Successive post-translational modifications of E-cadherin are required for InlA-mediated internalization of *Listeria monocytogenes*

Matteo Bonazzi,1,2,3 Esteban Veiga,1,2,3,† Javier Pizarro-Cerdá1,2,3 and Pascale Cossart1,2,3,*

1Institut Pasteur, Unité des Interactions Bactéries-Cellules, Paris F-75015, France.  
2INSERM, U604, Paris F-75015, France.  
3INRA, USC2020, Paris F-75015, France.

Summary

*Listeria monocytogenes* surface proteins internalin (InlA) and InlB interact with the junctional protein E-cadherin and the hepatocyte growth factor (HGF) receptor Met, respectively, on the surface of epithelial cells to mediate bacterial entry. Here we show that InlA triggers two successive E-cadherin post-translational modifications, i.e. the Src-mediated tyrosine phosphorylation of E-cadherin followed by its ubiquitination by the ubiquitin-ligase Hakai. E-cadherin ubiquitination induces the recruitment of clathrin that is required for optimal bacterial internalization. We also show that the initial clustering of E-cadherin at the bacterial entry site requires caveolin, a protein normally involved in clathrin-independent endocytosis. Strikingly clathrin and caveolin are also recruited at the site of entry of E-cadherin-coated sepharose beads and functional experiments demonstrate that these two proteins are required for bead entry. Together these results not only document how the endocytosis machinery is recruited and involved in the internalization of a zipping bacterium, but also strongly suggest a functional link between E-cadherin endocytosis and the formation of adherens junctions in epithelial cells.

Introduction

E-cadherin is a 120 kDa transmembrane glycoprotein that belongs to the family of classical cadherins and mediates adherens junction formation (Halbleib and Nelson, 2006). The extracellular domain of E-cadherin is involved in Ca²⁺-dependent intercellular homotypic interactions required for the formation of adherens junctions whereas its cytoplasmic domain interacts with the cell actin cytoskeleton that stabilizes cell junctions. Binding partners of the cytoplasmic domain of E-cadherin include α-, β- and p120-catenin (p120). β-Catenin binds to the C-terminal portion of E-cadherin and bridges E-cadherin with α-catenin that tightly controls the actin rearrangements required for adherens junction dynamics (Drees et al., 2005; Gates and Peifer, 2005). p120 is a Src substrate member of the catenin family (Reynolds et al., 1992) that binds to the juxtamembrane domain of E-cadherin and is involved in the maintenance of E-cadherin at the plasma membrane (Anastasiadis and Reynolds, 2000; Ireton et al., 2002; Xiao et al., 2005). In its E-cadherin-bound state p120 prevents the binding of the ubiquitin-ligase Hakai that mediates the ubiquitin-induced internalization of E-cadherin (Fujita et al., 2002; Pece and Gutkind, 2002). In its cytoplasmic state p120 binds the Vav exchange factor and regulates the activity of the small G-proteins Rac1, Cdc42 and RhoA (Noren et al., 2000). E-cadherin molecules are not stably exposed at the cell surface, rather they cycle on and off the plasma membrane in a highly dynamic fashion by exo- and endocytic events (Akhtar and Hotchin, 2001; Xiao et al., 2005). Internalization of E-cadherin from adherens junctions is initiated by the Src-mediated tyrosine phosphorylation of E-cadherin (Papkoff, 1997; McLachlan et al., 2007). This post-translational modification induces the dissociation of p120 from E-cadherin (Fujita et al., 2002; Potter et al., 2005) and the binding of the cbl-like ubiquitin-ligase Hakai that results in the ubiquitination of E-cadherin and its internalization within clathrin-coated endosomes (Fujita et al., 2002; Pece and Gutkind, 2002; Palacios et al., 2005). The extracellular domain of E-cadherin, in contrast to its cytoplasmic domain, has so far only few interactors: E-cadherin itself, αEβ7 integrin (Karecla et al., 1996; Higgins et al., 1998), the *Candida albicans* protein ALS3 (Phan et al., 2005; 2007) and the *Listeria monocytogenes* surface protein InlA (Mengaud et al., 1996). *L. monocytogenes* is a Gram-positive food-borne pathogen capable of invading non-phagocytic cells (Hamon et al., 2006). Upon infection *Listeria* crosses several host barriers leading to a wide range of symptoms associated...
with listeriosis, such as gastroenteritis, fetoplacental and central nervous system infections (Lecuit, 2005). Interactions of L. monocytogenes with non-phagocytic cells mainly occur via two bacterial surface proteins: internalin (Inl)A and InlB that have E-cadherin and Met as their respective major targets on host cells (Mengaud et al., 1996; Braun et al., 1998; Shen et al., 2000). InlA interaction with E-cadherin activates the β- and α-catenin-mediated signalling pathways involved in the formation of adherens junctions (Lecuit et al., 2000). Other proteins, e.g. the unconventional myosin VIIa, vezatin (Sousa et al., 2004) and ARHGAP10 Rho-GAP protein (Sousa et al., 2005), have been implicated in InlA-mediated infections and they are also involved in the actin-dependent remodelling of the plasma membrane during adherens junction formation. More recently we described a role for cortactin, Arp2/3 and Src in L. monocytogenes invasion of host cells, further underlining the fundamental role of the actin cytoskeleton during bacterial internalization (Sousa et al., 2007). InlB-mediated internalization of L. monocytogenes hijacks the Met receptor internalization pathway by inducing the ubiquitination of Met and its internalization in a clathrin-dependent manner (Veiga and Cossart, 2005). We have recently expanded the concept of the hijacking of clathrin-mediated endocytosis to the InlA-mediated internalization of Listeria as well as to other pathogenic bacteria and found it to be a typical feature of zippering bacteria (Veiga et al., 2007). In the present study we investigated the initial signals triggered by the interaction of InlA with E-cadherin to induce bacterial internalization. We first showed that purified InlA triggers the internalization of E-cadherin by inducing the Src-mediated tyrosine phosphorylation of E-cadherin followed by its ubiquitination by the ubiquitin-ligase Hakai. These post-translational modifications also occur during bacterial infections and are necessary for an efficient InlA-mediated bacterial internalization which we show to be dependent on both caveolin and clathrin. Finally we show that E-cadherin-coated latex beads are efficiently internalized within epithelial cells and require for this process the same endocytic proteins as those observed during InlA-mediated bacterial internalization. These observations shed light on how the formation of E-cadherin trans-dimers contribute to adherens junction formation. Together our data elucidate the mechanisms of the InlA-mediated internalization of L. monocytogenes and also provide new insight into the mechanisms of E-cadherin-mediated adherens junction formation.

Results

Purified InlA induces internalization of E-cadherin

To study whether E-cadherin is internalized upon interaction with soluble InlA, Jeg-3 cells were incubated with 50 nM InlA for 15, 30 and 60 min or left untreated and E-cadherin localization was analysed by immunofluorescence. At each time point, cells were fixed and incubated with the anti-E-cadherin monoclonal antibody HEC-D1 prior to permeabilization to label extracellular E-cadherin. Cells were then permeabilized and re-incubated with HEC-D1. This protocol allows to distinguish between extracellular and internalized E-cadherin as the detection of E-cadherin after permeabilization is indicative of internalized E-cadherin (Fig. 1A). The percentage of cells presenting internalized E-cadherin was then counted. On average, 15% of cells showed internalized E-cadherin through the time-course (Fig. 1B). This percentage increased to 35% after 15 min of incubation with purified InlA and decreased at approximately 25% after 60 min of incubation (Fig. 1B), still remaining significantly higher than that of control cells. Thus purified InlA is able to induce E-cadherin internalization.

InlA-mediated infection induces the tyrosine phosphorylation of E-cadherin

Our recent data have shown that InlA-mediated infection of L. monocytogenes induces the activation of the tyrosine kinase Src (Sousa et al., 2007). In addition, it has been reported that upon cell stimulation by hepatocyte growth factor (HGF) or by activation of the temperature-sensitive vSrc, E-cadherin endocytosis is triggered by Src-mediated phosphorylation on tyrosines 755 and 756 (Fujita et al., 2002). We thus decided to test whether InlA-mediated Listeria infection triggers E-cadherin phosphorylation by Src. Jeg-3 cells, as most epithelial cells, express both E-cadherin and Met and are thus permissive to both the InlA- and InlB-mediated entry pathways. We thus used for this study Listeria innocua expressing InlA [L. innocua(InlA)] as a mean to specifically study InlA-mediated infections. L. innocua is a non-pathogenic, non-invasive Listeria species, which upon InlA expression becomes invasive. Jeg-3 cells were incubated with L. innocua(InlA) and E-cadherin was immunoprecipitated at different time points of incubation with the specific antibody H108 (Fig. 1C, left panel). Blots were probed with the 4G10 antibody against tyrosine-phosphorylated proteins or with the anti-E-cadherin antibody after stripping (Fig. 1C, left panel). E-cadherin was not phosphorylated in immunoprecipitates from non-treated control cells whereas a clear phosphorylation signal detectable after 5 min of incubation peaked at 15 min and decreased at 30 min of incubation (Fig. 1C, left panel). When cells were pre-treated with the Src inhibitor PP1 (20 μM) prior to incubation with bacteria, E-cadherin was no longer tyrosine phosphorylated, suggesting that the phosphorylation of E-cadherin is Src-dependent (Fig. 1C,
left panel). The same results were obtained when alternatively, following *L. innocua*(InlA) incubation tyrosine-phosphorylated proteins were immunoprecipitated from Jeg-3 cells lysates and the presence of E-cadherin was assessed with the anti-E-cadherin antibody H108 and the blot was probed with the anti-tyrosine-phosphorylated proteins antibody 4G10 (top left panel). The blot was then stripped and labelled for E-cadherin (bottom left panel). Alternatively tyrosine-phosphorylated proteins were immunoprecipitated with the 4G10 antibody and the blot was probed with an anti-E-cadherin antibody (upper right panel). E-cadherin antibody was stripped and the blot was probed with an anti-4G10 antibody (lower right panel). Where needed, the Src inhibitor PP1 was added to cells 1 h prior to treatment and during cell incubation with bacteria to assess the role of Src in E-cadherin phosphorylation.

D. InlA-mediated infections activate Src. Jeg-3 cells were incubated with *L. innocua*(InlA) for the indicated times as in C and total cell lysates were probed with an anti-phospho Src antibody (top panel) and with an anti-total Src antibody after stripping as a loading control (bottom panel). Experiments were done in triplicate.

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InlA-mediated infection induces the ubiquitination of E-cadherin

E-cadherin phosphorylation is known to induce its interaction with the Cbl-like ubiquitin-ligase Hakai (Fujita et al., 2002). This event induces E-cadherin ubiquitination and internalization of E-cadherin from adherens junction, a process that could be exploited by Listeria to invade host cells. To investigate the possibility that during InlA-mediated internalization E-cadherin is ubiquitinated, Jeg-3 cells were incubated with either L. innocua or L. innocua(InlA) for several time points, fixed and immuno-labelled with an anti-ubiquitin antibody P4D1 and with the anti-L. innocua antibody R6. At 30 min of incubation ubiquitin is clearly concentrated around internalized bacteria expressing InlA (Fig. 2A, top panels) whereas incubation with the non-invasive strain L. innocua did not lead to any accumulation of ubiquitin (Fig. 2A, lower panels). The same results were obtained when Jeg-3 cells were transfected with HA-tagged ubiquitin prior to bacterial incubation and the recruitment of ubiquitin was followed using an anti-HA antibody (Fig S1A). These results, however, could not indicate whether E-cadherin was the ubiquitinated substrate recruited at the bacterial entry site.

Ubiquitination was thus monitored by immunoprecipitation of E-cadherin from Jeg-3 cells incubated with L. innocua(InlA). At several time points of incubation E-cadherin was immunoprecipitated with specific antibodies and ubiquitination was tested by Western blot. A clear band of ubiquitin, migrating at a size compatible with that of ubiquitinated E-cadherin, was detectable after 30 min of incubation whereas non-treated control cells showed basal levels of ubiquitination (Fig. 2B). At 45 min of incubation ubiquitination decreased significantly (Fig. 2B). To definitely establish that E-cadherin is ubiquitinated we dissociated protein complexes in the immunoprecipitates and performed a re-immunoprecipitation of E-cadherin. The ubiquitin signal was still detectable, confirming that ubiquitination does occur on E-cadherin (Fig. 2E).

Similarly, Jeg-3 cells were also incubated with increasing concentrations of purified InlA (10, 50, 100 nM) and ubiquitination of E-cadherin was again followed by immunoprecipitation. Ubiquitination of E-cadherin was detectable after 15 min of incubation with purified InlA. © 2008 The Authors
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(Fig. 2C) and was dependent on the concentration of InlA applied to cells (Fig. 2D).

The anti-ubiquitin antibody P4D1 used in this study recognizes both mono- and poly-ubiquitin moieties. In order to assess if E-cadherin is mono- or poly-ubiquitinated, immunoprecipitates from control cells and cells incubated with *L. innocua* (InlA) for 30 min were revealed with the anti-poly-ubiquitin antibody FK1 that is specific for poly-ubiquitin chains. After 30 min of incubation the ubiquitination of E-cadherin was also detectable with the anti-poly-ubiquitin antibody FK1 (Fig. 2F). This result indicated that E-cadherin is poly-ubiquitinated upon infection, although not excluding the possibility that E-cadherin is also mono-ubiquitinated.

Together these data demonstrated that InlA-mediated infection induces the ubiquitination of E-cadherin.

The ubiquitin-ligase Hakai mediates InlA-dependent internalization

Hakai is a Cbl-like ubiquitin-ligase able to ubiquitinate E-cadherin (Fujita *et al.*, 2002). To investigate a possible role of Hakai in the InlA-induced ubiquitination of E-cadherin, Jeg-3 cells were transfected with a plasmid expressing FLAG-tagged Hakai and incubated with *L. innocua* (InlA). At different time points of incubation cells were fixed and incubated with the monoclonal anti-InlA antibody L7.7 before permeabilization to visualize extracellular bacteria. Cells were then permeabilized and labelled with L7.7 to label both intra- and extracellular bacteria and with an anti-FLAG antibody to label Hakai. Hakai was clearly recruited at the bacterial entry site at 30 min of incubation (Fig. 3A). Localization of Hakai around the internalized bacteria was evident until 45 min of incubation and decreased with time as bacteria were internalized (not shown). To further study the role of Hakai in the InlA-mediated internalization of *Listeria*, Hakai and the ubiquitin-ligase Cbl, previously shown to have a role in the InlB-mediated internalization pathway (Veiga and Cossart, 2005), were knocked down in Jeg-3 cells by RNA interference. Seventy-two hours of siRNA treatment reduced Hakai and Cbl cellular levels to 30% as compared with cells treated with control, non-targeted siRNA sequences (Fig. S1A). Cells were then incubated with *L. innocua* (InlA) and invasion was assessed using the gentamicin assay. Bacterial entry was not affected by Cbl knocked-down cells whereas it was reduced by 70% in Hakai knocked-down cells as compared with control cells.
Successive post-translational modifications of E-cadherin

Ubiquitination of E-cadherin is required to recruit clathrin at the bacterial entry site

We have recently reported that similarly to InlB, InlA-dependent entry is partially mediated by clathrin (Veiga and Cossart, 2005; Veiga et al., 2007). We thus tested whether the ubiquitination of E-cadherin is the signal required to initiate the recruitment of clathrin at the bacterial entry site. Jeg-3 cells were treated with control or Hakai-targeted siRNA sequences to inhibit E-cadherin ubiquitination and incubated with L. innocua(InlA) for 15, 30 and 45 min. At each time point cells were fixed and processed for immunofluorescence where InlA and clathrin were labelled (Fig. 4A). For each condition, the number of bacteria recruiting clathrin was calculated as a ratio of the total number of bacteria counted. In control cells, 30% of bacteria recruited clathrin at the sites of internalization after 45 min of incubation (Fig. 4B). Clathrin recruitment was significantly impaired in cells knocked-down for Hakai with the percentage of bacteria recruiting clathrin dropping to 6% (Fig. 4B). Similarly, HeLa cells (which do not express E-cadherin) were transfected with clathrin dropping to 6% (Fig. 4B). Control and Hakai knocked-down cells were either left untreated or incubated with L. innocua(InlA) for 30 min (the time point corresponding to maximal ubiquitination of E-cadherin), lysed and E-cadherin was immunoprecipitated. At 30 min of incubation control cells showed a band of ubiquitinated E-cadherin whereas cells depleted for Hakai did not (Fig. 3C). These data indicate not only that Hakai localizes at the bacterial entry site but also that its function is required for an efficient, ubiquitin-mediated bacterial invasion.

Ubiquitination of E-cadherin is required to recruit clathrin at the bacterial entry site

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Caveolin1 is required for InlA-dependent internalization

As the inhibition of clathrin-dependent endocytosis only partially affects InlA-mediated internalization (Veiga et al., 2007), and HeLa cells overexpressing E-cad-ΔJM are still able to internalize bacteria independently of clathrin, we investigated the possibility that other endocytic pathways are activated upon InlA/E–cadherin interaction. Our laboratory has previously established that InlA preferentially binds to the pool of E-cadherin that resides with caveolin in cholesterol-rich domains at the plasma membrane and that cholesterol depletion prevents bacterial internalization (Seveau et al., 2004). Furthermore, caveolin has been implicated in the internalization of E-cadherin (Lu et al., 2003; Paterson et al., 2003). The recruitment of caveolin1 was thus followed in Jeg-3 cells incubated with either L. innocua or L. innocua(InlA). Caveolin recruitment was evident as soon as 15 min of incubation with L. innocua(InlA) (Fig. 5B), a time point that precedes E-cadherin ubiquitination, and was never observed when cells were incubated with L. innocua (Fig. 5A). Differently from clathrin that was found decorating a defined area surrounding entering bacteria (Veiga et al., 2007), caveolin was detected in larger patches around entering bacteria as well as newly internalized bacteria (Fig. 5B'). To better establish the relevance of caveolin in InlA-mediated infections, Jeg-3 cells were treated with siRNA directed against caveolin1. After 72 h caveolin1 cellular levels were reduced to 35% as compared with cells treated with control siRNA sequences (Fig. S1C) and infection was reduced by 60% in caveolin1 knocked-down cells as compared with control cells (Fig. 5C). Jeg-3 cells were then knocked down for both endocytic proteins and incubated with L. innocua(InlA) as above. Indeed the double knockdown further impaired the efficiency of infection of another 30% (Fig. 5C). These observations demonstrate that caveolin1 is implicated in InlA-mediated bacterial entry.

Caveolin is required to cluster E-cadherin at the bacterial entry site

Caveolin recruitment at the bacterial entry site early during infection and before E-cadherin ubiquitination suggested that caveolin plays a role during the early phases of bacterial adhesion. The dynamics of E-cadherin, caveolin and clathrin recruitment at the bacterial entry site were thus compared. Jeg-3 cells were incubated with
L. innocua(InlA) for 15, 30 and 45 min. At each time point cells were fixed and immunolabelled. For each condition, the number of bacteria recruiting E-cadherin, clathrin and caveolin was calculated as a ratio of the total number of bacteria counted. E-cadherin was recruited at the bacterial entry site starting from 15 min post infection (35% of bacteria) and peaking at 30 min post infection with approximately 50% of adhering bacteria surrounded by E-cadherin (Fig. 6A). Caveolin recruitment at the bacterial entry site reflected that of E-cadherin (Fig. 6A), suggesting an interplay between the two proteins. In contrast, the dynamics of clathrin recruitment largely differed: clathrin was detectable around approximately 10% of adhering bacteria at 30 min of incubation and in 30% of bacteria at 45 min post infection (Fig. 6A). To further investigate a possible link between E-cadherin and caveolin recruitments at the bacterial entry site, caveolin1 was knocked down by siRNA and the recruitment of E-cadherin was followed in L. innocua(InlA)-infected cells. As previously observed, E-cadherin was efficiently recruited around bacteria in control cells whereas it failed to do so in caveolin knocked-down cells (Fig. 6B). As expected, cells knocked down for caveolin also failed to recruit clathrin (Fig. 6B), highlighting an important role of caveolin in the clustering of E-cadherin at the bacterial entry site upon InlA/E–cadherin interaction.

Fig. 4. Post-translational modifications of E-cadherin are necessary for the recruitment of clathrin.
A and B. Jeg-3 cells were knocked-down for Hakai and incubated with L. innocua(InlA) for 45 min. Cells were then labelled (A) for InlA (green), and clathrin (red) and the percentage of bacteria recruiting clathrin was calculated (B). Bars: 5 μm.
C and D. HeLa cells were transfected with wt E-cadherin or with E-cad-DJM and incubated with L. innocua(InlA) for 45 min. Cells were then labelled (C) for E-cadherin (red), clathrin (green) and InlA (blue) and the percentage bacteria recruiting E-cadherin or clathrin was calculated (D). Bars: 3 μm.
E. HeLa cells treated as in C were incubated with L. innocua(InlA) for 45’. Bacteria were then double-labelled to identify intracellular and extracellular bacteria and the efficiency of infection was calculated. In all cases values are means (± standard deviation) of approximately 300 bacteria counted for each condition. Experiments were done in triplicate.
To study whether caveolin recruitment was dependent on E-cadherin phosphorylation, control or PP1-treated Jeg-3 cells were incubated with \textit{L. innocua} (InlA) and the recruitment of caveolin at the bacterial entry site was followed. PP1 treatment severely reduced the efficiency of bacterial internalization and the large majority of bacteria were still extracellular after 45 min of incubation (Fig. 6C). Nevertheless, caveolin1 was still recruited at the bacteria/cell interaction sites, indicating that the recruitment of caveolin is not dependent on E-cadherin phosphorylation. To further investigate this point, caveolin recruitment at the bacterial entry site was also followed in HeLa cells transfected with either wt E-cad or E-cad-\(\Delta\)JM where bacterial internalization occurs by a clathrin-independent mechanism. In both wt E-cad- and E-cad-\(\Delta\)JM-expressing cells, caveolin was found to be efficiently recruited around bacteria, strongly suggesting that caveolin mediates an alternative bacterial internalization pathway that is independent of E-cadherin ubiquitination (Fig. 6D).

\textbf{E-cadherin/E-cadherin interaction triggers the endocytic machinery}

Adherens junction formation is triggered by the interaction between the extracellular domains of E-cadherin molecules from neighbouring cells. This event initiates a signalling cascade that results in the actin-mediated remodelling of the plasma membrane (Bershadsky, 2004). Although reported to be characterized by a dynamic equilibrium between exocytosis and endocytosis, the fate
of E-cadherin, once initial intercellular interactions are established, remains elusive. In this study we have documented how InlA interaction with E-cadherin and alternatively caveolin and clathrin were labelled. The total number of cell-associated bacteria was counted and the percentage of those recruiting E-cadherin and caveolin or clathrin was calculated through the time-course.

B. Jeg-3 cells were knocked-down for caveolin1 and incubated with L. innocua(InlA). E-cadherin and clathrin recruitment at adherent bacteria was quantified at 45 min post infection as in A. In all cases values are means (±standard deviation) of approximately 300 bacteria counted for each condition. Experiments were done in triplicate.

C. Jeg-3 cells were pre-incubated for 1 h with DMSO (control) or with the Src inhibitor PP1 (PP1) and incubated with L. innocua(InlA). Bacteria were labelled before (extracellular Listeria) and after (total Listeria) cell permeabilization to distinguish extracellular and intracellular bacteria and caveolin1 was labelled after permeabilization (red). Bars: 10 μm. Experiments were done in triplicate.

D. HeLa cells were transfected with either wt E-cadherin or with E-cad-ΔJM and infected with L. innocua(InlA) for 30 min. Cells were then fixed and processed for immunofluorescence to visualize E-cadherin (red) and caveolin (green) recruitment around bacteria (blue).

To test this possibility we investigated the process of E-cadherin-coated bead internalization as a model of the initial engagement of E-cadherin molecules during the formation of adherens junctions. As a control and to consequently rule out that the effects observed during L. innocua(InlA) infections could be due to other L. innocua proteins, we incubated Jeg-3 cells with InlA-coated beads as well. We thus followed the recruitment of β-catenin, Hakai, ubiquitin, clathrin and caveolin by immunofluorescence (Fig. 7A). All of the proteins tested were
Fig. 7. E-cadherin/E-cadherin interaction induces E-cadherin internalization.

A and B. Jeg-3 cells were incubated with either InlA-coated (A) or E-cadherin-coated (B) beads fixed and processed for immunofluorescence to study the recruitment of different proteins at the site of entry of the beads. Both InlA-coated and E-cadherin-coated beads (green) recruit β-catenin (red) 15 min after incubation. The ubiquitin-ligase Hakai (red) is recruited around beads (green) at 30 min of incubation as well as ubiquitin (red) and clathrin (red). Caveolin1 (red) is also recruited around beads but earlier during incubation (15 min). Experiments were done in triplicate. Bars: 5 µm.

C. Jeg-3 cells were treated with control, clathrin-, caveolin- or Hakai-targeted siRNA sequences for 72 h and then incubated with E-cadherin-coated beads for 45 min. Beads were then labelled before and after cell permeabilization to distinguish between extracellular and intracellular beads and the efficiency of bead internalization was calculated. Values are means (±standard deviation) of three independent experiments where approximately 300 beads were counted for each condition.
efficiently recruited at the site of entry of InlA-coated beads. We then compared the internalization of InlA-coated beads with that of E-cadherin-coated beads. When incubated with Jeg-3 cells, E-cadherin-coated beads were efficiently internalized after 1 h whereas control Ig-coated beads were all extracellular (Fig. S1D). β-Catenin accumulation at sites where E-cadherin-coated beads contacted cells was already evident at 5 min of incubation (Fig. 7B), revealing that signalling had also occurred. Then, to test whether E-cadherin/E-cadherin interaction was able to induce the same signalisation cascade as that activated by InlA, Jeg-3 cells were transfected with FLAG-tagged Hakai prior to incubation with E-cadherin beads. Hakai was readily recruited around E-cadherin-coated beads between 15 and 30 min of incubation (Fig. 7B) and at 30 min of incubation, ubiquitin was also recruited at the site of entry of E-cadherin-coated beads (Fig. 7B). The presence of caveolin and clathrin around E-cadherin-coated beads was then tested. Caveolin localized around E-cadherin-coated beads from 15 min of incubation as observed during L. innocua(InlA) internalization (Fig. 7B). At the same time, as Hakai was observed around E-cadherin-coated beads clathrin was also present (Fig. 7B). To prove that Hakai, clathrin and caveolin also played a role in E-cadherin-coated bead internalization, each of the three proteins was knocked down with the respective siRNA sequences and the efficiency of bead internalization was quantified. In all cases, efficient knock down of either protein inhibited E-cadherin-coated bead internalization by 50–70% (Fig. 7C).

Discussion

As several other pathogens L. monocytogenes has developed the capability of invading non-phagocytic cells by expressing surface proteins that exploit host surface receptors (Cossart and Sansonetti, 2004, Pizarro-Cerdá and Cossart, 2006). During infections of human polarized epithelial cells the Listeria surface protein InlA uses E-cadherin as its sole binding partner (Mengaud et al., 1996). Our laboratory has recently documented two important findings: InlA-mediated internalization of Listeria activates the tyrosine kinase Src (Sousa et al., 2007) and the endocytic protein clathrin is, at least partially, involved in InlA-mediated bacterial entry into host cells (Veiga et al., 2007). Here we describe the initial successive events that lead to Listeria internalization by the InlA pathway and establish a functional link between Src activation and clathrin-mediated internalization. Upon interaction with InlA, E-cadherin clusters around bacteria and caveolin plays a key role in this event. The InlA/E-cadherin interaction triggers the Src-mediated tyrosine phosphorylation of E-cadherin. Phosphorylated E-cadherin then recruits the ubiquitin-ligase Hakai that mediates the ubiquitination of E-cadherin. Ubiquitination of E-cadherin is required to recruit clathrin at the bacterial entry site and mediates Listeria internalization. The presence of both clathrin and caveolin around entering bacteria, together with the observation that the inhibition of clathrin only partially affects InlA-mediated internalization, highlight that both caveolin and clathrin participate in bacterial internalization. We also present evidence that these two pathways are functional during adherens junction formation, as revealed by the analysis of E-cadherin-coated bead entry, raising the interesting possibility that endocytosis and the formation of cadherin-dependent cellular junctions are tightly linked events.

E-cadherin post-translational modifications, key events in Listeria internalization

In epithelial cells, E-cadherin is endocytosed via clathrin by a signalling pathway triggered by Src-mediated phosphorylation and ubiquitination of E-cadherin (Fujita et al., 2002). Similarly, purified InlA induces the Src-mediated tyrosine phosphorylation of E-cadherin that is detectable 5 min post infection, peaks at 15 min and decreases at 30 min post infection. When the recruitment of E-cadherin at the bacterium/cell interaction sites was monitored by immunofluorescence, it followed the same kinetics as E-cadherin phosphorylation, suggesting that tyrosine phosphorylation of E-cadherin and the clustering of the receptor at the bacterial entry site are linked. Here we show that caveolin1, a well-established marker of detergent-resistant membranes, is essential for E-cadherin clustering that occurs upstream of E-cadherin phosphorylation, in complete agreement with our previous studies demonstrating the role of DRMs in Listeria entry (Seveau et al., 2004).

E-cadherin phosphorylation is followed by its ubiquitination. Ubiquitination of E-cadherin has been reported to be dependent on the Cbl-like ubiquitin-ligase Hakai (Fujita et al., 2002). During InlA-mediated infections, Hakai is recruited at the bacterial entry site and its knock-down efficiently inhibits bacterial internalization. In contrast, the ubiquitin-ligase Cbl, which plays a role in InlB-mediated internalization (Veiga and Cossart, 2005), has no effect on the InlA-mediated internalization of Listeria. Altogether these data suggest that InlA interaction with E-cadherin allows Listeria to hijack a process that triggers E-cadherin internalization (Fujita et al., 2002).

Two pathways for the InlA-mediated internalization of Listeria

We have recently shown that clathrin and dynamin play a role in the InlA-mediated internalization of Listeria (Veiga et al., 2007). Here we characterize this process by
showing that the inhibition of InIA-induced E-cadherin phosphorylation and/or ubiquitination prevents clathrin recruitment at the bacterial entry site and efficiently inhibits infection, thus confirming a role for clathrin in InIA-mediated endocytosis. However, clathrin knock-down does not completely inhibit InIA-mediated bacterial invasion as it does for the InIB/Met internalization pathway (Veiga et al., 2007), suggesting that other clathrin-independent endocytic mechanisms can mediate bacterial and E-cadherin endocytosis. It is known that, in subconfluent cellular cultures, the endocytosis of E-cadherin molecules not engaged in the formation of adherens junctions is mediated by clathrin-independent mechanisms (Akhtar and Hotchin, 2001; Lu et al., 2003; Paterson et al., 2003). We have shown that caveolin1 localizes around internalized bacteria and plays a role in the InIA/E-cadherin internalization pathway. Whether clathrin and caveolin mediate two distinct bacterial endocytic pathways and/or take part in the same internalization process is a complex issue. Caveolin is required for E-cadherin clustering around invading bacteria, and in turn E-cadherin clustering and activation are prerequisites for clathrin recruitment. These data show that caveolin is implicated in the very first steps of the clathrin-mediated bacterial internalization pathway. In addition, we show here that HeLa cells transiently expressing the E-cadherin ΔJM mutant are unable to recruit clathrin but this does not affect bacterial internalization, in agreement with that we had previously reported using L2071 fibroblasts stably expressing the E-cadherin ΔJM (Lecuit et al., 2000). These data suggest that, when fully functional, the juxtamembrane domain of E-cadherin can play a regulatory role in the clathrin-mediated internalization of the receptor, but when this domain is absent, E-cadherin-mediated bacterial internalization can still occur in a clathrin-independent pathways. Here we show that caveolin is efficiently recruited at the bacterial entry site in both HeLa cells expressing wt E-cadherin or the E-cadherin ΔJM mutant, indicating that caveolin can also mediate an alternative bacterial internalization pathway, which is not dependent on E-cadherin ubiquitination and is highlighted when the clathrin-dependent pathway is inactive. The fact that bacterial internalization is inhibited when Src activity is blocked by PP1 and it is not in HeLa cells expressing E-cadherin ΔJM mutant (that lacks the E-cadherin phosphorylation sites targeted by Src) might indicate that the caveolin-mediated pathway is dependent on the activity of Src.

Listeria infections as a model to study the formation of adherens junctions

During InIA-mediated infections of epithelial cells a panel of proteins, normally recruited and involved in adherens junction formation, are recruited at the bacterial entry site (Lecuit et al., 2000; Sousa et al., 2004; Sousa et al., 2005; Hamon et al., 2006). Here we show that in addition, InIA-mediated invasion also triggers post-translational modifications normally occurring during the endocytosis of E-cadherin. Phosphorylation of E-cadherin following Src activation has been so far associated with the opening of adherens junctions (Fujita et al., 2002). Our present work, together with the recent evidence that the formation of E-cadherin homotypic interactions can trigger Src activity (McLachlan et al., 2007), represent a first indication that E-cadherin post-translational modifications can be triggered not only by Src activation or HGF treatment during the opening of adherens junctions, but also directly by E-cadherin/E-cadherin interactions. Together our results suggest that adherens junction formation involves at the same time the formation of E-cadherin homotypic interactions and the recruitment of the endocytic machinery that can promote the internalization of E-cadherin-coated beads and bacteria. How the endocytic machinery regulates adherens junction formation or opening is an issue that deserves further investigation. Due to their long co-evolution with their host, bacterial pathogens provide invaluable tools to investigate eukaryotic cell functions. In this regard, the InLA-mediated internalization pathway of L. monocytogenes appears yet again as a powerful tool for studying adherens junction formation.

Experimental procedures

Bacterial strains, cell lines, plasmids, siRNA sequences and antibodies

*Listeria innocua* (BUG499) was grown in brain–heart infusion (Difco laboratories, Detroit, Michigan). *L. innocua* transformed with pRB474 harbouring the *inlA* gene (BUG 1489) was grown in brain–heart infusion with chloramphenicol (7 μg ml⁻¹). Jeg-3 cells (human epithelial placental cells ATCC n°: HTB-36) were grown in MEM medium containing Glutamax, non-essential aminoacids, sodium pyruvate and 10% fetal bovine serum (Biowest, France). FLAG-tagged Hakai and anti-Hakai polyclonal antibody were kindly provided by Dr Yasuyuki Fujita (UCL, London); HA-Ubiquitin was kindly provided by Prof. Ivan Dikic. WT HE-cadherin (BUG1654) and HE-cadherin-ΔJM (BUG1663) were all constructed in the laboratory. HEC1Δ anti-E-cadherin antibody was obtained by Takara (Shiga, Japan); H108 polyclonal anti-E-cadherin, monoclonal anti-HA and anti-β-catenin H102 polyclonal antibodies were all obtained by Santa Cruz (Santa Cruz, CA); anti-InIA monoclonal antibody L 7.7 and anti-L. innocua polyclonal antibody R6 were produced in our laboratory; anti-caveolin1 polyclonal antibody was obtained by Transduction Laboratories (Becton Dickinson, NJ); anti-phospho-tyrosine monoclonal antibody clone 4G10 was obtained from Upstate (Lake Placid, NY); anti-ubiquitin monoclonal antibodies P4D1 and FK1 were obtained from Cell Signaling Technology (Beverly, MA) and Affinity Research Products (Exeter, UK) respectively; anti-FLAG monoclonal antibody-Cy3 conjugate and anti-β-actin AC15 monoclonal antibody were obtained from Sigma (St Louis,
anti-clathrin heavy chain monoclonal antibody was obtained from Affinity BioReagents (Golden, CO). Anti-Src and Phospho-Src antibody were from Abcam (Cambridge, UK) and Calbiochem (San Diego, CA) respectively. Hakai-targeted double-stranded RNA sense: 5′-CUC GAU CGG UCA GUC AGG AAA-3′ and antisense: 5′-UUU CUC GAC UGA CCG AUC GAG-3′ were obtained by Eurogentech (Seraing, Belgium); caveolin1-targeted double-stranded RNA sense: 5′-GGA UAG AAG UAU ACC UGA UTT-3′ and antisense: 5′-AUC AGG UAU ACU UCU AUC CTT-3′; Cbl-targeted double-stranded RNA sense: 5′-GGG AAA AGA AAG AAU GUA Utt-3′ and antisense: 5′-AUA CAU UCU UUC UCU Ctc-3′; clathrin-targeted double-stranded RNA sense: 5′-GCC CCA GGU GGU AAU CAU UTT-3′ and antisense: 5′-AAU GAU UAC CAC CUG GCC Ctg-3′ and control siRNA sequences were obtained from Ambion (Austin, TX). Src inhibitor PP1 was from Calbiochem. Paraformaldehyde was from EMS (PA).

Protein purification and coating of beads

InlA lacking its cell-wall anchor was purified from BUG1290 as described previously (Sousa et al., 2005). Coated beads were prepared as previously described (Veiga et al., 2007).

Transient transfections

For DNA transient transfection Jeg-3 cells were plated to 80% confluency on glass coverslips in 24-well plates or on glass-bottomed Petri dishes. Cells were transfected using JetPei (Obiogene, Irvine, CA) according to manufacturer’s instructions. Cells were incubated 24 h with the transfectant before the experiment. For siRNA transfection 105 Jeg-3 cells were plated in six-well plate 24 h before transfection using Dharmafect1 siRNA transfection reagent (Dharmacon) according to manufacturer’s instructions. Cells were incubated with the transfectant 3–4 h and complete medium was added to cells. Cells were probed for siRNA efficacy 72 h after transfection.

InlA-mediated infections

Semiconfluent cell layers were washed twice in serum-free culture medium and incubated with L. innocua(InlA) or L. innocua as a control at a multiplicity of infection (MOI) of 50. Cells were then centrifuged for 2 min at 1000 r.p.m. and then incubated at 37°C for the indicated times. At each time point cells were fixed in 4% paraformaldehyde for 30 min and processed for immunofluorescence. Where applicable, cells were pre-treated with 20 μM PP1 for 1 h at 37°C and then incubated with L. innocua(InlA) always in the presence of PP1.

Bead internalization assay

Jeg-3 cells were grown to confluency on glass coverslip in a 24-well plate. E-cadherin- or InlA-coated 1 μm beads were diluted in culture medium, mixed by vortexing, sonicated and added to cells. Cells were centrifuged for 2 min at 1000 r.p.m. and incubated at 37°C for the indicated times. At each time point cells were washed three times in PBS, fixed with 4% paraformaldehyde and processed for immunofluorescence.

Invasion assay

Jeg-3 cells were grown to confluency on six-well plates and invasion assay was performed using the gentamicin survival assay as previously described (Veiga et al., 2007). Bacteria were added to cells at an MOI of 50. Cells were incubated with the bacteria for 45 min at 37°C and 10% CO2 and 1 h in the presence of gentamicin (10 μg ml⁻¹). Cells were then lysed with 0.1% Triton X-100 in sterile water and lysates were plated for colony counting.

Immunoprecipitations

Jeg-3 cells were grown to confluency in 75 mm² flasks and either incubated with L. innocua wt or L. innocua(InlA) at an MOI of 50 or left uninfected. Alternatively cells were incubated with 50 nM purified InlA in FCS-free medium. After treatment cells were rinsed three times in pre-chilled PBS at 4°C and incubated in 500 μl of lysis buffer (1% NP-40, 20 mM Tris pH 8.0, 150 mM NaCl, 10% glycerol, 20 mM NaF, 5 mM Na3VO4, complete protease inhibitors cocktail, Roche). Cells were then scraped and collected in 1.5 ml eppendorf tubes and further incubated in the lysis buffer for 15 min at 4°C on a spinning wheel. Lysates were then centrifuged 15 min at 4°C at maximum speed and the supernatant was collected in 1.5 ml eppendorf tubes. The protein content of each sample was measured and cleared lysates were incubated 1 h at 4°C on a spinning wheel with protein A sepharose beads to eliminate unspecific binding of proteins to beads. Lysates were then centrifuged 2 min at maximum speed to eliminate beads and incubated overnight at 4°C on a spinning wheel either with 1 μg of anti-E-cadherin H108 polyclonal antibody or 2 μg of 4G10 monoclonal antibody accordingly. The following day protein A sepharose beads were added to each samples and incubated at 4°C on a spinning wheel for 3 h. Samples were then centrifuged 2 min at 4°C at maximum speed and the supernatants were discarded. Sepharose beads were then washed three times in lysis buffer and re-suspended in laemli-loading buffer. Samples were then loaded on an 8% polyacrylamide gel and blotted for protein analysis.

Re-immunoprecipitations were performed as previously described (Ireton et al., 1999).

E-cadherin internalization assay

Jeg-3 cells were grown to confluency on glass coverslip in a 24-well plate, washed twice in fetal bovine serum-free culture medium and incubated at 37°C with or without 50 nM InlA. Cells were then rinsed in PBS and incubated on ice with the anti-E-cadherin antibody HEC1D. Cells were then washed in PBS, fixed in 4% paraformaldehyde and incubated with the Cy3-conjugated secondary antibody to label extracellular E-cadherin. Cells were then permeabilized with saponin and further incubated with the anti-E-cadherin HEC1D antibody and an Alexa 488-conjugated secondary antibody to label total E-cadherin. Images were acquired with a Zeiss Axiovert 135 epifluorescence microscope and the Cy3 channel was subtracted to the Alexa 488 channel to select for internalized E-cadherin. Cells positive for internalized E-cadherin were counted and their percentage with respect to the total number of cells sampled was plotted over time.
Immunofluorescence analysis

Cells were fixed in 4% paraformaldehyde in PBS for 20 min at room temperature. Cells were then rinsed in PBS before an incubation in blocking solution (0.5% BSA, 50 mM NH₄Cl in PBS, pH 7.4). 0.05% saponin was added to the blocking solution where needed. Cells were then incubated with the primary antibodies diluted in the blocking solution for 1 h at room temperature, rinsed five times in PBS and further incubated for 45 min with the secondary antibodies diluted in the blocking solution. Cells were then rinsed five times in PBS and mounted on glass coverslips using Fluoromount mounting medium (EMS, PA). Where needed, Zenon antibody labelling kit (Invitrogen, OR) was used according to manufacturer's instructions to avoid secondary antibody cross-reactivity. Samples were analysed either with a Zeiss Axiovert 135 epifluorescence microscope (Carl Zeiss, Germany) connected to a CCD camera or with a Zeiss scanning confocal microscope (LSM 510, Carl Zeiss, Germany). Images were acquired alternatively with a 100× or 63× oil immersion objectives and images were processed with MetaMorph (Universal Imaging) and LSM (Carl Zeiss) software. For the quantification of protein recruitment around adherent bacteria, an average of 30 images was acquired from each condition allowing the sampling of approximately 300 bacteria. Within these the fraction of bacteria recruiting the protein of interest was expressed as the percentage of the totality of bacteria counted.

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Supporting Information

Fig. S1. A. Jeg-3 cells were transfected with HA-tagged ubiquitin (red) and incubated with either L. innocua or L. innocua(InlA). Bacteria were labelled before (blue) and after (red) permeabilization to distinguish between extracellular and intracellular bacteria. After 30 min of incubation ubiquitin was localized around L. innocua(InlA) but not around L. innocua (arrows). Bar: 10 μm.

B. Jeg-3 cells were transfected with control, Hakai-targeted and Cbl-targeted siRNA sequences. 72 h post transfection the protein content was assessed by Western blot. Actin was used as a loading control.

C. Jeg-3 cells were transfected with control or caveolin1-targeted siRNA sequences. 72 h post transfection the protein content was assessed by Western blot. Actin was used as a loading control.

D. Jeg-3 cells were incubated with E-cadherin-coated or Ig-coated beads as control. Cells were fixed and double-labelled for E-cadherin or Ig before and after permeabilization to determine the efficiency of bead internalization. The image shows confocal orthogonal views (xy, xz, yz from a single optical section) of intracellular beads (green) and actin (red). The squares represent the xy view corresponding to the z plane indicated by the small arrow-heads in the lateral rectangles. The upper and lateral rectangles represent the xz and yz views respectively. The x and y planes shown correspond to those indicated by the small arrowhead presented in the square. In both, the apical part of the cell looks inward and the lower part, in contact with the slide, to the outside of the figure.

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