Src, cortactin and Arp2/3 complex are required for E-cadherin-mediated internalization of *Listeria* into cells

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**Summary**

*Listeria monocytogenes* is a food-borne pathogen able to invade non-phagocytic cells. InlA, a *L. monocytogenes* surface protein, interacts with human E-cadherin to promote bacterial entry. *L. monocytogenes* internalization is a dynamic process involving co-ordinated actin cytoskeleton rearrangements and host cell membrane remodelling at the site of bacterial attachment. Interaction between E-cadherin and catenins is required to promote *Listeria* entry, and for the establishment of adherens junctions in epithelial cells. Although several molecular factors promoting E-cadherin-mediated *Listeria* internalization have been identified, the proteins regulating the transient actin polymerization required at the bacterial entry site are unknown. Here we show that the Arp2/3 complex acts as an actin nucleator during the InlA/E-cadherin-dependent internalization. Using a variety of approaches including siRNA, expression of dominant negative derivatives and pharmacological inhibitors, we demonstrate the crucial role of cortactin in the activation of the Arp2/3 complex during InlA-mediated entry. We also show the requirement of the small GTPase Rac1 and that of Src-tyrosine kinase activity to promote *Listeria* internalization. Together, these data suggest a model in which Src tyrosine kinase and Rac1 promote recruitment of cortactin and activation of Arp2/3 at *Listeria* entry site, mimicking events that occur during adherens junction formation.

**Introduction**

*Listeria monocytogenes* is able to cross the intestinal, fetoplacental and blood–brain barriers, causing gastroenteritis, septicaemia, abortions and central nervous system infections (Vazquez-Boland et al., 2001; Khelef et al., 2006). *L. monocytogenes* enters and replicates into cultured non-phagocytic cells. Two major modes of *Listeria* internalization have been described and are referred as InlA- and InlB-dependent pathways (Cossart et al., 2003; Cossart and Sansonetti, 2004; Hamon et al., 2006; Pizarro-Cerda and Cossart, 2006). InlA and InlB are two *L. monocytogenes* surface proteins that interact with specific receptors at the host cell surface. InlA interacts with E-cadherin whereas InlB binds c-Met, gC1qR and proteoglycans (Mengaud et al., 1996a; Jonquieres et al., 1999; 2001; Braun et al., 2000; Shen et al., 2000). Both InlA and InlB-mediated entry pathways require actin cytoskeleton rearrangements (Gaillard et al., 2001), whose activation mechanism is cell type-dependent and may result from the cooperation between Rac/WAVE and Cdc42/N-WASP signalling cascades (Bierne et al., 2005). During the InlA-dependent uptake, as for adherens junction formation, E-cadherin signals through β- and α-catenins and induce actin cytoskeleton rearrangements (Yap et al., 1997; Lecuit et al., 2000). We have previously shown that the unconventional myosin VIIa and vezatin, two actin cytoskeleton-associated proteins localized at adherens junctions, are required for InlA-dependent entry (Sousa et al., 2000)
et al., 2004). Recently, we have also demonstrated the critical role of ARHGAP10, a new Rho-GAP protein, in InlA-dependent entry and in the formation of adherens junctions (Sousa et al., 2005). However, the cellular proteins that drive actin polymerization during InlA-mediated entry of *Listeria* are unknown.

In eukaryotic cells, actin polymerization is not a spontaneous process. A nucleator molecule is needed to initiate efficient actin filament formation and elongation. The Arp2/3 complex is a highly conserved actin nucleator, abundantly expressed in all eukaryotic cells and localized at sites of dynamic actin assembly (Pollard and Cooper, 1986; Higgs and Pollard, 2001). It is a stable protein complex composed of two actin-related proteins (Arp2 and Arp3) and five other unrelated proteins (Goley and Welch, 2006). The Arp2/3 complex is essential for the actin rearrangements that mediate the formation of actin rich structures such as lamellipodia and filopodia (Machesky et al., 1997; Welch et al., 1997; Svitkina and Borisy, 1999). Arp2/3 alone has a low intrinsic actin nucleation activity, allowing a tight control of the actin network generation. Indeed, to initiate actin polymerization, Arp2/3 needs to be activated (Mullins et al., 1998; Welch et al., 1998; Pollard and Borisy, 2003). The first identified activator of Arp2/3 was the ActA protein from *L. monocytogenes* (Welch et al., 1998; Gouin et al., 2005). Other activators have since been identified, including the SCAR/WASP/WAVE protein family (Egile et al., 1999; Machesky et al., 1999; Rohatgi et al., 1999), the *Dictyostelium* CARMIL proteins (Jung et al., 2001), the yeast Abp1 protein (Goode et al., 2001) and the actin-binding protein cortactin (Weed et al., 2000; Weaver et al., 2001; Uruno et al., 2005). Arp2/3 complex mediates signals from Cdc42/N-WASP (Machesky and Insall, 1998; Rohatgi et al., 1999) and Rac/WAVE (Miki et al., 2000) pathways to induce cortical actin polymerization (Bompard and Caron, 2004).

In this work, we investigated whether factors involved in initiating actin polymerization within the cell were involved in actin polymerization at the InlA/E-cadherin-dependent entry site of *Listeria* and were needed for entry. We used a series of approaches to disrupt signalling pathways previously shown to directly or indirectly regulate actin polymerization. We found that the Arp2/3 complex is recruited together with actin at the entry site of bacteria and that it is required for the entry process. We further show that in E-cadherin-expressing cells, InlA/ E-cadherin-dependent entry of *Listeria* requires signalling pathways involving Rac1 and cortactin. We also demonstrate the requirement of Src kinase activity in cortactin tyrosine phosphorylation to promote entry. Because Rac, cortactin and the Arp2/3 complex are necessary for efficient formation of nascent cell–cell contacts (Kovacs et al., 2002a,b; Helwani et al., 2004; Verma et al., 2004), this work provides a further illustration of the converging mechanisms for InlA/E-cadherin-mediated entry and E-cadherin-mediated adherens junction formation.

**Results**

**A role for the Arp2/3 complex in *Listeria* InlA/E-cadherin-mediated entry**

In this study, we use a variety of bacterial tools and cell types in order to precisely focus our study on the InlA/E-cadherin-dependent entry. These bacterial strains characterized and successfully used in a number of previous studies (Lecuit et al., 1997; 2000; Sousa et al., 2004; 2005) are *Listeria innocua* expressing InlA (*L. innocua* (InlA)), a non-pathogenic species engineered to express InlA and *L. monocytogenes* ∆inIB, an isogenic derivative of *L. monocytogenes* reference strain EGD expressing InlA but not InlB. We also showed used latex beads coated with purified InlA (Lecuit et al., 1997). In these three experimental systems, InlA induces internalization in cells expressing E-cadherin, and our earlier studies have indicated that these three different tools behave similarly regarding the entry process (Lecuit et al., 1997; 2000; Sousa et al., 2004; 2005). These systems exhibit the following respective advantages: *L. innocua* (InlA) enters only via the InlA pathway and does not secrete the *L. monocytogenes* virulence factor LLO and thus allows the use of high multiplicity of infection (moi) without detrimental effect on cell viability; InlA-coated beads have a regular shape and detection of protein recruitment around them is thus easier; finally *L. monocytogenes* ∆inIB allows the assessment of the relevance in the *L. monocytogenes* species of results obtained in the two other systems. In the present study, these complementary tools were used in parallel and always behaved similarly, as far as entry was concerned (see below). We also use here a number of different cell types which have different specific properties: as InlA/E-cadherin interaction is critical for the crossing of the intestinal barrier, the intestinal epithelial cell line Caco-2 is considered as the most relevant model. However, these cells are difficult to transfect and cannot be used to assess in a quantitative manner the effect of overexpression of dominant negative derivatives on bacterial uptake. We thus used additional well-defined and previously described cell lines including L2071 fibroblasts stably transduced with human E-cadherin (Lecuit et al., 2000), and HEK293, that also express E-cadherin and can be easily transfected. Here again, these different tools gave concordant results.

To investigate the role of Arp2/3 complex in InlA/ E-cadherin-mediated entry, we first analysed the localization of Arp2/3 complex in *Listeria*-infected cells. Caco-2 cells, in which *Listeria* enters mainly via the InlA-pathway, were infected with *L. innocua* (InlA). Infected Caco-2 cells

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were fixed and incubated with antibodies raised against Arp3. We observed that Arp3 was recruited at bacterial entry sites 45 min after infection (Fig. 1A). Moreover, Arp3 colocalized with actin at these sites, as well as at cell–cell contacts.

We next analysed the respective kinetics of actin and Arp2/3 recruitment during entry mediated by the interaction of InlA with E-cadherin. To synchronize entry, Caco-2 cells were infected with \( \text{L. innocua} \) (InlA) at 4°C submitted to mild centrifugation and then shifted to 37°C. Infected cells were fixed at different time points after temperature shift (10, 20, 30, 45, 60 and 90 min). We then monitored the entry of bacteria, the presence of an actin cup and the recruitment of Arp3. For each time point, 150 bacteria were analysed (Fig. 1B). The recruitment of Arp2/3 complex at the bacterial entry site reached a peak 30 min after temperature shift and then decreased rapidly (Fig. 1B). The formation of F-actin cups around bacteria peaked at 45 min. At this time point, 78% of the bacteria had been internalized (Fig. 1B). These results suggest that the recruitment of Arp2/3 complex at the bacterial entry site precedes actin polymerization leading to actin cup formation and uptake of \textit{Listeria}.

To investigate the role of the Arp2/3 complex in the entry process, we quantified the entry of InlA-coated beads into transiently transfected \( \text{L.2071hEcad} \) cells (fibroblasts expressing human E-cadherin) expressing ScarWA, corresponding to the C-terminal WASP-homology and acidic domains of Scar1, an Arp2/3 activator (Machesky and Insall, 1998). This truncated protein binds the Arp2/3 complex and its overexpression sequesters Arp2/3 in the cytosol. ScarW that does not contain the acidic domain and does not bind Arp2/3 was used as a negative control (Machesky and Insall, 1998). Expression levels of ScarW and ScarWA proteins in transfected cells were similar (Fig. 1C). Beads uptake was significantly inhibited in ScarWA-expressing \( \text{L.2071hEcad} \) cells, with a 60% decrease when compared with non-transfected cells (Fig. 1D). Scar WA construct was also expressed in HEK293 cells and the entry of \( \text{L. innocua} \) (InlA) and \( \text{L. monocytogenes} \) \( \Delta \text{inlB} \) was quantified. In accordance with InlA-beads uptake, the entry of both bacterial strains was strongly reduced in HEK293 cells expressing ScarWA (Fig. 1E). Expression of ScarW had no inhibitory effect on either beads or bacterial uptake (Fig. 1D and E). Together, these results indicate that the actin nucleation activity of the Arp2/3 complex is required for \textit{Listeria} InlA/E-cadherin-dependent entry.

\textbf{A role for the small GTPase Rac1 in InlA/E-cadherin-dependent entry}

The implication of Arp2/3-dependent actin polymerization led us to investigate the role of RhoGTPases in \textit{Listeria} invasion. Rho-GTPases are upstream activators of the Arp2/3 complex in many actin polymerization events such as cell–cell adhesion, cell movement and phagocytosis (Jaffe and Hall, 2005). To investigate the role of the Rho-GTPases Rac1 and Cdc42 in InlA-mediated internalization, we quantified the entry level of InlA-coated beads in \( \text{L.2071hEcad} \) cells expressing the dominant negative construct RacN17 or Cdc42N17 fused to GFP and \( \text{L. innocua} \) (InlA) and \( \text{L. monocytogenes} \) \( \Delta \text{inlB} \) in HEK193 cells expressing the same dominant negative derivatives. Expression of GFP-RacN17 inhibited the entry of InlA-coated beads and bacteria, compared with that observed in non-transfected \( \text{L.2071hEcad} \) or HEK293 control cells (Ctr) (Fig. 2A, B and D). Moreover, InlA-coated beads mediated the recruitment of GFP-RacN17 (Fig. 2C). In contrast, Cdc42N17 or GFP alone were not recruited at beads entry sites and their expression had no significant inhibitory effect on InlA-mediated uptake (Fig. 2A, B and D). The RhoA dominant-negative construct (GFP-RhoN19) had no inhibitory effect on InlA-beads uptake (data not shown).

Together, these results suggest that in \( \text{L.2071hEcad} \) and HEK293 cells, InlA-mediated phagocytosis requires Rac1 activity.

\textbf{WAVE is not involved in InlA/E-cadherin-dependent uptake}

We searched for downstream effector(s) of Rac1 able to recruit and activate the Arp2/3 complex at the InlA/E-cadherin-dependent entry site of \textit{Listeria}. WAVE proteins have been described to activate Arp2/3 complex downstream of Rac1 (Goley and Welch, 2006; Takenawa and Suetsugu, 2007). First, using polyclonal antibodies recognizing the three isoforms of WAVE, we examined their intracellular distribution in Caco-2 cells infected with \( \text{L. innocua} \) (InlA). To our surprise, we did not observe any recruitment of WAVE at the entry site of bacteria. Moreover, in \( \text{L.2071hEcad} \) cells expressing WAVE2-GFP, we did not detect any accumulation of WAVE2-GFP protein around InlA-coated beads (data not shown). As the proteins involved in the uptake of \textit{Listeria} are usually recruited and detectable at the entry site, these results suggested that WAVE is not involved in InlA-dependent uptake of \textit{Listeria}. To confirm this hypothesis, \( \text{L.2071hEcad} \) cells expressing WAVE2-\( \Delta V \), a dominant negative form of WAVE2 unable to bind and activate Arp2/3 complex (Miki et al., 1998), were incubated with InlA-coated beads and the entry level monitored. Overexpression of the WAVE2 dominant negative form had no inhibitory effect on beads uptake, suggesting that, at least in \( \text{L.2071hEcad} \) cells, WAVE is not required for InlA-dependent entry (Fig. 3). As a control, we used the construct N-WASP-\( \Delta V \), encoding the
Fig. 1. The Arp2/3 complex is necessary for InlA-dependent entry of *Listeria*.

A. Recruitment of Arp3 during entry of *L. innocua* (InlA) in Caco-2 cells. Arp3 and actin colocalize at the bacterial entry sites (white arrows) and at cell–cell junctions (open arrows).

B. Kinetic analysis of Arp2/3 recruitment (red), F-actin cup formation (green) and bacteria uptake (black) during InlA-dependent entry in Caco-2 cells. Internalized, Arp2/3 and F-actin associated *L. innocua* (InlA) were quantified by immunofluorescence at different times after infection.

C. Western blot on HEK293 cells overexpressing either ScarW or ScarWA myc-tagged constructs. ScarW and ScarWA were detected using an anti-c-myc antibody (C-19, Santa Cruz Biotechnology). Total amount of loaded proteins was controlled revealing actin in the same membrane.

D. Effect of the overexpression of ScarW and ScarWA on the internalization of InlA-coated beads into L2071hEcad cells. The uptake of beads was quantified by immunofluorescence in non-transfected cells (Ctr) or cells expressing either ScarW or ScarWA constructs. Entry values are expressed as the percentage of intracellular beads compared with the total number of beads associated with cells. Results are means ± SD from three independent experiments.

E. Effect of the overexpression of ScarW and ScarWA on entry of *L. innocua* (InlA) and *L. monocytogenes ΔinlB* in HEK293 cells. Entry levels of bacteria were quantified by gentamicin survival assay in non-transfected cells (Ctr) or cells expressing either ScarW or ScarWA constructs. Entry into Ctr cells has been normalized to 100 and the levels of entry in transfected cells are expressed as relative values. Results are means ± SD from three independent experiments.
dominant negative form of N-WASP, an Arp2/3 activator that is itself activated by Cdc42 (Takenawa and Suetsugu, 2007). In agreement with results obtained for Cdc42N17, the overexpression of N-WASP-DV had no effect on beads uptake (Fig. 3). Together, these results indicate that neither WAVE nor N-WASP are involved in the InlA-dependent entry.

A role for cortactin in InlA/E-cadherin-mediated entry of Listeria

Cortactin has emerged as a critical regulator of actin assembly in a variety of contexts including the tight regulation of membrane movements (Weed et al., 2000; Olazabal and Machesky, 2001; Higgs, 2002; Selbach and Backert, 2005). Direct interaction of cortactin with the Arp2/3 complex activates actin nucleation in vitro (Uruno et al., 2001; Weaver et al., 2001). In addition, upon activation of Rac1, cortactin is recruited from the cytosol to

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the cell periphery (Weed et al., 1998). We investigated the role of cortactin in the nucleation and assembly of actin downstream InlA/E-cadherin interaction. We first analysed the localization of cortactin in Caco-2 cells infected or not with L. innocua (InlA). In non-infected cells, cortactin colocalized with Arp2/3 and actin at the adherens junctions (Fig. 4A, upper panels). Upon infection of Caco-2 cells, cortactin was abundantly recruited around entering bacteria along with the Arp2/3 complex (Fig. 4A, lower panels). In order to analyse the functional role of cortactin in Listeria uptake, we quantified the entry of InlA-coated beads in L2071hEcad cells expressing cortactin variants bearing point mutations (W22A and DD2021AA) that abolish its ability to bind and activate Arp2/3 (Weed et al., 2000; Bougneres et al., 2004). The entry of InlA-coated beads was reduced by 70% in cells expressing either one of the cortactin mutants as compared with mock-transfected control (Ctr) cells (Fig. 4B and D). We also evaluated the entry level of L. monocytogenes ΔinlB in HEK293 expressing different cortactin variants. As observed for the uptake of InlA-coated beads, the entry of bacteria in cells expressing mutants W22A or DD2021AA was reduced compared with control (Ctr) cells (Fig. 4C). Overexpression of the cortactin N-terminus acidic domain (NTA) that binds to the Arp2/3 complex, reduced by 50% the uptake of bacteria, whereas the overexpression of full-length cortactin (FL) had no effect.

To confirm these results, we inhibited cortactin expression and evaluated the entry level of L. monocytogenes ΔinlB. HEK293 cells were transfected with two different siRNA duplexes (RNA#1, RNA#2). The efficiency of cortactin siRNA was assessed at the protein level by Western blot analysis (Fig. 5A). Expression of cortactin was strongly reduced in siRNA-transfected compared with control non-transfected cells (Ctr) or cells transfected with scrambled siRNAs (Scr1, Scr2) (Fig. 5A). The entry level of L. monocytogenes ΔinlB was reduced twofold in HEK293 cells transfected with cortactin siRNAs (Fig. 5B). Cells transfected with control siRNA (Scr1, Scr2) did not show any defect in entry compared with non-transfected cells (Ctr) (Fig. 5B).

Altogether these results strongly suggest a role for cortactin in the activation of Arp2/3 complex during InlA/E-cadherin-mediated entry of Listeria.

Activity of the Src kinase is required for InlA/E-cadherin-dependent uptake

Cortactin is a substrate for the tyrosine kinase Src (Wu et al., 1991). Cortactin tyrosine phosphorylation is closely correlated with Src activity in a number of signalling pathways, such as integrin-mediated cell adhesion (Weed and Parsons, 2001), growth factor stimulation and pathogen invasion of epithelial cells (Dehio et al., 1995; Bougneres et al., 2004; Martinez and Cossart, 2004). The Src kinase can phosphorylate cortactin on three tyrosine residues (Y421, Y466 and Y482) (Huang et al., 1997; Huang et al., 1998). To address the contribution of cortactin tyrosine phosphorylation on bacterial entry, we used a tyrosine-mutated (TM) cortactin construct (Huang et al., 1998). In HEK293 cells expressing TM-cortactin, the entry of L. monocytogenes ΔinlB was significantly reduced compared with Ctr and FL-cortactin expressing cells (Fig. 4C). In addition, phosphorylated cortactin was recruited at the entry site of L. innocua (InlA) in Caco-2 cells (Fig. 6A, upper panel). Together, these results indicate a role for cortactin tyrosine phosphorylation in InlA/E-cadherin-mediated internalization. To evaluate whether cortactin phosphorylation during InlA-mediated entry correlates with Src kinase activity, we also analysed the localization of Src in Caco-2 cells infected with L. innocua (InlA). We also observed the recruitment of Src and active phosphorylated Src at the site of bacterial entry (Fig. 6A), suggesting a role for Src activity in InlA-dependent uptake.

Studies with other pathogens have demonstrated that invasion of host cells stimulates the tyrosine phosphorylation of cortactin (Selbach and Backert, 2005). We investigated whether this was also the case for the InlA-dependent entry of Listeria. L2071hEcad and Caco-2 cells were infected with L. monocytogenes ΔinlB and tyrosine phosphorylated proteins were immunoprecipitated at different time points after infection. In Caco-2 (Fig. 6C), as well as in L2071hEcad cells, we failed to detect any global change in cortactin phosphorylation upon InlA/E-cadherin-mediated invasion. However, in these cell types, a high level of constitutive phosphorylated cortactin is detected in non-infected serum-starved cells (time point 0), possibly explaining why no further increase could be observed in the course of infection (Fig. 6C). In contrast, Src was transiently tyrosine-phosphorylated (Fig. 6C), definitely showing that Src is activated upon entry and thus strongly suggesting a role for Src kinase activity on cortactin during the InlA-dependent entry.

We further evaluated the entry of Listeria in presence or absence of different concentrations of PP1, a pharmacological inhibitor of Scr tyrosine kinases. Caco-2 cells were infected either with wild-type L. monocytogenes, L. monocytogenes ΔinlB or L. innocua (InlA) (Fig. 7A). Infections were performed in absence or in presence of 20 or 50 μM PP1. We observed that inhibition of Src family tyrosine kinases by PP1 drastically reduced Listeria entry in Caco-2 cells, in a concentration-dependent manner (Fig. 7A). The same bacterial strains were used to infect L2071hEcad cells, in which WT L. monocytogenes is internalized via both InlA- and InlB-mediated pathways. As shown in Fig. 7B, in these cells L. monocytogenes internalization is not affected by PP1, whereas the
Fig. 4. Cortactin is involved in the InlA-mediated uptake of Listeria.
A. Cortactin localizes at the cell–cell junctions in Caco-2 cells along with the Arp2/3 complex and actin (upper panels). Cortactin is recruited at the entry site of L. innocua (InlA) in Caco-2 cells. Cortactin, Arp3 and actin colocalize at the bacterial entry site (lower panels).
B. Effect of the overexpression of cortactin mutants deficient for Arp2/3-binding (W22A and DD2021AA) on the internalization of InlA-coated beads into L2071hEcad cells. The uptake of beads was quantified by immunofluorescence in non-transfected cells (Ctr) or cells expressing either W22A or DD2021AA cortactin mutants. Entry values are expressed as the percentage of intracellular beads compared with the total number of beads associated with cells. Results are means ± SD from three independent experiments.
C. Effect of the overexpression of cortactin mutants on the internalization of L. monocytogenes ΔinlB in HEK293 cells. Bacterial entry levels were quantified by gentamicin survival assay in non-transfected cells (Ctr) or cells expressing either cortactin variants unable to bind Arp2/3 (W22A or DD2021AA), or the N-terminal domain of cortactin (NTA), or the cortactin full-length (FL) or a cortactin mutant in which the tyrosines substrates for Src have been mutated (TM). Entry into Ctr cells has been normalized to 100 and the levels of entry in transfected cells are expressed as relative values. Results are means ± SD from three independent experiments.
D. Western blots on HEK293 cells overexpressing several cortactin constructs. W22A, DD2021AA and NTA cortactin (left panel) were detected using an anti-c-myc antibody (C-19, Santa Cruz Biotechnology). FL and TM cortactin were detected using an anti-cortactin antibody (clone 4F11). As loading control actin was revealed in the same membranes.
InlA-restricted internalization of L. monocytogenes ΔinlB and L. innocua (InIA) is strongly affected. These results indicate that the tyrosine phosphorylation activity of Src is specifically required for InIA/E-cadherin-dependent entry of Listeria. As a control, Caco-2 cells were infected in absence or in presence of different concentration of AG1478, a specific inhibitor of the epidermal growth factor (EGF) receptor tyrosine kinase, and no effect on entry levels was observed (Fig. 7C). In addition, we found that in cells overexpressing the tyrosine-mutated cortactin, the entry levels were similar in absence or presence of PP1 (Fig. 8), indicating that the main effect of Src inhibition is on cortactin phosphorylation, although Src could still also regulate other targets. Interestingly, in Caco-2 cells infected with L. innocua (InIA) in presence of PP1, cortactin was not recruited at the entry site (Fig. 6B), showing that recruitment of cortactin around entering bacteria requires Src kinase activity.

These results strongly indicate that tyrosine phosphorylation of cortactin and the kinase activity of Src are both required during InlA-mediated entry.

Discussion

We show here that the signal transduction events following InlA/E-cadherin-mediated docking of the bacteria at the cell surface lead to the activation of the Arp2/3 complex and transient actin cytoskeleton reorganization. Moreover, we demonstrate that cortactin and Src kinase activity are key elements of the signalling cascade leading to Arp2/3 activation and actin polymerization during InlA/E-cadherin-induced internalization of Listeria.

Host actin cytoskeleton rearrangements are required for the internalization of a number of invasive pathogens. The Arp2/3 complex has been previously implicated in the uptake of enteropathogens such as Yersinia enterocolitica (Wiedemann et al., 2001), Salmonella enterica (Criss and Casanova, 2003), Shigella flexneri (Bougnères et al., 2004) (L. Bougnères, P.J. Sansonetti and G. Tran-Van-Nhieu, unpubl. res.), Rickettsia conorii (Martinez and Cossart, 2004), as well as in the InIB-mediated entry of Listeria (Biere et al., 2001). The Arp2/3 complex is so far the best characterized cellular initiator of actin filament nucleation and is localized at sites of dynamic actin assembly (Pollard and Borisy, 2003; Goley and Welch, 2006). The site of attachment of L. monocytogenes to the host cell corresponds to a restricted area, where F-actin polymerization is locally triggered. We show here that the Arp2/3 complex is recruited at the bacterial entry site, and our kinetics experiments indicate that the recruitment of Arp2/3 to the InIA-dependent entry site precedes the formation of phagocytic actin-cups, strongly suggesting that Arp2/3 recruitment at the entry site promotes the local actin polymerization required for bacterial engulfment. This actin polymerization is tightly co-ordinated in time and space, as both Arp2/3 recruitment and actin rearrangements are local and transient during the InIA/E-cadherin-mediated internalization. Such dynamic actin assembly at sites of E-cadherin adhesion has already been described, particularly when nascent contact zones are being established and undergo extension (Vasioukhin et al., 2000; Kovacs et al., 2002b; Verma et al., 2004). The Arp2/3 complex is recruited to newly formed E-cadherin contacts, as it is to the entry site of Listeria upon the interaction InIA/E-cadherin. As shown here for InIA/E-cadherin interaction, it has been demonstrated that E-cadherin homophilic interaction is necessary to trigger Arp2/3 recruitment to the plasma membrane (Kovacs et al., 2002b). Upon homophilic interaction, E-cadherin associates with the Arp2/3 complex during nascent adhesive contacts (Kovacs et al., 2002b). Both branched actin networks generated by Arp2/3 complex and linear actin
Fig. 6. Src kinase is activated during the InlA-mediated uptake of Listeria.
A. Immunofluorescence analysis of Caco-2 cells infected with L. innocua (InlA), showed tyrosine-phosphorylated cortactin (upper panel), Src kinase (middle panel) and tyrosine-phosphorylated Src (lower panel) recruitment at the bacterial entry site and colocalization with actin.
B. Immunofluorescence analysis of cortactin and actin recruitment to the L. innocua (InlA) entry site in PP1-treated Caco-2 cells.
C. Immunoprecipitation of total tyrosine-phosphorylated proteins using anti-4G10 and Western blot analysis using anti-cortactin [pY421] or anti-Src [pY418] revealed that InlA-dependent invasion of Caco-2 cells induces the transient phosphorylation and activation of Src 2 min after infection.

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cables are involved in intercellular adhesion (Vasioukhin et al., 2000; Vaezi et al., 2002). Formin-1 has been identified as a binding partner of α-catenin playing a crucial role in the generation of such radial actin cables (Kobielak et al., 2004). Whether formin-1 is involved in *Listeria* internalization is currently unknown.

Recruitment and activation of the Arp2/3 complex at sites of dynamic actin assembly is tightly regulated by the Rho family GTPases (Goley and Welch, 2006; Takenawa and Suetsugu, 2007). Here we show, that Rac1 activity is necessary for the *InlA*-dependent uptake. In agreement, Rac1 also plays a key role in the process of adherens junctions formation (Braga et al., 1997; 2000). During this process, the homophilic interaction of E-cadherin directly activates Rac1 (Kovacs et al., 2002a). One could thus envision that upon InlA binding, Rac1 is locally activated at the site of bacterial attachment, and in turn stimulates Arp2/3 to drive actin assembly necessary for *Listeria* uptake. In a ‘classical’ scenario, one would expect that activated Rac1 stimulates Arp2/3 via the activation of WAVE proteins (Takenawa and Suetsugu, 2007), however, we show here that WAVE plays no role in *InlA*-dependent uptake and the role of Rac1 in this process remains to be clarified.

Cortactin has recently emerged as a critical regulator of actin assembly, able to bind directly both Arp2/3 and

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**Fig. 7.** Activity of Src kinase is required for *InlA*-dependent entry of *Listeria*. A and B. Entry of wild-type *L. monocytogenes*, *L. monocytogenes* Δ*InIB* and *L. innocua* (*InlA*) into Caco-2 (A) or L2071hEcad (B) was evaluated in the presence of 20 or 50 μM of PP1 and compared with entry in untreated cells (0). Entry into untreated cells has been normalized to 100 and the levels of entry in treated cells are expressed as relative values. Results are means ± SD from three independent experiments. C. As control, entry levels of the same bacterial strains in Caco-2 cells were evaluated in absence or presence of different concentrations of AG1478.
F-actin (Weed et al., 2000). Direct binding of cortactin to actin activates Arp2/3-driven actin nucleation in vitro (Urano et al., 2001; Weaver et al., 2001) and potentially stabilizes the cortical actin network by inhibiting the disassembly of actin filaments (Weaver et al., 2001). The subcellular localization of cortactin has been shown to be regulated by Rac1 (Weed et al., 1998). Upon growth factor stimulation, cortactin redistributes from the cytoplasm to the cell cortex and membrane ruffles in a Rac1-dependent manner (Weed et al., 1998). Moreover, cortactin tyrosine phosphorylation occurring at the cell periphery requires Rac1 activity (Head et al., 2003). It is thus possible that the role of Rac1 in InlA-dependent entry is to allow cortactin phosphorylation. Importantly, cortactin is recruited along with the Arp2/3 complex to the E-cadherin-based cell–cell contacts and plays a role in the formation of stable E-cadherin-based junctions, and the inhibition of cortactin function prevents E-cadherin and catenins accumulation at the cell contacts (Helwani et al., 2004). Here, we have shown the role of cortactin in the actin rearrangements necessary to promote InlA-mediated Listeria entry into cells, highlighting again that Listeria, during entry, mimics the events the events occurring during adherens junction formation.

Cortactin interacts with the GTPase dynamin and triggers lamellipodia formation, by mediating the remodelling of cortical actin (Krueger et al., 2003). In addition, dynamin has been shown to localize to the phagocytic cup of mouse macrophages and is required for complete enclosure of the phagosome (Gold et al., 1999). Dynamin-cortactin complexes thus appear to be associated with actin-mediated processes that require the active extension of membrane. In the case of InlA-induced phagocytosis, dynamin may also help to recruit cortactin at the entry site.

Interestingly, cortactin was recently reported to be necessary to the InlB-mediated invasion of Listeria (Veiga and Cossart, 2005; Barroso et al., 2006). As the clathrin-mediated endocytic machinery seems to play a key role in this entry pathway, it will be important to test whether it is also the case in the InlA pathway, and whether cortactin recruitment also contributes to InlA-mediated internalization. In addition, cortactin also contributes to cell infection by other invasive pathogens such as S. flexneri (Dehio et al., 1995; Dumenil et al., 1998; Bougneres et al., 2004), Cryptosporidium parvum (Chen et al., 2003), Chlamydia trachomatis (Fawaz et al., 1997), R. conorii (Martinez and Cossart, 2004) and Escherichia coli (EPEC and EHEC) (Cantarelli et al., 2000; 2002; 2006). Together, cortactin thus appears as a key protein in different types of internalization.

Upon cell infection by Shigella, Rickettsia and Cryptosporidium, cortactin is tyrosine phosphorylated. Moreover, inhibition of Src kinase, and hence cortactin tyrosine phosphorylation, blocks Shigella and Cryptosporidium invasion, implicating a Src/cortactin-dependent pathway in these infectious processes (Dehio et al., 1995; Dumenil et al., 1998; Chen et al., 2003; Bougneres et al., 2004). Although we show here that Src is activated upon Listeria infection and Src kinase activity is necessary for InlA-dependent uptake, we did not detect any significant increase in global cortactin tyrosine phosphorylation during Listeria entry. It is possible that cortactin tyrosine phosphorylation only occurs at the site of entry and in a transient fashion, explaining why we could not detect such an increase at a global level. In addition, in our experimental system, the endogenous phosphorylation level of cortactin is high, thus preventing the detection of subtle modifications of its phosphorylation status. Cortactin is not the only Src substrate at adherens junction. The catenin p120 is another Src substrate that may be critical for entry and deserves further investigations.

Experimental procedures

Bacterial strains, cell types, antibodies and other reagents

Listeria monocytogenes wild-type strain (EGD-BUG600) and L. monocytogenes ΔinlB (BUG 1047) were used and grown in brain heart infusion (Difco laboratories, Detroit, MI). L. innocua transformed with pRB474 harbouring the inlA gene (BUG 1489) (Lecuit et al., 1997) was grown in brain heart infusion (Difco laboratories, Detroit, MI) in presence of chloramphenicol (7 μg ml⁻¹). Caco-2 cells were grown as described previously (Mengaud et al., 1996a). Transfected L2071 cells expressing human E-cadherin have been described (Lecuit et al., 2000). Mousse anti-InlA (L7.7) was used as described (Mengaud et al., 1996b). Appropriate AlexaFluor 488- and 546-conjugated secondary antibodies diluted in PBS containing 1% BSA and 1% gelatin were used following manufacturer’s instructions (Molecular Probes). Alexa-Phalloidin-647 or 488 (Molecular Probes) was used to label actin filaments. Monoclonal antibody against c-Src

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(clone GD11) and monoclonal antibody against cortactin (clone 4F11) were purchased from Upstate Biotechnology (UBI). Monoclonal antisera against actin (AC-15) was obtained from Sigma. Polyclonal anti-cortactin ([pY421] phosphospecific antibody and anti-Src [pY418] phosphospecific antibody were purchased from BioSource International. Protein phosphatase 1 (PP1) and AG1478 were obtained from Calbiochem.

Protein purification and coating on latex beads
InlA lacking its cell-wall anchor were purified as described previously (Pizarro-Cerdà et al., 2002). Purified InlA was covalently coupled to 1.0 μm carboxylate-modified latex beads (blue fluorescent Fluospheres, Molecular Probes). A total amount of 400 μg of purified protein was coated on the surface of 200 μl of a 2% aqueous suspension of latex beads as described (Lecuit et al., 1997).

Transient transfections and quantifying beads uptake
24 h before transfection 5 × 10⁵ L2071 hEcad cells were plated in glass coverslips in 6-well plates. Transient transfections were performed at 80% confluence using Lipofectamine 2000 (Invitrogen) and 4 μg DNA/well following the manufacturer’s protocol. 24–36 h after transfection, InlA-coated beads were added to cells at an moi of 50. After 1 h incubation at 37°C 10% CO₂, cells were fixed samples were permeabilized with 0.1% Triton X-100 in PBS 15 min at room temperature (RT). Extracellular beads were labelled with a mouse monoclonal antibody raised against InlA (L7.7) and revealed by anti-mouse Alexa-546 secondary antibody (Molecular Probes). The cells were then permeabilized 5 min at RT with 0.1% Triton X-100 in PBS. Transfected cells were labelled with different antibodies and revealed by Alexa-488 secondary antibody (Molecular probes). Each experiment was carried out in triplicate each time and three times independently. For each coverslip, 50 transfected cells were selected randomly and the number of total beads evaluated under phase contrast observation. The number of extracellular beads labelled by Alexa-546 was evaluated. The number of intracellular ones was obtained by subtracting the number of extracellular beads labelled by Alexa-546 from the number of total beads.

Invasion assays
Invasion assays were performed in 6-well plate using the gentamicin survival assay as described previously (Mengaud et al., 1996a). When indicated, 1 h prior to infection, cells were incubated with serum-free medium containing PP1 or AG1478 at the indicated concentrations. As control, cells were incubated with DMEM serum-free medium containing 0.5% DMSO. Bacteria were added to cells at an moi of 100. Cells were incubated with bacteria during 1 h at 37°C and 10% CO₂ and 2 h in presence of gentamicin (10 μg ml⁻¹). Cells were then lysed with Triton X-100 0.2% in PBS and the lysates were plated for bacterial counting.

Immunofluorescence analysis
Cells were fixed with 3.5% paraformaldehyde (40 min at 4°C). Fixed samples were permeabilized with 0.1% Triton X-100 in PBS for 5 min at RT and then blocked in PBS containing 1% BSA and 1% gelatin (45 min at RT). Primary and secondary antibodies were diluted in PBS containing 1% BSA and 1% gelatin. Preparations were observed with a Zeiss Axiovert 135 epifluorescence microscope connected to a CCD camera (Princeton) or with a confocal laser-scanning microscope (LSM 510, Carl Zeiss Microlmaging). Images were acquired with a 63× objective and processed with the MetaMorph software, version 6.1 (Universal Imaging).

RNA#1 resulted from the annealing of a sense strand (5′-GACUGAGAAGCAUGCCUCCTT-3′) and the corresponding anti-sense strand designed to match the 5′ of human cortactin mRNA. The scrambled version of RNA#1 (Scr1) corresponded to the sense strand 5′-AGGCCAGUGCCAGAUGUC-3′ and was tested for the absence of matching with any GenBank DNA sequence. HEK293 cells were transfected using the jetSI reagent as indicated by manufacturer’s instructions (Eurogentec). RNA#2 corresponded to a double-stranded RNA against cortactin (sense strand 5′-GGGAGAUGUCUUCAAGATT-3′) purchased from Ambion (Austin, TX). Control RNA (silent negative control 1 siRNA) (Scr2) was purchased from Ambion. HEK293 cells dsRNA2 transfections were performed used oligofectamine (Invitrogen), as recommended by the manufacturer. Cells were tested 24 h after transfection.

Immunoprecipitation
Cells were seeded onto 75 cm² flasks and incubated at 37°C 5% CO₂ (approximately 5 × 10⁶ cells ml⁻¹). The day of the experiment, cells were washed twice and serum starved for 5 h at 37°C and 5% CO₂. Cells were either left uninfected or infected with L. monocytogenes ΔinlB (moi = 50), during different times. After each time point, cells were washed twice with ice-cold PBS and lysed in 1 ml of 1%NP-40 lysis buffer (1% NP-40, 50 mM Tris pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM AEBSF, 3 mM Na3VO⁴, 1× Complete Protease Inhibitor Cocktail). Lysates were centrifuged at 15 000 g for 10 min at 4°C and the supernatants were clarified with 60 μl of a 20% (v/v) suspension of Protein G Sepharose beads (Amersham) for 1 h at 4°C. Lysates were then immunoprecipitated with 2 μg of antiphosphotyrosine antisera (clone 4G10, Upstate Biotechnology UBI) overnight at 4°C. Immune complexes were captured with 60 μl of a 20% (v/v) suspension of Protein G Sepharose at 4°C, washed three times (Wash buffer 0.2% NP-40, 50 mM Tris pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM AEBSF, 3 mM Na3VO⁴). Immunoprecipitated proteins were eluted and boiled in Laemmli buffer containing 0.1 mM DTT and 5% β-mercaptoethanol. Samples were resolved by SDS-PAGE, transferred to nitrocellulose membrane blocked overnight (20 mM Tris pH 7.5, 0.9% NaCl and 0.1% Triton X-100 containing 5% milk) and immunoblotted with primary antibody and with HRP conjugate.

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