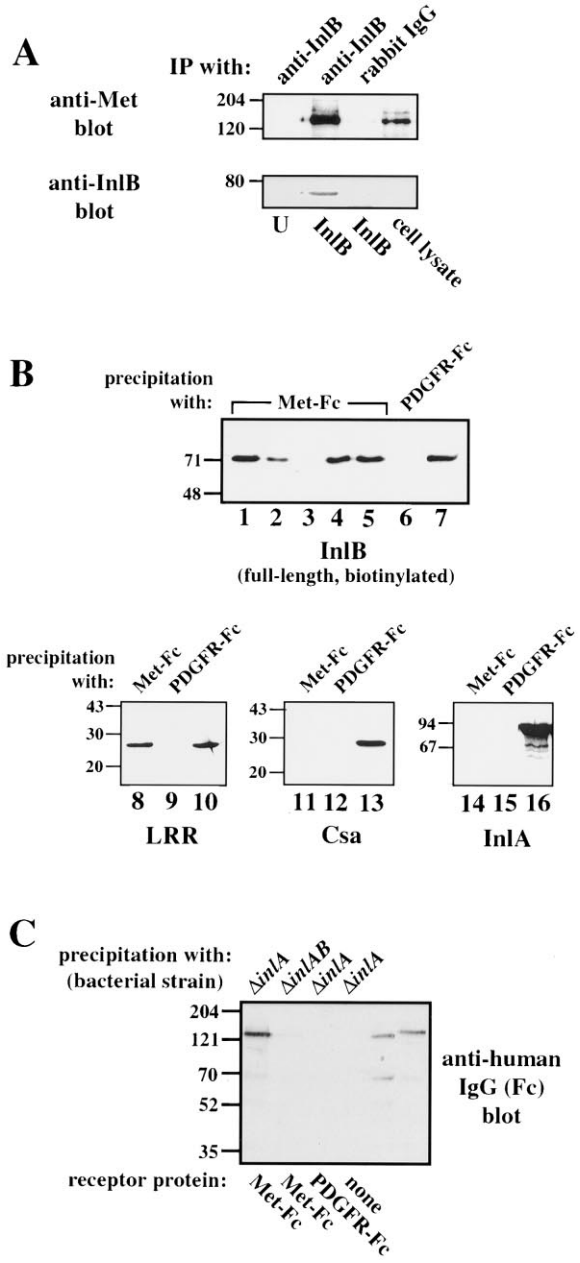

Figure 5. InlB Interacts with the Extracellular Domain of Met

(A) InlB associates with Met on mammalian cells. Vero cells were chilled to 4°C and left untreated (U), or incubated with 3 nM InlB for 15 min. Cells were solubilized and lysates were subjected to immunoprecipitation with polyclonal antibodies against InlB or with an equivalent amount of preimmune rabbit IgG. Immunoprecipitates were Western blotted with antibodies against Met (upper panel) or InlB (lower panel).

(B) InlB co-precipitates with purified Met receptor. 1.5 nM of biotinylated InlB was incubated with 1.3 nM of Met-Fc (lanes 1–5) or PDGFR-Fc fusion protein (lane 6) for 2 hr at 4°C. The sample in lane 1 contained only biotinylated InlB and Met-Fc. Samples in lanes 2 and 3 also contained a ten- and fifty-fold excess, respectively, of non-biotinylated competitor InlB added at the same time as the biotinylated protein. In lanes 4 and 5, a fifty-fold molar excess of non-biotinylated InlA (lane 4) or HGF (lane 5) was added with biotinylated InlB. After precipitation with protein A-sepharose beads, bound (biotinylated) InlB was detected by probing with streptavidin-alkaline phosphatase. Binding of HGF to Met-Fc (lane 5) was verified by probing the precipitates with an anti-HGF antibody (data not shown). Lanes 8–10, 11–13, and 14–16: 4 nM of the LRR derivative, the Csa protein, or InlA were incubated with Met-Fc or PDGFR-Fc (3 nM). InlB derivatives or InlA in precipitates were detected with antibodies against InlB or InlA. In lanes 7, 10, 13, and 16, the same amount of (biotinylated) InlB (100 ng), LRR (100 ng), Csa (100 ng), or InlA (300 ng) used for binding was loaded as a reference for the maximum amount of protein that could be precipitated. Each binding assay was performed at least three times, with similar results.

(C) Met binds to *L. monocytogenes* strains expressing *inlB*. Δ*inlA* or Δ*inlAB* mutant bacterial strains were incubated with or without Met-Fc or PDGFR-Fc proteins, as indicated. Binding was performed as described in the Experimental Procedures, and fusion proteins that co-precipitated with bacteria were detected by Western blotting with rabbit anti-human IgG. In the last two lanes, 25 ng of purified Met-Fc or PDGFR-Fc were loaded as references for migration of the receptor proteins.

Table 1. Expression of Met Renders Mammalian Cells Susceptible to InlB-dependent Entry

Bacterial Strain	Genotype	Mammalian Cells	Relative Entry
wt	*inlA* + *inlB*+	T47D	1.0
Δ*inlA*	*inlA*− *inlB*+	T47D	0.045 ± 0.011
Δ*inlB*	*inlA*+ *inlB*−	T47D	1.0 ± 0.17
Δ*inlAB*	*inlA*− *inlB*−	T47D	0.040 ± 0.0072
wt	*inlA*+ *inlB*+	T47D/Met	1.6 ± 0.52
Δ*inlA*	*inlA*− *inlB*+	T47D/Met	0.41 ± 0.15
Δ*inlB*	*inlA*+ *inlB*−	T47D/Met	1.1 ± 0.29
Δ*inlAB*	*inlA*− *inlB*−	T47D/Met	0.049 ± 0.016

Shown are the average, relative entry values ± standard deviations of seven independent experiments. In each experiment, infection with each bacterial strain was performed in duplicate. Percent entry is the percentage of the initial inoculum that survived gentamicin treatment after one hr infection in the absence of antibiotic, followed by two hr incubation in the presence of gentamicin (Mengaud et al., 1996). To obtain the relative entry values, the absolute percent entry values in a given experiment were normalized to that of the wt strain EGD in T47D cells. Absolute percent entry for EGD in the seven experiments ranged from 22% to 88% in T47D cells and 50% to 190% in T47D/Met cells. The greater than 100% values are due to replication of bacteria during infection. (Bacteria undergo one to two doublings in 3 hr).

entry, but did not affect entry dependent on InlA or other bacterial factors. Entry of the Δ*inlA* strain into T47D cells was not inhibited by DO-24 (data not shown), indicating that this antibody affects uptake by interacting with Met. The effect was specific to *L. monocytogenes,* since DO-24 did not inhibit internalization of the unrelated invasive bacterium *Yersinia pseudotuberculosis* (Isberg and Tran Van Nhieu, 1994) (Figure 6A).

Since the Met-Fc protein bound to *L. monocytogenes* strains expressing *inlB* (Figure 5C), we asked whether incubation with soluble Met-Fc affects bacterial entry by competing with Met on the surface of host cells. Preincubation of Δ*inlA* mutant bacteria with 10 nM Met-Fc caused an ~80% inhibition in entry into T47D/Met cells, whereas pre-incubation with 10 nM PDGFR-Fc protein had no effect (Figure 6B). Taken together with the data in Table 1 and Figure 6A, these results demonstrate that Met is required for InlB-mediated entry of *L. monocytogenes*.

Discussion

In this work, we demonstrated that Met, the receptor for HGF/scatter factor, is needed for pathogen entry and host signal transduction promoted by the bacterial protein InlB. Like HGF, InlB induces tyrosine-phosphorylation of Met. InlB also stimulates signaling and biological processes known to occur downstream of Met, including phosphorylation of Gab1 and Cbl and epithelial cell scattering. These results suggest that InlB and HGF elicit similar signaling pathways, albeit with different kinetics (Figure 2A). Met activation is likely to result from a direct interaction with InlB. Using purified proteins, we found that InlB binds to the extracellular domain of Met. Binding to Met in vitro and phosphorylation of Met in vivo was mediated primarily by the LRR domain. These results are consistent with previous findings that indicated the LRR region is critical for bacterial entry and activation of PI 3-kinase (Braun et al., 1999).

InlB and HGF do not share significant amino acid similarity (Shen and Ireton, our unpublished results). HGF is a disulfide-linked α/β heterodimer homologous to the blood protease plasminogen (Trusolino et al., 1998). The α chain of HGF contains an N-terminal (N) hairpin loop and four Kringle (K) domains, and the β chain has homology to the catalytic domain in serine proteases. The N and first Kringle domains (NK1) in the α chain are sufficient to bind Met with high affinity (Trusolino et al., 1998). As expected from the lack of sequence similarity between InlB and HGF, a comparison of recently solved crystal structures indicates that the LRR region in InlB (Marino et al., 1999) and NK1 in HGF (Chirgadze et al., 1999; Ultsch et al., 1998) are structurally unrelated. Interestingly, excess HGF does not inhibit binding of InlB to Met, suggesting that InlB and HGF may interact with distinct sites on this RTK. Despite the lack of competition between HGF and InlB, prolonged (4 hr) pretreatment of mammalian cells with high concentrations of HGF ($\sim$3 nM) results in inhibition in entry of wt *L. monocytogenes* (Shen and Ireton, our unpublished results). Under these conditions, HGF causes a substantial decrease in steady state levels of Met protein. Thus HGF-induced inhibition in entry may be due to downregulation of surface-bound Met.

How does interaction of InlB with Met lead to entry of *L. monocytogenes*? One likely role of Met is to mediate host cytosolic signaling events, including activation of p85-p110. Experiments with wortmannin or LY294002 demonstrate that host PI 3-kinase activity is required for InlB-mediated entry into Vero, T47D/Met, and other cell lines (Ireton et al., 1996). Interaction of InlB with host cells leads to an activation of PI 3-kinase that coincides with the formation of complexes containing p85 and the tyrosine-phosphorylated adapters Gab1 and Cbl (Ireton et al., 1999). In this work, we found that Met is needed for InlB-induced phosphorylation of Gab1 and Cbl, and formation of Gab1.p85 complexes. Thus Met acts upstream of these adapters and p85-p110 in InlB-mediated signaling.

Stimulation of cells with HGF results in the recruitment of Gab1, Cbl, p85, and other signaling molecules to activated Met (Nguyen et al., 1997; Trusolino et al., 1998). Recruitment of these proteins is dependent on the intrinsic kinase activity of Met and requires phosphorylation of two 'docking site' tyrosines (Y1349 and Y1356) in the Met cytoplasmic domain. Phosphorylation of Y1349 and Y1356 is also needed for all of the known biological functions of Met (Birchmeier and Gherardi, 1998; Trusolino et al., 1998). It will be interesting to determine whether entry of *L. monocytogenes* requires phosphorylation of docking site tyrosines in Met.

How might interaction of InlB with Met affect pathogenesis in vivo? In non-immune mice, virulent *L. monocytogenes* replicates primarily in the liver after infection of hepatocytes (Cossart and Lecuit, 1998). Efficient liver infection and bacterial entry into mouse hepatocytes in vitro requires *inlB*, but not *inlA* (Cossart and Lecuit, 1998; Dramsi et al., 1995). Our data indicates that InlB induces tyrosine phosphorylation of Met in the mouse hepatocyte cell line TIB73 (Figure 1C). Incubation of *L. monocytogenes* with soluble Met-Fc protein inhibits uptake into TIB73 cells (Shen and Ireton, our unpublished results), suggesting that bacterial entry requires interaction with Met. Our findings are consistent with the possibility that Met mediates infection of mouse hepatocytes in vivo.

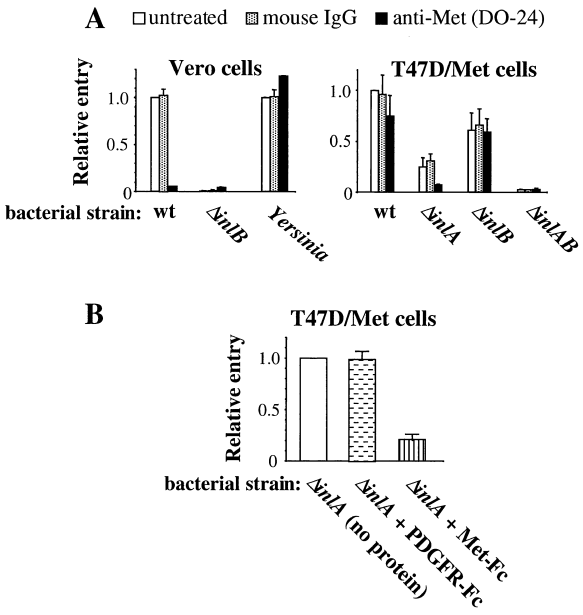

Figure 6. Inhibition of InlB-Mediated Entry

(A) Inhibition of entry by incubation of mammalian cells with an antibody against the extracellular domain of Met. Vero or T47D/Met cells were incubated with DMEM alone (untreated), DMEM containing mouse IgG (50 mg/l), or DMEM containing the anti-Met monoclonal antibody DO-24 (50 mg/l) for 45 min. Cells were then infected with the indicated *L. monocytogenes* strains or with *Y. pseudotuberculosis* strain YPIIIC (Mengaud et al., 1996) (X axis), and gentamicin protection assays were performed as described in the Experimental Procedures. On the Y axis is the relative percent entry ($\pm$ SD) values from two (Vero) or three (T47D/Met) experiments in which infection with each bacterial strain was done in duplicate.

(B) Inhibition of entry by incubation of bacteria with a soluble protein containing the Met extracellular domain. The Δ*inlA* mutant of *L. monocytogenes* was diluted in DMEM and incubated in the absence of protein or in the presence of 10 nM control PDGFR-Fc or Met-Fc protein as described (Experimental Procedures). The bacterial suspensions were used to infect T47D/Met cells and entry was measured as described. The results are from three experiments in which each infection condition was performed in duplicate.

In contrast to the situation in mice, Listeria pathogenesis in humans is characterized by meningitis/meningoencephalitis or perinatal infections. One way in which bacteria could traverse the blood-brain barrier and/or infect the fetus is through penetration of endothelial cells lining blood vessels in the brain and/or placenta. Importantly, *L. monocytogenes* enters directly into human endothelial cells in an InlB-dependent (and InlA-independent) manner (Greiffenberg et al., 1998; Parida et al., 1998). Endothelial cells in the brain, placental villi, and umbilical cord express Met (Bussolino et al., 1992; Kilby et al., 1996). We are currently testing whether Met is essential for entry of *L. monocytogenes* into human endothelial cells.

While our results demonstrate that Met is required for InlB-mediated entry, it is possible that other mammalian receptors also participate in this process. A recent report suggests a role for the mammalian gC1q-receptor (R) in InlB-dependent bacterial internalization (Braun et al., 2000). gC1q-R, previously identified as a protein that binds the complement component C1q (Eggleton et al., 1998), does not contain a typical hydrophobic transmembrane sequence or a glycosylphosphatidyl inositol anchoring motif. Thus, it is unclear how this protein is localized to the mammalian plasma membrane. gC1q-R also lacks an identifiable cytoplasmic region, indicating that it is unlikely to play a direct role in signaling downstream of InlB. It has been suggested that gC1q-R interacts with an unidentified membrane-spanning receptor to mediate bacterial entry (Braun et al., 2000). Further work will clarify whether Met and gC1q-R act together or independently to promote uptake of *L. monocytogenes*.

Many microbial pathogens adhere to or enter into mammalian cells through interaction with host surface receptors of the Integrin or Immunoglobulin superfamilies (Finlay and Falkow, 1997; Virji, 1996). More recently, G-Protein Coupled Receptors have been shown to mediate entry of the HIV virus (Berger et al., 1999). InlB/Met interaction is a novel example in which entry of a pathogenic microorganism is mediated through direct engagement of a host RTK. Further work should elucidate the role of Met in *L. monocytogenes* pathogenesis and contribute to a better understanding of the general mechanisms through which this receptor is activated by extracellular signals.

Experimental Procedures

Bacterial Strains and Mammalian Cell Lines

The *L. monocytogenes* Δ*inlA*, Δ*inlB*, and Δ*inlAB* mutant strains are isogenic with the wt strain EGD and contain in-frame deletions in the *inlA* and/or *inlB* genes. (Dramsi et al., 1995). Growth of these strains and of the *Y. pseudotuberculosis* strain YPIIIC were as described (Dramsi et al., 1995; Mengaud et al., 1996).

The mammalian cell lines used are the African Green Monkey kidney cell line Vero (ATTC CRL-1587), the dog Madin Darby Canine kidney (MDCK) cell line, the human mammary epithelial cell line T47D (ATTC HTG-133), and the mouse hepatocyte cell line TIB-73 (ATTC TIB-73). T47D/Met cells containing the human Met cDNA in the mammalian expression vector pSV-A-MLV were generated by retroviral infection of T47D cells essentially as described for construction of MDCK cells expressing the CSF-Met receptor (Zhu et al., 1994). All cell lines were grown at 37°C in 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 g/l glucose, 2 mM glutamine, and 10% fetal calf serum. For all experiments except the entry assays, T47D/Met cells were grown in medium with 200 mg/l G418 to select for the Met cDNA.

Antibodies, Proteins, and Other Reagents

The polyclonal antibodies used were preimmune rabbit IgG (Sigma), preimmune mouse IgG (Sigma), rabbit anti-human IgG (Fc fragment specific) (Jackson Immunolabs), rabbit anti-mouse IgG (Sigma), anti-Cbl (sc-170; Santa Cruz Biotechnology), anti-Gab1 (06–579; Upstate Biotechnology [UBI]), anti-p85 (06–195; UBI), anti-human Met C-12 (sc-10, Santa Cruz Biotechnology), anti-mouse Met 101, and anti-InlB (Braun et al., 1997). The monoclonal antibodies used were anti-Met DO-24 (05–237; UBI), anti-Met DL-21 (05–238; UBI), anti-phosphotyrosine 4G10, and anti-InlA K18.4 (Braun et al., 1997). DO-24 and DL-21 recognize epitopes in the extracellular domain of human and monkey Met. Secondary antibody horse radish peroxidase (HRPO) and alkaline phosphatase conjugates were from Jackson Immunolabs. The streptavidin-alkaline phosphatase conjugate and recombinant human EGF were from Sigma. Recombinant human HGF was purified as described (Zhu et al., 1994) or obtained from Sigma. Recombinant proteins containing the extracellular domains of human Met or Platelet Derived Growth Factor Receptor (PDGF-R) fused to the Fc region of human IgG1 were from R&D systems (258-MT and 385-PR).

Purification of Recombinant InlB and InlA Proteins

Recombinant full-length InlB protein is from the same preparation as that in Ireton et al. (Ireton et al., 1999). Biotinylated InlB was prepared using Sulfo-NHS-LC-Biotin (Pierce), according to the manufacturer's protocol for IgG. Biotinylated protein was separated from free biotin on micro-spin columns, and remaining unreacted biotin was quenched with a one-tenth volume of 1 M ethanolamine (pH 8.5). The final biotinylated preparation was active in stimulating Met phosphorylation. The LRR-IR-BR derivative of InlB (previously called InlB1–398) was purified as described (Braun et al., 1997). For purification of proteins consisting of only the LRR or Csa domains in InlB or of full-length InlA, we used the Polymerase Chain Reaction (PCR) and a mixture of Taq and Pwo DNA polymerases (Expand Long Template PCR system; Boehringer Mannheim) to amplify the corresponding DNA regions from chromosomal DNA of wt *L. monocytogenes* strain EGD. The primers used for amplification of the LRR and Csa regions were the same as those in a previous study (Braun et al., 1999). The primers used for InlA were 5′-GGAATTCCATATG GCTACAATTACACAAGATACTCCT-3′ (positions 1638 to 1661; [Gaillard et al., 1991]) and 5′-GGCCTCGAGCTATTTACTAGCACGTGCTTT-3′ (positions 3934 to 3914) (underlined sequences indicate NdeI and XhoI sites, respectively). PCR products were ligated into the expression plasmid pET28a(+) (Novagen) after digestion with NheI/HindIII (LRR and Csa) or NdeI/XhoI (InlA). DNA sequencing indicated that the resulting plasmids, pET28a-LRR, pET28a-Csa, and pET28a-InlA coded for proteins identical to the LRR or Csa regions in InlB or to full-length mature InlA with the expected 6x histidine tag added to the NH_2-terminus. Cultures of transformants of *E. coli* strain BL21 λDE3 containing pET28a-LRR, pET28a-Csa, or pET28a-InlA were induced by addition of 1 mM IPTG for 3 hr, and the corresponding 6x histidine tagged proteins were purified as described for full-length InlB (Ireton et al., 1999). InlA was further purified with a HiTrap Q column (Amersham Pharmacia Biotech) as described (Mengaud et al., 1996). Finally, the 6x histidine tag in the LRR-IR-BR, LRR, Csa, or InlA proteins was removed by thrombin treatment and traces of thrombin were eliminated (Ireton et al., 1999). Purified InlA was active in binding mammalian cells (Caco-2) expressing E-cadherin (Mengaud et al., 1996). Purified Csa was shown to be functional by demonstrating binding to intact *L. monocytogenes*.

Immunoprecipitation and Western Blotting

$7x10^5$ Vero, $7x10^5$ TIB73, $9x10^5$ T47D, or $9x10^5$ T47D/Met cells were seeded in 10 cm plates and grown for 40 hr. Cells were starved for 4–5 hr in DMEM containing 0.2% fetal calf serum before treatment with InlB, InlA, HGF, or EGF, or before infection with *L. monocytogenes* strains. Immunoprecipitation of proteins from solubilized lysates of mammalian cells was as described (Ireton et al., 1999), except that the lysis buffer contained 1% Triton X-100, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 3 mM sodium orthovanadate, 20 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and 10 mg/l each of aprotinin, leupeptin, and pepstatin. Protein concentrations of lysates were determined using a BCA kit (Pierce), and equal

quantities of total protein were used in each immunoprecipitation in a given experiment (typically 0.70 mg of lysate per precipitation). Incubations of lysates with antibodies were done overnight. For efficient immunoprecipitation with antibody DO-24, 2 μg of rabbit anti-mouse IgG was added per precipitation one hr after DO-24. Immune complexes were collected on protein A-sepharose CL-4B beads, and proteins in immunoprecipitates were separated on 7.5% SDS-polyacrylamide gels. Transfer of proteins to nitrocellulose membranes, incubation of membranes with antibodies, detection with ECL, ECL Plus, or CDP Star chemiluminescent systems (Amersham Pharmacia Biotech), and exposure to film were all as described (Ireton et al., 1999). Unless otherwise stated, all immunoprecipitation experiments in Figures 1–5 were performed at least three times, with similar results.

Coprecipitations with Met-Fc

Met-Fc or PDGFR-Fc fusion proteins were added to the concentrations indicated in the legend to Figure 5B in 1 ml of binding buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 0.2% Bovine Serum Albumin, 0.2% Triton X-100, 1 mM PMSF, and 10 mg/l each of aprotinin and leupeptin) in 1.5 ml plastic tubes. The tubes were incubated on a rotating wheel at 4°C for 30 min, and InlB, LRR, Csa, or InlA proteins were added to the concentrations indicated in Figure 5B. In competition experiments, excess non-biotinylated InlB, InlA, or HGF was added at the same time as biotinylated InlB. HGF used for competition was pre-incubated at 37°C for 2 hr in 5% fetal calf serum to allow processing to the disulfide-linked heterodimeric form. Tubes were then incubated with rotation for 2 hr at 4°C. One hundred microliters of a 10% slurry of Protein A-sepharose was added to each tube, samples were incubated for an additional hr with rotation at 4°C, and the Met-Fc or PDGFR-Fc proteins were precipitated by centrifugation. Precipitates were washed four times in buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1 mM PMSF) with 0.1% Tween 20 and twice in buffer without Tween 20, followed by addition of sample buffer, boiling (5 min), and storage at ~80°C. Samples were later migrated on 7.5% (full-length InlB and InlA) or 12% (LRR and Csa proteins) SDS/PAGE gels, transferred to nitrocellulose, and bound proteins were detected as described in the legend to Figure 5B.

For binding of bacteria to Met-Fc, Δ*inlA* or Δ*inlAB* mutant strains of *L. monocytogenes* were grown in Brain Heart Infusion (BHI) broth to an OD600 of 0.80, and 1 ml of each culture was taken for each binding reaction. Bacterial cells were washed twice in buffer with 20 mM HEPES pH 7.5, 150 mM NaCl, resuspended in 1 ml of binding buffer (20 mM HEPES pH 7.5, 150 mM NaCl, 1 mM PMSF, 1 mM $CaCl_2$, 1mM $MgCl_2$, and 0.2% BSA), and incubated at room temperature on a rotating wheel for 30 min. Where indicated, Met-Fc or PDGFR-Fc proteins were added to a final concentration of 1 μg/ml (~6 nM) and samples were incubated with rotation for an additional hr. Samples were centrifuged to pellet bacteria, and washed three times in 20 mM HEPES pH 7.5, 300 mM NaCl, 0.05% Tween 20 and three times in buffer lacking Tween 20. The final bacterial pellets were resuspended in 20 microliters of PBS containing sample buffer, boiled for 5 min, and stored at ~80°C before migration on 7.5% SDS/PAGE gels and Western blotting.

Scattering Assays

Scattering assays were performed on serum-starved MDCK cells essentially as described (Royal and Park, 1995). Photographs were taken ~8 hr after addition of HGF, InlB, or InlA.

Infection of Cell Monolayers and Measurement of Bacterial Entry

Infection of mammalian cells with the *L. monocytogenes* strains EGD (wt), Δ*inlA*, Δ*inlB*, and Δ*inlAB* was as described (Ireton et al., 1999). A multiplicity of infection of ~50:1 was used for both immunoprecipitation and entry experiments. For entry assays, $2x10^4$ Vero cells or $4.5x10^4$ T47D or T47D/Met cells were seeded per well of 24 well plates two days before infection. In experiments with T47D and T47D/Met cells, the number of cells present per well before infection were similar for the two different cell lines. Gentamicin protection assays to measure intracellular (viable) bacteria were as described (Mengaud et al., 1996), except that 20 mg/l and 100 mg/ml gentamicin was used for *L. monocytogenes* strains and *Y. pseudotuberculosis* strain YPIIIC, respectively. For antibody inhibition experiments, cells in 24 well plates were incubated with 0.5 ml of DMEM lacking antibody, containing antibody DO-24, or containing control mouse IgG at a final concentration of 50 mg/l approximately 45 min before infection. Bacterial suspensions in DMEM (0.3 ml) were then added to each well and cells were left in contact with bacteria and antibodies during the 60 min infection period. Cells were then washed with DMEM and incubated with DMEM containing gentamicin as described (Mengaud et al., 1996). For experiments with the Met-Fc and PDGFR-Fc proteins, suspensions of bacteria diluted in DMEM were incubated for 20 min at room temperature in the presence or absence of 10 nM of either receptor. Then suspensions containing bacteria alone or bacteria and receptor were added to wells, and the gentamicin assay was performed as usual.

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