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A single amino acid in E-cadherin responsible for host specificity towards the human pathogen *Listeria monocytogenes*

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Human E-cadherin promotes entry of the bacterial pathogen *Listeria monocytogenes* into mammalian cells by interacting with internalin (InlA), a bacterial surface protein. Here we show that mouse E-cadherin, although very similar to human E-cadherin (85% identity), is not a receptor for internalin. By a series of domain-swapping and mutagenesis experiments, we identify Pro16 of E-cadherin as a residue critical for specificity: a Pro→Glu substitution in human E-cadherin totally abrogates interaction, whereas a Glu→Pro substitution in mouse E-cadherin results in a complete gain of function. A correlation between cell permissivity and the nature of residue 16 in E-cadherins from several species is established. The location of this key specificity residue in a region of E-cadherin not involved in cell–cell adhesion and the stringency of the interaction demonstrated here have important consequences not only for the understanding of internalin function but also for the choice of the animal model to be used to study human listeriosis: mouse, albeit previously widely used, and rat appear as inappropriate animal models to study all aspects of human listeriosis, as opposed to guinea-pig, which now stands as a small animal of choice for future *in vivo* studies.

Keywords: E-cadherin/internalin/invasion/*Listeria*/specificity

Introduction

Listeria monocytogenes is the etiological agent of listeriosis, a severe human food-borne infection characterized by bacterial dissemination to the central nervous system and the fetoplacental unit, due to its capacity to cross the intestinal barrier, the blood–brain barrier and the feto-placental barrier (Lorber, 1996). The molecular basis of these crucial steps is unknown. In contrast, the infectious process at the cellular level is better understood (Cossart and Lecuit, 1998). One important feature of this bacterium is its ability to induce its own internalization into cells that normally are non-phagocytic, such as epithelial cells (Ireton and Cossart, 1997; Cossart and Lecuit, 1998). Two invasion proteins have been characterized in detail. These two proteins, internalin (InlA) and InlB, are leucine-rich repeat (LRR) proteins and mediate entry in different cell types (Ireton and Cossart, 1997; Cossart and Lecuit, 1998).

Internalin, which is a surface protein of *L.monocytogenes*, is necessary and sufficient to promote bacterial internalization into the human enterocyte-like epithelial cell line Caco-2 (Gaillard *et al.*, 1991; Lecuit *et al.*, 1997). In these cells, human E-cadherin (hEcad) was shown to be the receptor for internalin (Mengaud *et al.*, 1996). In addition, fibroblastic cells transfected with the cDNA for LCAM, the chicken hEcad homolog, allow entry of not only *L.monocytogenes*, but also of *L.innocua*, a non-invasive species of the genus *Listeria*, when expressing internalin, or of internalin-coated beads (Mengaud *et al.*, 1996; Lecuit *et al.*, 1997). Untransfected cells or cells expressing N-cadherin do not allow *Listeria* internalization, demonstrating that the internalin–E-cadherin interaction is specific and promotes entry (Mengaud *et al.*, 1996). InlB mediates entry into a wide variety of cells, such as fibroblasts, hepatocytes, epithelioid and endothelial cells (Ireton and Cossart, 1997; Cossart and Lecuit, 1998). The receptor for InlB is currently being investigated.

E-cadherin is a calcium-dependent cell adhesion molecule composed of five extracellular domains and a cytoplasmic tail (Takeichi, 1990; Geiger and Ayalon, 1992; Kemler, 1993; Yap *et al.*, 1997). It plays a key role in embryogenesis by mediating the sorting of cells in tissues (Larue *et al.*, 1994). In adult life, it contributes to cell cohesion and tissue architecture (Hermiston and Gordon, 1995). E-cadherin mediates adhesion between epithelial cells through homophilic interactions which require the first extracellular domain (EC1). Both lateral dimerization of the ectodomain and connection of the cytoplasmic tail of E-cadherin to the actin cytoskeleton via catenins are required for strong homophilic interactions and formation of 'adherens junctions' between epithelial cells (Nagafuchi and Takeichi, 1988; Ozawa *et al.*, 1990; Yap *et al.*, 1998). E-cadherin is not only expressed at the 'adherens junctions' but also on the basolateral face of polarized epithelial cells in the intestine and choroid plexus, as well as at the cell–cell contacts of intracerebral microvascular endothelial cells (Gallin *et al.*, 1983; Thiery *et al.*, 1984; Rubin *et al.*, 1991; Fenyves *et al.*, 1993; Figarella-Branger *et al.*, 1995). It is also present on chorionic villi of placenta, on hepatocytes and on dendritic cells (Shimoyama *et al.*, 1989; Tang *et al.*, 1993; Borkowski *et al.*, 1994). Interestingly, all these E-cadherin-expressing cells are potential *Listeria* targets during the infectious process *in vivo*.

Mouse E-cadherin (mEcad) has been used widely to analyze E-cadherin function during embryonic development and adult life (Larue *et al.*, 1994; Hermiston and Gordon, 1995). It has also been used widely to study homophilic interactions at the molecular level and to

identify the cytoplasmic protein partners of E-cadherin, the α, β, γ and p120 catenins (Nagafuchi and Takeichi, 1988; Ozawa *et al.*, 1990; Yap *et al.*, 1998). The tertiary structure of the first two extracellular domains of mEcad has been established (Overduin *et al.*, 1995; Shapiro *et al.*, 1995; Nagar *et al.*, 1996; Tamura *et al.*, 1998). In addition, mouse has been the most extensively used animal model to study *Listeria* pathophysiology and the immune response to *L.monocytogenes*.

When we identified E-cadherin as a receptor for internalin, our next goal was to identify the regions of E-cadherin required for internalization. Preliminary experiments towards this goal resulted in the intriguing discovery that cells expressing mouse E-cadherin did not promote entry in the way in which cells expressing human E-cadherin do. This observation led us to investigate the lack of function of mouse E-cadherin at the molecular level. Here we describe the identification of a residue critical for human/mouse specificity. These data, in addition to providing a molecular explanation for the stringent specificity of internalin for human E-cadherin, identify a residue critical for the internalin–E-cadherin interaction, which is located in a region not involved in cell–cell adhesion. This discovery is a key step in the understanding of internalin function. In addition, determination and analysis of the sequences of E-cadherins of other animal species have led to the very important finding that the mouse model cannot be used to study all aspects of human listeriosis; the guinea-pig now appears to be the model of choice for future *in vivo* studies.

Results

Mouse E-cadherin does not allow internalin-dependent entry into mammalian cells

We had long observed that in cells of mouse origin, no 'internalin-dependent entry' could be detected, i.e. no difference in entry was observed between *L.monocytogenes* and its isogenic internalin mutant, or between *L.innocua* and *L.innocua* expressing internalin, or between latex beads covalently coated with internalin and beads coated with bovine serum albumin (BSA) (unpublished data). When E-cadherin was identified as the internalin receptor, we tested whether mouse cells known to express a high level of E-cadherin, such as NMe cells (Vleminckx *et al.*, 1991), would promote internalin-dependent entry; they do not (Figure 1). We then tested a series of transfected cell lines expressing mEcad that previously were used to study E-cadherin homophilic interactions (Nagafuchi *et al.*, 1987; Nose *et al.*, 1988; Chen *et al.*, 1997) or interaction with αE-β7 integrin (Karecla *et al.*, 1996), another reported heterophilic ligand of E-cadherin expressed on intraepithelial lymphocytes (Cepek *et al.*, 1994; Karecla *et al.*, 1995). None of these cells allow internalin-dependent entry (Table I). In contrast, all human cell lines expressing hEcad that we have tested so far, such as LoVo, HCT8 or HepG-2 cells (Drewinko *et al.*, 1976; Aden *et al.*, 1979; Vermeulen *et al.*, 1995), allow internalin-dependent entry, as do the Caco-2 cells originally used to identify the internalin receptor (Figure 1; Dramsi *et al.*, 1995). These results suggested that internalin does not interact with mEcad although it interacts with hEcad in Caco-2 cells and LCAM in LCAM-transfected S180 fibroblasts (Mengaud

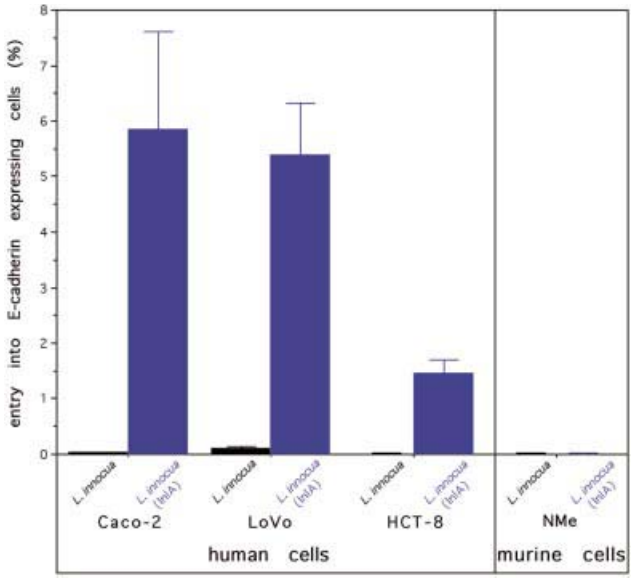

Fig. 1. Invasivity assays in cells expressing hEcad and mEcad. Entry of *L.innocua* transformed with a control plasmid (black bars) and of *L.innocua* (InlA) expressing internalin (blue bars) in human cells (Caco-2, LoVo and HCT8) and murine cells (NMe). Values are expressed as the percentage of bacteria resistant to gentamicin and are the mean ± SD of three independent assays.

et al., 1996). This finding was unexpected since mEcad and hEcad share 85% identity, whereas hEcad and LCAM share 66.5% identity. To clarify these observations, we compared in the same genetic background the ability of LCAM, hEcad or mEcad expression to promote internalin-dependent entry. L2071 fibroblasts stably transfected with LCAM, hEcad or mEcad cDNAs were first tested for their capacity to adhere to purified internalin (see Materials and methods). Fibroblasts expressing LCAM and hEcad bind to internalin in a concentration-dependent manner, whereas non-transfected and mEcad-expressing L2071 fibroblasts do not (Figure 2A). We then tested in these cells adhesion and entry of internalin-coated beads (Figure 2B) and entry of *L.innocua* expressing internalin (Figure 2C). LCAM- and hEcad-expressing L2071 cells promote adhesion and entry of both internalin-coated beads and *L.innocua* expressing internalin, whereas L2071 cells expressing mEcad behave as non-transfected cells. Taken together, these data clearly establish that hEcad and its chicken homolog LCAM are both receptors for internalin, whereas mEcad is not.

The first extracellular domain of E-cadherin (EC1) is responsible for specificity

To determine the molecular basis of the specificity of E-cadherin towards internalin, we generated a series of E-cadherin chimeras by swapping hEcad and mEcad domains (Figure 3A). These chimeric E-cadherins were transiently expressed in L2071 cells, and transfected cells were tested for their ability to promote adhesion and entry of internalin-coated beads. The results reported in Figure 3A provide evidence that specificity resides within EC1 of hEcad, and more precisely within the first 94 amino acids of this domain. All the other domains of hEcad and mEcad are interchangeable.

Table I. Stably transfected cells expressing mouse E-cadherin do not allow entry of *Listeria innocua* expressing internalin

Cell lines	E-cadherin species	Type	Origin	Internalin-dependent entry
Els8	murine	L cell	Nagafuchi *et al.* (1987)	no
Elβ1	murine	L cell (SZL)	Nose *et al.* (1988)	no
L KB	murine	L cell (KB)	Karecla *et al.* (1996)	no
WCE2	murine	WC5 cell	Chen *et al.* (1997)	no
CHO(mEcad)	murine	CHO cell	B.Geiger (unpublished)	no

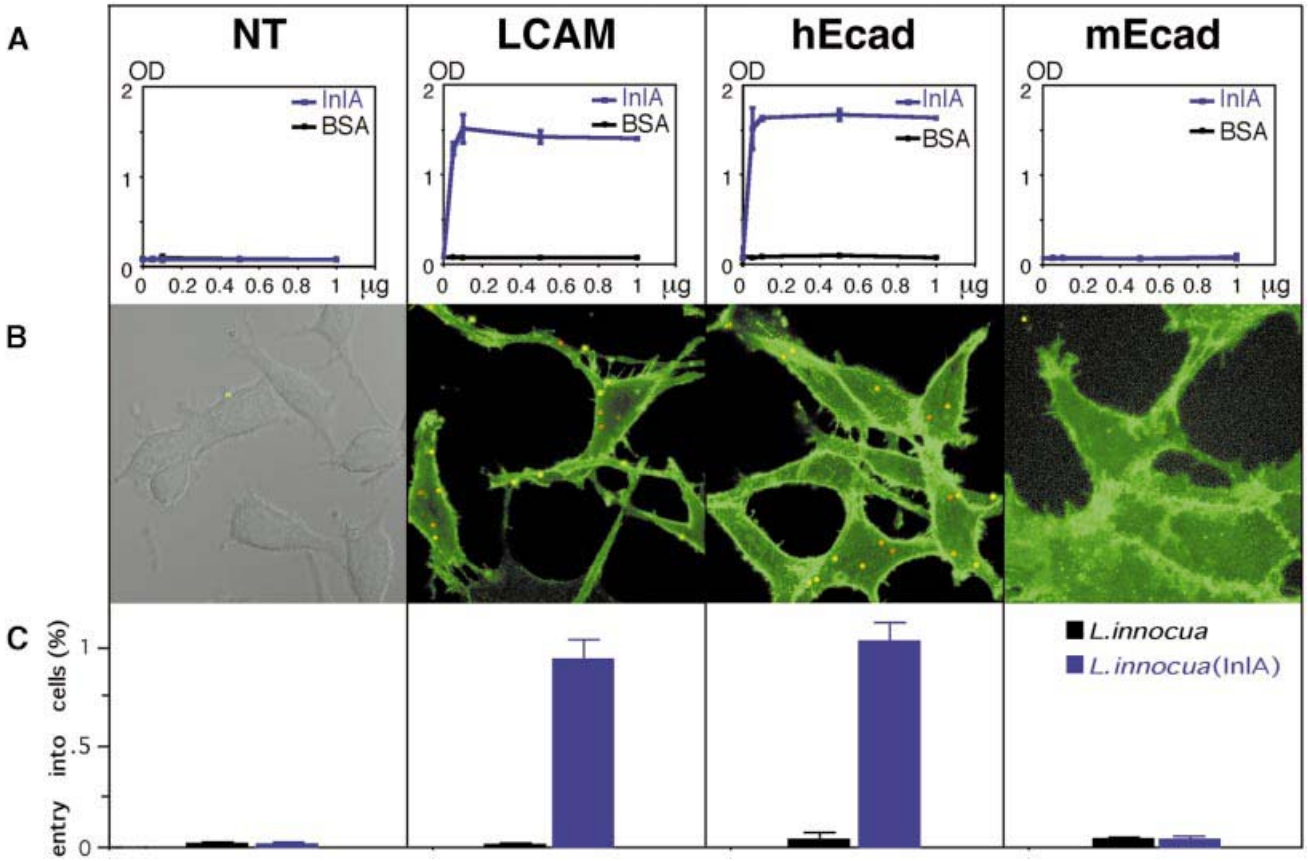

Fig. 2. mEcad is not a receptor for internalin. (**A**) Cell adhesion to purified internalin. LCAM- and hEcad-transfected cells interact with internalin in a dose-dependent manner, whereas non-transfected (NT) and mEcad-transfected L2071 cells do not. (BSA is used as a negative control; mean ± SD of four wells for each protein concentration.) (**B**) Confocal view of internalin-coated beads interacting with cells. All beads are far-red fluorescent, extracellular beads are labeled with an anti-internalin antibody and an FITC-conjugated anti-mouse IgG. Surface E-cadherin of L2071-transfected cells is labeled with anti-E-cadherin antibodies revealed with an FITC-conjugated antibody. Non-transfected cells were subjected to a mix of these anti-E-cadherin and conjugated antibodies. For NT, Nomarski, red and green channels were merged. For the three other cell lines, red and green channels were merged. Cells expressing E-cadherin appear in green, extracellular beads in yellow and intracellular beads in red. (**C**) Invasivity tests in non-transfected L2071 cells and stably transfected L2071 cells expressing LCAM, hEcad or mEcad. Values are expressed as the percentage of bacteria resistant to gentamicin and are the mean ± SD of three independent assays.

Pro16 is critical for specificity of the internalin–E-cadherin interaction

Among the 10 amino acid positions different in hEcad and mEcad in the 1–94 EC1 region, only four are identical in hEcad and LCAM (Figure 3B). Among those, only one, residue 16, is located in an exposed loop in the structure of mEcad and thus appeared to be a potential critical residue (Figure 3B and D). This amino acid is a proline in hEcad and LCAM, and a glutamic acid in mEcad (Figure 3B). We decided to change Pro16 of hEcad into glutamic acid, and Glu16 of mEcad into proline (Figure 3C). These mutated E-cadherins were expressed in L2071 cells and their ability to allow adhesion and entry of internalin-coated beads was determined and compared with that of wild-type hEcad and mEcad. The P16E substitution in hEcad results in a complete loss of function (Figure 3C), whereas E16P substitution in mEcad leads to a complete gain of function (Figure 3C). These results clearly identify Pro16 of the exposed loop located between the two first β-sheets of the first extracellular domain of human E-cadherin as crucial for interaction with internalin (Figure 3D).

We then tested whether this position 16 is important for homophilic interaction. We thus performed aggregation assays as previously described (Murphy-Erdosh *et al.*, 1995), by mixing hEcad-expressing and mEcad-expressing L2071 cells; we could demonstrate that hEcad and mEcad induce the formation of mixed aggregates, indistinguishable from homophilic aggregates, indicating that hEcad and mEcad can interact in an heterospecific manner as

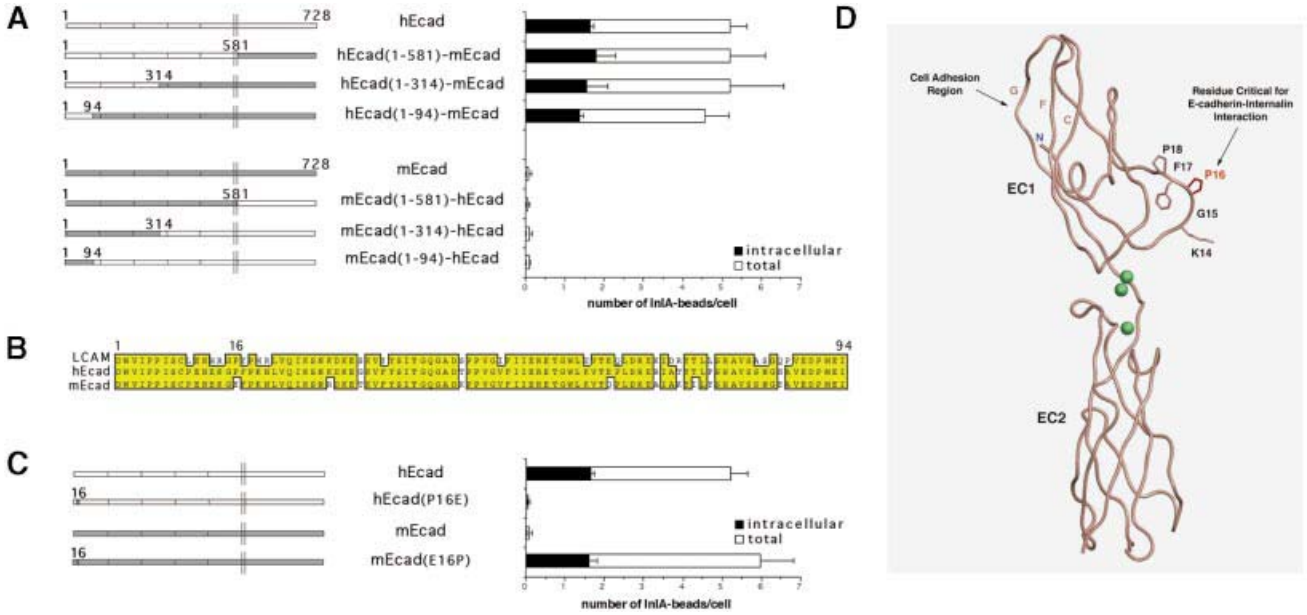

Fig. 3. Determination of the molecular basis of the specificity of the internalin–E-cadherin interaction. (**A**) Left panel: schematic drawing of E-cadherin chimeras. White boxes indicate E-cadherin regions of human origin and gray boxes E-cadherin regions of mouse origin in the different chimeric proteins. Center panel: denomination of the different chimeras. Right panel: adhesion and invasivity of internalin-coated beads to cells expressing E-cadherin chimeras. Mean ± SD of total (white bars) and intracellular (black bars) internalin-coated beads (InlA-beads). (**B**) Alignment of the first 94 amino acids of LCAM, hEcad and mEcad (performed using the Clustal program). (**C**) Left panel: schematic drawing of wild-type and mutated E-cadherins. Center panel: denomination of the mutated E-cadherins. Right panel: adhesion and invasivity of internalin-coated beads to cells expressing wild-type and mutated E-cadherins. Mean ± SD of total (white bars) and intracellular (black bars) internalin-coated beads (InlA-beads). (**D**) A backbone worm diagram of mEcad(E16P). This diagram was drawn from the crystal structure of the EC1–EC2 fragment of mEcad (PDB entry 1EDH). Side chains are drawn only for amino acids in the putative internalin-binding loop. Calcium ions are shown as green spheres. C, F and G β-strands are indicated. The N-terminus is indicated by a blue N.

well as in an homophilic manner (data not shown). Thus the nature of the amino acid at position 16 of E-cadherin is not crucial for cadherin–cadherin interaction, in agreement with previous similar data showing that chicken B-cadherin and mEcad, which harbor a proline or a glutamic acid in that position, respectively, interact in a heterophilic manner (Murphy-Erdosh *et al.*, 1995). We also showed that antibodies inhibiting hEcad homophilic interaction, such as HECD1 and MB2 (Shimoyama *et al.*, 1989; Bracke *et al.*, 1993), have no inhibitory effect on internalin–E-cadherin interaction (data not shown). Taken together, these results show that the region responsible for the specificity of internalin–E-cadherin interaction is different from the regions involved in E-cadherin–E-cadherin homophilic interaction (Figure 3D).

Internalin-dependent entry in cell lines from various species relies on the nature of residue 16

The fact that mEcad is not recognized by internalin *in vitro*, as shown in this study, provides an explanation for the fact that no role for internalin *in vivo* could be identified in a mouse model. Indeed, oral and intravenous infections of mice with *L.monocytogenes* and its isogenic internalin mutant led to the same results: wild-type *L.monocytogenes* and the internalin mutant translocate across the intestinal barrier and reach the liver and spleen with the same low efficiency, and their LD_{50}s are identical (data not shown; see Materials and methods).

Since a recent report using another system, the rat ligated ileal loop system, did not detect a role for internalin in the crossing of the epithelial intestinal barrier (Pron *et al.*, 1998), we tested the permissivity of cells expressing rat E-cadherin. We chose the NBT2 cell line (Tucker *et al.*, 1990). This cell line does not allow internalin-dependent entry (Figure 4A). The sequence of rat E-cadherin was unknown. We thus determined the sequence of the region encoding the rat E-cadherin EC1 using RT–PCR with degenerate primers deduced from previously reported E-cadherin-coding sequences. The rat E-cadherin displays a glutamic acid in position 16 (Figure 4B), thus placing the rat in the same group as mouse (Figure 4C).

It had been reported in the early 1970s that in guinea-pigs, following oral infection, *Listeria* are detected in enterocytes (Racz *et al.*, 1972). We thus anticipated that guinea-pig epithelial cells expressing E-cadherin should be permissive to bacteria expressing internalin. We infected GPC16 cells, which are guinea-pig cells of epithelial origin. These cells are recognized by anti-E-cadherin antibodies (HECD1 and ECCD2; data not shown). They do exhibit a high level of internalin-dependent entry (Figure 4A). Using the same strategy as for rat, we sequenced the guinea-pig E-cadherin EC1-coding region. Guinea-pig E-cadherin harbors a proline at position 16 (Figure 4B).

Discussion

This study is the first report describing at the molecular level the host specificity of a bacterial invasion protein. We have shown that internalin-mediated entry of *L.monocytogenes* into mammalian cells stringently requires a proline residue at position 16 of E-cadherin. However, the exact amino acids interacting with each other in each of

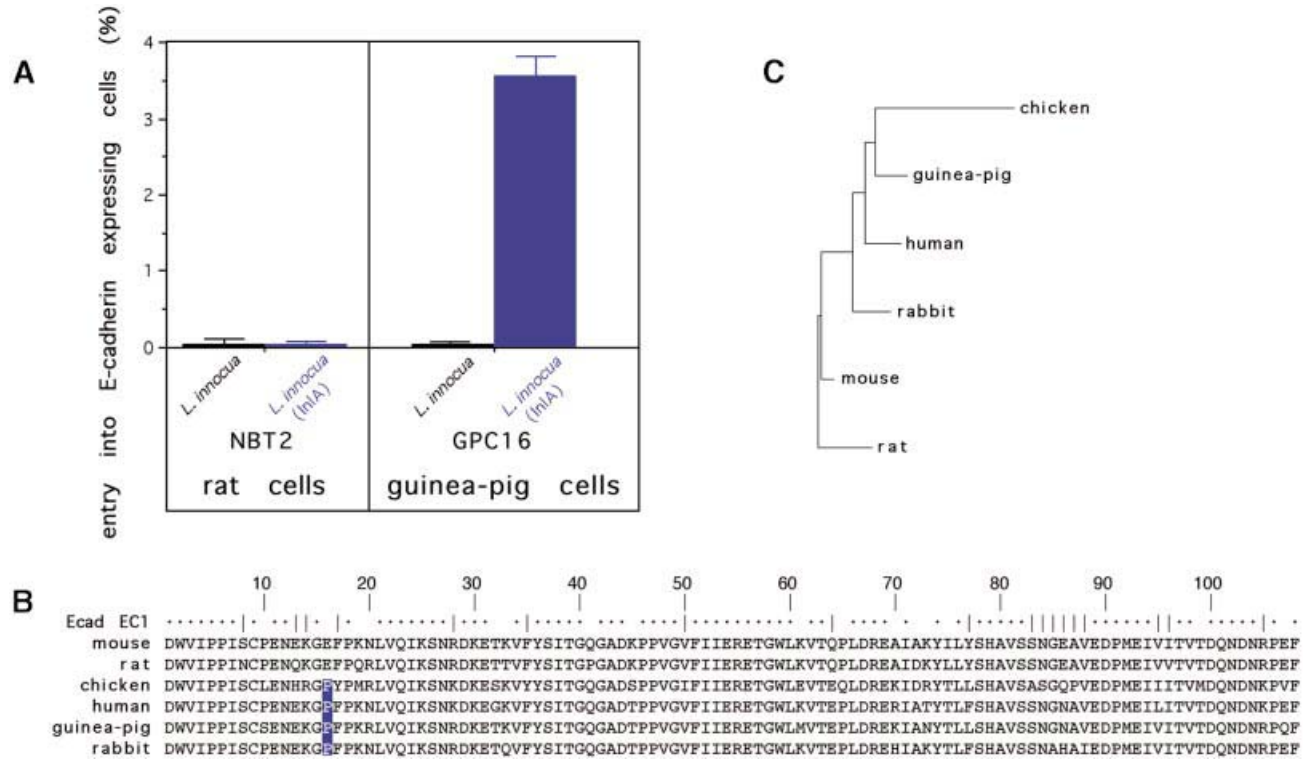

Fig. 4. Guinea-pig E-cadherin but not rat E-cadherin is a receptor for internalin and harbors a proline in amino acid position 16. (**A**) Invasivity tests in guinea-pig cells (GPC16) and rat cells (NBT2). Values are expressed as the percentage of bacteria resistant to gentamicin and are the mean ± SD of three independent assays. (**B**) Mouse and rat E-cadherins harbor a glutamic acid in position 16, whereas chicken, human, guinea-pig and rabbit E-cadherins harbor a proline (Mohan *et al.*, 1995). Multiple alignment was performed with the Clustal program. Black dots indicate identity, vertical lines similarity; proline in position 16 is boxed in blue. (**C**) Dendrogram of the E-cadherin EC1 domain from different species. E-cadherin EC1 domains interacting with internalin (chicken, guinea-pig and human) cluster in the same group. E-cadherin EC1 domains not interacting with internalin (rat and mouse) cluster in a separate group (this dendrogram was made using the NJplot program).

the two partners have not been identified. Residue 16 is located in the close vicinity of the first E-cadherin 'calcium-binding pocket', and the structure of the loop encompassing this critical amino acid has been shown to be strongly influenced by calcium concentration (Overduin *et al.*, 1995). Interestingly, in good agreement with these new data, internalin–E-cadherin interaction has already been shown to be calcium dependent (Mengaud *et al.*, 1996). In addition, in hEcad, the loop harboring residue 16 is primarily hydrophobic and uncharged, whereas in mEcad it is more hydrophilic and charged (Figure 3B), suggesting that internalin–hEcad interaction involves hydrophobic interactions. These results are in line with our recent findings that the region in internalin involved in the interaction is the N-terminal 330 amino acid LRR region (Lecuit *et al.*, 1997), although, as mentioned above, the precise region of internalin interacting with E-cadherin and the precise region of E-cadherin interacting with internalin are unknown.

The region of E-cadherin involved in cell–cell adhesion as shown in Figure 4D is different from the region critical for internalin–hEcad interaction, strongly suggesting that E-cadherins engaged in homophilic interactions may still be accessible to internalin. A challenging issue in understanding the role of internalin *in vivo* will be to determine whether such an interaction can take place and whether interaction of internalin with a molecule of E-cadherin engaged in a homophilic interaction destabilizes this interaction and has the capacity to disrupt the structure of an epithelium.

Internalin was first identified as an invasion protein, by an *in vitro* approach, i.e. by a search for non-invasive mutants in the human epithelial cell line Caco2 (Gaillard *et al.*, 1991). Its receptor was then identified using an affinity chromatography approach (Mengaud *et al.*, 1996). However, the *in vivo* role of internalin has not been identified. The work described here explains why the function of internalin could not be identified using mouse and rat models. Guinea-pig now appears to be the animal model of choice to address not only the *in vivo* function of internalin, but also probably various aspects of human listeriosis, such as dissemination to the central nervous system and to the fetoplacental unit, which are both bordered by E-cadherin-expressing cells. Interestingly, guinea-pigs and rabbits were the two animal species in which Murray first discovered *L.monocytogenes* in 1926 during an epidemic in animal care houses (Murray *et al.*, 1926). These two species are natural hosts for this pathogen, and guinea-pig and rabbit E-cadherins both exhibit a proline at position 16 (Mohan *et al.*, 1995; this study). Experiments in guinea-pigs (or rabbits) will thus help us to unravel the role of internalin–E-cadherin interaction. However, it still remains possible that redundancy exists and that the function of internalin even in a guinea-pig model may be hidden by other listerial proteins.

Internalin belongs to a large family of surface or secreted proteins in *L.monocytogenes*. These proteins share in common the presence of LRRs of 22 amino acids. A function has been identified for only two members, internalin, which has 15 LRRs, and InlB, which has

20 LRRs. InlB mediates entry into a wide variety of cells. Its receptor is under current investigation and is not E-cadherin (L.Braun and P.Cossart, unpublished results). Whether InlB also displays a species specificity is a challenging issue. Preliminary experiments indicate that it could be the case, at least in cultured cell lines (M.Lecuit and P.Cossart, unpublished results).

It is worth mentioning that in the case of αE-β7 integrin, the other heterophilic ligand of E-cadherin, its interaction with E-cadherin has been shown to involve the two first extracellular domains of E-cadherin, with Glu31 crucial for this interaction (Karecla *et al.*, 1996). Glu31 is predicted to be located at the top of EC1 (Overduin *et al.*, 1995), while Pro16 is located at the inferior face of EC1 (Overduin *et al.*, 1995), indicating that internalin and αE-β7 integrin recognize opposite sides of EC1. In contrast to position 16, Glu31 is conserved among all E-cadherins and, in agreement with these data, αE-β7 integrin was shown to interact with both mouse and human E-cadherins (Cepek *et al.*, 1994; Karecla *et al.*, 1995).

In conclusion, the molecular basis of host specificity has already been reported for several viruses such as poliovirus, human immunodeficiency virus and hepatitis C virus (Clayton *et al.*, 1988; Ren *et al.*, 1990; Pileri *et al.*, 1998) and the diphtheria toxin (Cha *et al.*, 1998); we report here the first example of a specificity (i.e. complete loss or gain of function) depending on a single amino acid and involving proteins with a particularly high level of similarity (85%) between the 'permissive' and the 'non-permissive' proteins. Solving a problem of specificity has led us to identify a zone in E-cadherin critical for the internalin–E-cadherin interaction. This region is not involved in cell–cell adhesion, suggesting possible interaction of internalin with E-cadherin molecules engaged in cell–cell interactions. This finding may have very important consequences in the understanding of the infection *in vivo*. Our results provide a molecular explanation for the fact that no role in virulence could be attributed to internalin *in vivo* using mouse or rat models. They strongly indicate that the mouse model, which has been the most widely used animal model for the study of listeriosis including its immunological aspects, is inappropriate to study specific features of human listeriosis, as opposed to the guinea-pig, which now appears to be the model of choice for future *in vivo* studies. Alternatively, transgenic mice expressing E-cadherin may be very instrumental. Taken together, these results clearly illustrate how molecular approaches and apparently reductionist *in vitro* studies can assist in rationalizing the choice of an animal model for studying human disease, as recently discussed (Finlay, 1999).

Materials and methods

Invasivity and adhesion assays

Gentamicin survival assays were performed as previously described (Lecuit *et al.*, 1997) with *L.innocua* transformed with pRB474 without insert, and *L.innocua* transformed with pRB474 harboring the *inlA* gene. Internalin purification and cell adhesion assays were performed as previously described (Mengaud *et al.*, 1996).

DNA constructs

mEcad full-length cDNA was obtained from P.J.Kilshaw (Karecla *et al.*, 1996) and cloned at the *Hin*dIII site in the T7 promoter orientation in the pBluescript SK– vector (Stratagene) and in the mammalian expression vector pcDNA3 (InVitrogen), thus giving rise to pSK–(mEcad) and pcDNA3(mEcad), respectively. hEcad partial cDNA encoding hEcad lacking its last 35 amino acids was obtained from D.Rimm (Cepek *et al.* 1994) and cloned at *Hin*dIII and *Xho*I sites in pcDNA3, thus giving rise to pcDNA3(hEcadΔ35).

To obtain hEcad full-length cDNA, mRNA from human A431 cells (provided by K.Wary) was used to make a cDNA library using oligo(dT) primers and Superscript II reverse transcriptase (Gibco-BRL). A PCR fragment was obtained using oligonucleotides CytoA (5′-TGACACCC-GGGACAACGTTTATTA-3′) and CytoB (5′-CTAGTCTAGACCCCTA-GTGGTCCTCG-3′). This 425 bp PCR fragment was digested with *Sma*I and *Xba*I and cloned at these sites in pcDNA3(hEcadΔ35), thus giving rise to pcDNA3(hEcad), which harbors hEcad full-length cDNA. The structure of this construct was verified by sequencing. hEcad full-length cDNA was also cloned at *Hin*dIII and *Xba*I sites into pBluescript SK–, thus giving rise to pSK–(hEcad).

For hEcad(1–581)–mEcad, a PCR product obtained with oligonucleotides OML36 (5′-GGCTTGGATTTTGAGGCCAAGC-3′) and OML37 (5′-TCCCCCCGGGCTACACTGCAGCTCTCCTCCGAAGA-AACAGC-3′) using pSK–(hEcad) as a template was digested by *Kpn*I and *Sma*I and subcloned in pBluescript SK–. A second PCR product obtained with oligonucleotides OML44 (5′-AACTGCAGTGGTCAAA-GAGCCCCTGCTGCC-3′) and OML40 (5′-CAATTAACCCTCACT-AAAGGG-3′) using pSK–(mEcad) as a template was digested by *Pst*I and subcloned in this plasmid. This new plasmid was then digested by *Kpn*I and *Xba*I, and the restriction fragment obtained was subcloned in pcDNA3(hEcad), thus giving rise to pcDNA3[hEcad(1–581)–mEcad].

For mEcad(1–581)–hEcad, a PCR product obtained with oligonucleotides OML36 and OML38b (5′-TCCCCCCGGGCTACACTGCAGCT-CTCCTCCGTAGAAACAGTAGG-3′) using pSK–(mEcad) as a template was digested by *Xho*I and *Sma*I and subcloned in pBluescript SK–. A second PCR product obtained with oligonucleotides OML43 (5′-AACTGCAGTGGTCAAAGAGCCCTTACTGCC-3′) and OML40 using pSK–(hEcad) as a template was digested by *Pst*I and *Xba*I and subcloned in this plasmid. This new plasmid was then digested by *Xho*I and *Xba*I, and the restriction fragment obtained was subcloned in pcDNA3(mEcad), thus giving rise to pcDNA3[mEcad(1–581)–hEcad].

For hEcad(1–314)–mEcad and mEcad(1–314)–hEcad, we took advantage of the presence of a unique *Xcm*I site in hEcad and mEcad cDNAs. pcDNA3(hEcad) and pcDNA3(mEcad) were double digested with *Hin*dIII and *Xcm*I, and restriction fragments were purified and ligated to give rise to pcDNA3[hEcad(1–314)–mEcad] and pcDNA3[mEcad(1–314)–hEcad].

For hEcad(1–94)–mEcad and mEcad(1–94)–hEcad, we followed a similar strategy, taking advantage of a unique *Bsa*BI site conserved in hEcad and mEcad cDNAs to construct pcDNA3[hEcad(1–94)–mEcad] and pcDNA3[mEcad(1–94)–hEcad].

All PCR and ligation products were verified by sequencing. The chimeric and mutated E-cadherin cDNAs are all subcloned in the same pcDNA3 mammalian expression vector, their 5′ end is cloned at the same *Hin*dIII site, and they are under the control of the strong cytomegalovirus enhancer–promoter.

Mutagenesis was performed using the Chameleon double-stranded, site-directed mutagenesis kit (Stratagene), following the manufacturer's instructions. Oligonucleotide mut mE-P (5′-AATGAAAAGGGT-CCATTCCCAAAGAACC-3′) was used to obtain mEcad(E16P), and oligonucleotide mut hP-E (5′-AATGAAAAAGGCGAATTTCCTAAA-AACC-3′) was used to obtain hEcad(P16E). Mutagenic codons are underlined. Mutagenized regions were verified by sequencing.

Stable transfection experiments

L2071 are described in the ATCC catalog under reference CCL1.1, LCAM-transfected L2071 cells (LE6) and mEcad-transfected L2071 cells (L2E2) have been described and characterized previously (Chen *et al.*, 1997); they express similar levels of E-cadherin. hEcad-expressing L2071 cells were obtained as follows: L2071 cells were transfected using the calcium phosphate method with the plasmid pcDNA3(hEcad). Transfected cells were selected by incubation in medium containing 800 μg/ml of G418 (Gibco-BRL). Stably transfected L2071 cells expressing hEcad were labeled with anti-hEcad HECD1 monoclonal antibody (Shimoyama *et al.*, 1989) revealed by an anti-mouse fluorescein isothiocyanate (FITC)-conjugated antibody and isolated by fluorescence activated cell sorting (FACS; Coulter).

Transient transfection experiments, immunofluorescent labelings and quantification of invasivity of internalin-coated beads

pcDNA3-derived plasmids were purified using the Nucleobond AX kit (Macherey-Nagel) and transfections were carried out using the calcium phosphate method with 2×10^5 L2071 cells, grown for 24 h on coverslips. At 48 h post-transfection, 2×10^7 internalin-coated beads, prepared as previously described (Lecuit *et al.*, 1997) and diluted in Dulbecco's modified Eagle's medium (DMEM), were added to these cells. Following 1 h of incubation at 37°C in 10% CO_2, cells were rinsed three times with DMEM and fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS). Transfected cells were detected using an anti-E-cadherin monoclonal antibody [either a mouse HECD1 anti-hEcad antibody (Shimoyama *et al.*, 1989) or a rat ECCD2 anti-mEcad and hEcad antibody (Takara)] and the appropriate conjugated antibody. (This allowed us to locate the HECD1 epitope in hEcad between amino acid positions 94 and 314.) Extracellular beads were labeled with a mouse monoclonal antibody directed against internalin revealed by an FITC-conjugated secondary antibody.

For numeration of total and intracellular beads in transfected cells, three coverslips per transfected chimeric construction were observed. On each coverslip, 50 transfected cells were selected randomly by immunofluorescence. The number of total beads per transfected cell was evaluated under phase contrast observation, the number of extracellular beads by numerating among these beads those that were FITC labeled, and the number of intracellular beads by subtracting the number of extracellular FITC-labeled beads from the number of total beads.

RT–PCR and sequencing

NBT2 cells were obtained from A.M.Valles and previously were shown to express E-cadherin (Tucker *et al.*, 1990). For determination of the coding sequence of rat E-cadherin EC1, total RNA was extracted using the High Pure RNA isolation kit (Boehringer Mannheim) from confluent NBT2 cells trypsinized from a 75 cm^2 culture flask. A 5 µg aliquot of RNA was subjected to reverse transcription using the degenerate oligonucleotide P′1 (5′-AGCTCRGGMTCYTGGCTGA-3′) and Superscript II reverse transcriptase (Gibco-BRL). Half of the reverse transcription product was then subjected to PCR (94°C 1 min 30 s; 40 cycles 94°C 30 s, 45°C 30 s and 72°C 1 min 30 s; 72°C 10 min), using degenerate oligonucleotides EC1for (5′-GRAGRCAGAARMGRGAYTGGGT-3′) and P′3 (5′-GATGGCRGCRTTGTAGGTGTT-3′) and Vent polymerase (Biolabs). The 452 bp PCR product obtained was sequenced directly.

GPC16 cells were obtained from ATCC and are described under reference CCL 242. The same procedure and oligonucleotides as for determination of the coding sequence of rat E-cadherin EC1 were used to determine the guinea-pig E-cadherin EC1-coding region from GPC16 cells.

Animal experiments

Animal experiments were performed using 7-week-old female BALB/c mice obtained from IFA-CREDO, according to the Institut Pasteur guidelines for laboratory animal husbandry and as previously described (Dramsi *et al.*, 1997). Two groups of 12 mice were infected via the oral route with 3×10^9 of either *L.monocytogenes* (EGD strain) or its isogenic internalin mutant. Bacterial counts of homogeneates of liver, spleen and mesenteric lymph nodes were evaluated 24 and 48 h after infection by serial dilutions on BHI agar plates. LD_{50}s were determined by the probit method after intravenous injection of groups of five mice with various dilutions of bacteria. The LD_{50} was estimated as 10^4 for *L.monocytogenes* (EGD strain) and as 1.2×10^4 for its isogenic internalin mutant.

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