Unconventional myosin VIIa and vezatin, two proteins crucial for *Listeria* entry into epithelial cells

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Summary

Listeria monocytogenes **is a bacterial pathogen with the capacity to invade non-phagocytic cells. This dynamic process involves coordinated membrane remodelling and actin cytoskeleton rearrangements. Although some of the molecular factors promoting these events have been identified, the driving force allowing internalization is unknown. One of the receptors for *L. monocytogenes* on epithelial cells is E-cadherin, a transmembrane protein normally involved in homophilic interactions that allow cell-cell contacts at the adherens junctions. E-cadherin has to be connected to the actin cytoskeleton to mediate strong cell-cell adhesion and to trigger *Listeria* entry; α- and β-catenins play key roles in these processes. We have recently identified an unconventional myosin, myosin VIIa and its ligand vezatin, at the adherens junctions of polarized epithelial cells. Here, we demonstrate by pharmacological and genetic approaches that both myosin VIIa and vezatin are crucial for *Listeria* internalization. These results provide the first evidence for the role of an unconventional myosin in bacterial internalization and a novel example of the exploitation of mammalian proteins, by a pathogen, to establish a successful infection.**

Key words: E-cadherin, *Listeria*, Myosin, Vezatin, Phagocytosis

Introduction

Listeria monocytogenes is a food-borne pathogen that has the ability to cross three barriers during infection: the intestinal, blood-brain and foetoplacental barriers. It causes gastroenteritis, septicaemia and lethal central nervous system and maternofetal infections (Vazquez-Boland et al., 2001). In infected tissues, *Listeria* is intracellular owing to its capacity to invade and induce its own uptake into non-phagocytic eukaryotic cells. *Listeria* entry into mammalian cells is a highly dynamic process requiring actin polymerization and membrane remodelling (Cossart et al., 2003). To date, two surface proteins of *L. monocytogenes*, internalin A and B (InlA, InlB), are known to play a major role in its internalization (Cossart et al., 2003). InlB mediates entry into various cell types, reflecting the wide expression of its receptors Met, gC1q-R and proteoglycans (Braun et al., 2000; Braun et al., 1998; Jonquieres et al., 1999; Jonquieres et al., 2001; Shen et al., 2000). By contrast, InlA has a restricted tropism for cells expressing its receptor E-cadherin (Mengaud et al., 1996b). Latex beads coated with purified InlA or the non-invasive species *Listeria innocua* expressing InlA are able to invade mammalian cells expressing E-cadherin (Lecuit et al., 1997; Mengaud et al., 1996b), demonstrating that InlA is sufficient to promote entry. The InlA/E-cadherin interaction is highly species specific. This specificity relies on the nature of the 16th amino acid of mature E-cadherin, which is a proline in permissive species such as humans and a glutamic acid in non-permissive species such as mice (Lecuit et al., 1999). The generation of transgenic mice expressing human E-cadherin has showed that the InlA/E-cadherin interaction on enterocytes is crucial for *L. monocytogenes* translocation across the intestinal barrier and the onset of listeriosis in vivo (Lecuit et al., 2001). A recent X-ray study of the InlA/E-cadherin complex has magnificently confirmed the specificity of these interactions (Schubert et al., 2002).

E-cadherin is a well-characterized cell-adhesion molecule involved in cell sorting and the formation of adherens junction between polarized epithelial cells during embryonic development (Yap et al., 1997). In adult life, it maintains the cohesion of epithelia. This transmembrane protein contains an extracellular ectodomain involved in homophilic interactions with E-cadherin molecules expressed by neighbouring cells. The C-terminus of its cytoplasmic domain interacts with β-catenin, which itself binds α-catenin, an actin-binding protein, thereby providing a link between E-cadherin and the actin cytoskeleton. The connection of E-cadherin to the actin cytoskeleton is required for adherens junction formation (Yap et al., 1997), as well as for the InlA-dependent entry of *Listeria* (Lecuit et al., 2000).

It has been proposed that E-cadherin molecules engaged in homophilic interactions linked to actin filaments might use a myosin to generate the tension necessary to maintain adherens junctions between adjacent cells (Adams et al., 1998; Krendel and Bonder, 1999; Vasioukhin and Fuchs, 2001). Myosins belong to a superfamily that comprises two types of myosins: the conventional and the unconventional myosins (Berg et al., 2001; Hodge and Cope, 2000). All myosins are actin-filament motor proteins and contain a highly conserved ATP- and actin-binding motor domain (or head) located at their N-terminus, and a divergent C-terminal domain (or tail) interacting with specific ligands determining their specific function (Berg et al., 2001; Hodge and Cope, 2000). Conventional myosin, also

called myosin II, forms bipolar filaments that are crucial for muscle contraction. Unconventional myosins do not assemble into filaments and participate in the transport of intracellular organelles, the actin-based rearrangement of plasma membrane and the maintenance of cell polarity, and are involved in basic cellular processes such as endocytosis, phagocytosis and cell migration (Wu et al., 2000).

Myosin VIIa is an unconventional myosin that possess the hallmarks of a myosin motor, namely Mg^{2+}-dependent ATPase activity and the ability to move along actin filaments (Inoue and Ikebe, 2003; Udovichenko et al., 2002). Myosin VIIa is crucial for ear and eye development and function in humans and mice (Gibson et al., 1995; Petit, 2001; Weil et al., 1995). It is mainly expressed in epithelial cells and is proposed to be involved in the morphogenesis of structures like microvilli and cilia (Sahly et al., 1997). Myosin VIIa contains the highly conserved motor domain present in all myosins, followed by a neck region with five isoleucine-glutamine (IQ) motifs (which are expected to bind calmodulin) and a long tail containing two large repeats composed of a myosin-tail-homology 4 (MyTH4) and a Band-4.1/ezrin/radixin/moesin (FERM) domain. These two repeats are separated by a weakly conserved SH3 domain (Petit, 2001) (Fig. 1). The FERM domain of the myosin VIIa tail was recently shown to interact specifically with a widely expressed transmembrane protein named vezatin (Kussel-Andermann et al., 2000). Strikingly, vezatin mediates the recruitment of myosin VIIa to the adherens junctions of epithelial cells in a process that is not completely understood but involves the C-terminal domain of α-catenin (Kussel-Andermann et al., 2000).

Given the apparent similarities between the molecular machineries involved in InlA-mediated entry and adherens-junction formation and maintenance (Lecuit et al., 2000), as well as the recent localization of myosin VIIa to adherens junctions (Kussel-Andermann et al., 2000), we tested the hypothesis that a myosin, possibly myosin VIIa, also generates the force necessary to pull the plasma membrane around the bacteria and/or to allow its internalization.

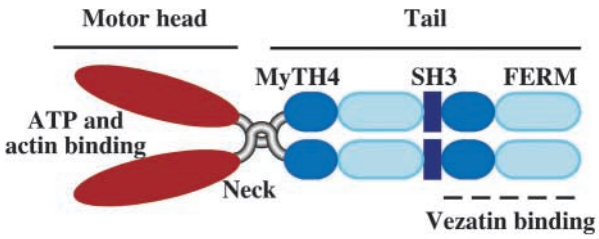

Fig. 1. Schematic representation of the myosin VIIa. The N-terminus of myosin VIIa consists of a conserved motor head containing the ATP- and actin-binding sites. The neck region, composed of five isoleucine-glutamine (IQ) motifs, is followed by a long tail of 1360 amino acids. The tail contains a short coiled-coil domain, which is implicated in the formation of homodimers, and two large repeats arranged in tandem separated by a putative SH3 domain. Each of these large repeats contains a MyTH4 and a FERM domain. The region interacting with vezatin is indicated.

Materials and Methods

Bacterial strains, cell lines, culture media

L. monocytogenes strain EGD (BUG 600), *L. monocytogenes* Δ*inlA* (BUG 947) and *L. monocytogenes* Δ*inlB* (BUG 1047) were 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 in presence of chloramphenicol (7 μg ml^{-1}). L2071 cells expressing human E-cadherin have been described (Lecuit et al., 2000). Caco-2 cells were grown as described previously (Mengaud et al., 1996b).

Protein purification and coating on latex beads

InlA lacking its cell-wall anchor and InlB coupled to a N-terminal six-His tag were purified as described previously (Pizarro-Cerdà, 2002). Purified InlA or InlB were 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).

Antibodies and other reagents

The monoclonal antibody that recognizes the extracellular domain of human E-cadherin (HECD1) was obtained from M. Takeichi (Kyoto University) (Shimoyama et al., 1989), anti-α- and β-catenin antibodies were purchased from Transduction Laboratories (Lexington, KY). Mouse monoclonal anti-InlA (L7.7) and anti-InlB (B4-6) antibodies, rabbit polyclonal anti-*L.-monocytogenes* (R11), anti-myosin VIIa (anti-SSI) and anti-vezatin (anti-mA34) antibodies were used as described (Braun et al., 1999; Kussel-Andermann et al., 2000; Mengaud et al., 1996a).

Cells labelled with anti-vezatin antibody were fixed in cold methanol for 10 minutes at –20°C. For other immunolabellings, cells were fixed in 3% paraformaldehyde in PBS for 15 minutes at room temperature. For actin-filament labelling, cells were fixed in 3% paraformaldehyde in PBS, permeabilized with 0.1% Triton X-100 in PBS and incubated for 45 minutes with Alexa-Phalloidin-488 (Molecular Probes), or fixed in cold methanol and incubated with monoclonal anti-β-actin antibody (clone AC-15; Sigma, St Louis, MO).

2,3-Butanedione monoxime (BDM) (Sigma, St Louis, MO) 0.5 M solution was made fresh for each experiment as described (Cramer and Mitchison, 1995).

Invasion assays

Invasion assays were performed in 24-well plates using the gentamicin survival assay as described previously (Pizarro-Cerdà, 2002). Bacteria were added to cells at a multiplicity of infection (MOI) of 100. Cells were incubated with bacteria for 1 hour at 37°C and 10% CO_2, and for 2 hours in presence of gentamicin (10 μg ml^{-1}). Cells were then lysed with 0.2% Triton X-100 in PBS and the lysates were plated for bacterial counting. These tests were performed in the absence or in presence of 10 mM or 50 mM BDM.

Transient transfections and quantifying the uptake of beads or bacteria

Transient transfections were performed using Lipofectamine Plus (Gibco BRL) following the manufacturer's instructions. 24 hours before transfection, 5×10^5 cells were plated on glass coverslips in six-well plates. When cells reached around 80% confluence, they were transfected using 1.5 μg DNA per well. 36-48 hours after transfection, InlA- or InlB-coated beads or bacteria were added to cells at an MOI of 50. After 1 hour of incubation at 37°C and 10% CO_2, cells were rinsed three times with PBS containing calcium and magnesium (GIBCO, Ref. 14080), and fixed in 3% paraformaldehyde in PBS for 15 minutes at room temperature. Green fluorescent protein (GFP) detection was used to identify transfected cells. Fibroblasts expressing human E-cadherin/α-catenin chimeras were detected using the HECD1 antibody. Extracellular beads were labelled with a mouse

monoclonal antibody raised against InlA (L7.7) or InlB (B4-6) and revealed by a secondary antibody coupled to Alexa-546 (Molecular Probes). Extracellular bacteria were labelled with a rabbit polyclonal antibody raised against *L. monocytogenes* (R11) and revealed by a secondary antibody coupled to Alexa-546. Cells were then permeabilized with 0.1% Triton X-100 in PBS and all the bacteria were labelled with R11 antibody revealed with a secondary antibody coupled to Alexa-488 (Molecular Probes).

Each experiment was done in triplicate each time, and performed three times independently. For each coverslip, 50 transfected cells were selected randomly and the number of total beads evaluated under phase contrast observation. The total number of bacteria was evaluated by the labelling with R11-Alexa-488. The number of extracellular beads or bacteria labelled with R11, L7.7 or B4-6 and then secondary antibody coupled to Alexa-546 was evaluated. The number of intracellular beads or bacteria was obtained by subtracting the number of extracellular beads or bacteria labelled by Alexa-546 from the total number associated with cells.

Immunofluorescence analysis

Preparations were observed with a Zeiss Axiovert 135 microscope or a laser scanning confocal microscope (Zeiss LSM510). Image acquisition from the Zeiss Axiovert 135 was made with a CCD camera (Princeton) and the images were processed with the Metamorph software, version 4.01 (Universal Imaging). The expression level of the different transfected constructs was measured by epifluorescence microscopy. For E-cadherin/α-catenin chimeras, transfected L2071 fibroblasts were fixed and labelled using the HECD1 antibody, which recognizes the ectodomain of E-cadherin. Images of 150 transfected cells (50 cells per coverslip) were acquired under the same conditions. The 150 images were analysed and the integrated fluorescence intensity was measured for each transfected cell. The same procedure was applied to quantify the intensity of GFP fluorescence in L2071 hEcad fibroblasts expressing the vezatin constructs.

DNA constructs

hEcad and hEcad-Δcyto constructs were cloned in pcDNA3 and have been described previously (Lecuit et al., 2000). hEcad-Cαcat is a chimeric protein fusing the ectodomain of human E-cadherin to the C-terminal 398 amino acids of mouse α-catenin (Cαcat) and is encoded by pcDNA3(hEcad-Cαcat) (Lecuit et al., 2000).

hEcad-αcat-AMD harbours the extracellular domain of human E-cadherin (amino acids 1-580) fused to a part of the C-terminus of α-catenin (amino acids 509-643). It was constructed by replacing the *Cla*I-*Xba*I digestion fragment of pcDNA3(hEcad-Cαcat) with a PCR-amplified product digested by the same restriction enzymes, thus giving rise to pcDNA3(hEcad-αcat-AMD) (BUG 1972). This PCR product was obtained using primers 509D (5′-TTTCTTCGGAGG-AGAGCTATCGATGG-3′), which contains a *Cla*I site, and 643E (5′-GCTCTAGATCAGAAGTCAGAGTCGTCC-3′), which contains an *Xba*I site. pcDNA3 (hEcad-Cαcat) was used as template.

In hEcad-ABD, the α-catenin fragment fused to the ectodomain of human E-cadherin contains the distal actin-binding site (ABD) (amino acids 631-906). To obtain pcDNA3(hEcad-αcat-ABD) (BUG 1971), the PCR product obtained with the primers 631D (5′-CCATC-GATATCAGGACCCCC-3′), which contains a *Cla*I site, and 906E (5′-ACAGTCGAGGCTGATCAGCG-3′), and pcDNA3 (hEcad-Cαcat) as template was digested by *Cla*I and *Xba*I, and subcloned in pcDNA3 (hEcad-Cαcat) digested by the same enzymes.

Three vezatin-GFP fusions retaining different regions of vezatin have been constructed using the pEGFP-N2 vector (Clontech), allowing the generation of GFP-fusion proteins in which the GFP is located at the C-terminus. The full-length vezatin cDNA (AF16644 in GenBank) was cloned into the *Eco*RI and *Sal*I sites of pEGFP-N2, giving rise to pEGFP-N2(Vez FL) (BUG 1973).

Using pEGFP-N2(VezFL) as a template and specific primers, we amplified two different fragments of vezatin using PCR. These fragments were digested by *Eco*RI and *Sal*I, and cloned into pEGFP-N2 at the same restriction sites.

To obtain pEGFP-N2(VezC-ter) (BUG 1976), we used the primers VEZ1 (5′-ATCCGGAATTCATGTCTGCCTGTTTGGAAGAGC-3′) and VEZ2 (5′-TACGTCGCCGTCCAGCTCGACCAGGATGGG-3′). VEZ3 (5′-ATCCGGAATTCATGAGCAACAATTGCTGGGAAGA-GG-3′) and VEZ2 were used to construct pEGFP-N2(VezC-terT) (BUG 1977). All the final DNA constructs were verified by sequencing.

Results

A myosin is implicated in *L. monocytogenes* internalization

In order to evaluate the involvement of myosins in *L. monocytogenes* internalization, we first tested the inhibitory effect of the general myosin inhibitor BDM on bacterial entry. Although this compound was recently shown not to be completely specific for myosin function inhibition, it is well accepted that it inhibits several cellular functions consistent with myosin activity (Ostap, 2002; Titus, 2003). BDM inhibits the ATPase activity of myosins (Cramer and Mitchison, 1995) and has been shown to inhibit the phagocytosis of immunoglobulin-opsonized erythrocytes in macrophages but not the formation of actin-rich phagocytic cups (Swanson et al., 1999). We compared the level of *Listeria* entry into different cell types in the absence or presence of BDM. To analyse the effect of BDM on InlA-dependent entry, three types of infections were performed: (1) Caco-2 cells were infected with *L. monocytogenes*; (2) stably transfected L2071 fibroblasts expressing human E-cadherin (L2071 hEcad) were infected with *L. innocua* expressing InlA; and (3) the same cells were incubated with InlA-coated beads. In addition, Vero cells, in which the entry of *Listeria* is purely InlB dependent, were infected with *L. monocytogenes* and with *L. monocytogenes* Δ*inlA*. BDM inhibited both InlA- and InlB-mediated entry (Fig. 2). In Caco-2 cells, mainly infected via the InlA pathway, the entry level was reduced to 40% when cells were treated with 10 mM BDM and to 1% when treated with 50 mM BDM. Similar results were obtained in L2071 hEcad infected with *L. innocua* expressing InlA or incubated with InlA-coated beads (Fig. 2). In Vero cells, *Listeria* uptake was also strongly affected in the presence of BDM (Fig. 2).

Because the cellular target of BDM remains elusive, we verified that BDM treatment had no effect on the actin cytoskeleton. Cells were labelled for F-actin with phalloidin/Alexa-488 before and after BDM treatment. At the concentrations and incubation times used to inhibit *Listeria* entry, BDM did not affect the actin cytoskeleton (data not shown). These results suggest that the inhibitory effect of BDM on *Listeria* uptake is due to the inactivation of one or more myosins and not to the disruption of the actin cytoskeleton.

Myosin VIIa is required for *Listeria* InlA-dependent entry

Myosin VIIa, together with its ligand vezatin, has been shown to be recruited to the junctional complex (Kussel-Andermann et al., 2000). In the search for a myosin implicated in InlA-dependent entry, myosin VIIa appeared as a candidate of choice. We anticipated that, if myosin VIIa is required for

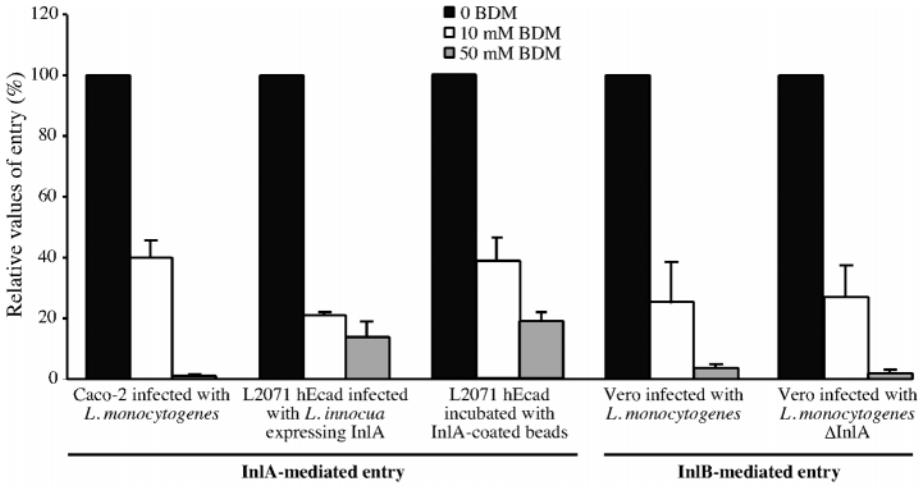

Fig. 2. Effect of BDM on *Listeria* and InlA-coated beads internalization. Entry of wild-type *L. monocytogenes* into human Caco-2 cells and of *L. innocua* expressing InlA and of InlA-coated beads into L2071 fibroblasts expressing human E-cadherin, in the presence of 10 mM or 50 mM BDM was evaluated and compared with entry in untreated cells. Vero cells were infected with wild-type *L. monocytogenes* and *L. monocytogenes* Δ*inlA* in the absence or presence of BDM and the entry levels were determined. Entry to untreated cells has been normalized to 100 and the levels of entry in cells treated with BDM are expressed as relative values. Results are means±SD from three independent experiments, each done in triplicate.

Listeria internalization, overexpression of the myosin VIIa tail, lacking its ATPase and actin-binding domain (Fig. 1), would have a dominant negative effect on entry. To test this hypothesis, L2071 hEcad cells were transfected with a construct encoding myosin VIIa tail fused to GFP (GFP/myosin-VIIa-tail). We first examined the localization of GFP/myosin-VIIa-tail in transfected cells. This chimera was highly enriched at the adherens junctions in Caco-2 cells and colocalized with its known ligand vezatin (Fig. 3). We confirmed by western blot that endogenous myosin VIIa is expressed in L2071 hEcad fibroblasts (data not shown), and analysed by epifluorescence microscopy the localization of GFP-myosin VIIa tail in transfected fibroblasts that had been incubated with either InlA- or InlB-coated beads. GFP/myosin-VIIa-tail was recruited around InlA-coated, but not InlB-coated, beads (Fig. 4A, top). 72±5% of InlA-coated beads associated with transfected cells recruited GFP/myosin-VIIa-tail, whereas only 7±2.4% of the InlB-coated beads did (Fig. 4A, bottom), suggesting that myosin VIIa plays a role in InlA-mediated but not InlB-mediated entry. In cells expressing GFP alone, 8±3% of InlA-coated beads recruited GFP (Fig. 4A, bottom). These observations prompted us to evaluate the rate of entry in these cells. Strikingly, and in agreement with the myosin VIIa localization studies (Fig. 4A), the entry of InlA-coated, but not of InlB-coated, beads was inhibited threefold in cells overexpressing the myosin VIIa tail compared with L2071 hEcad control cells (Fig. 4B). These results were confirmed using two different strains of *Listeria* – *L. monocytogenes* Δ*inlB*, which retains the capacity to enter via the InlA-dependent entry, and *L. monocytogenes* Δ*inlA*, which enters into the cells via the InlB-mediated pathway. The entry of *L. monocytogenes* Δ*inlB* was affected by the overexpression of the myosin VIIa tail (Fig. 4B), whereas that of *L. monocytogenes* Δ*inlA* was not (Fig. 4B). Expression of GFP alone had no inhibitory effect (Fig. 4B). Taken together, these results provide evidence that myosin VIIa is required for InlA/E-cadherin-dependent entry of *Listeria* but does not affect the InlB-mediated entry.

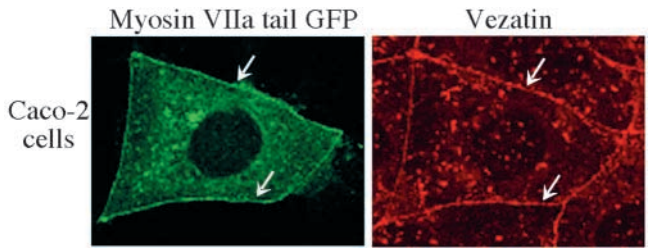

Fig. 3. Localization of the myosin VIIa tail fused to GFP in transfected Caco-2 cells. The myosin VIIa tail fused to GFP localizes with endogenous vezatin at the cell-cell contacts. Arrows show regions where GFP/myosin-VIIa-tail and vezatin colocalize.

Myosin VIIa and vezatin colocalize with actin at the site of InlA-dependent entry

Cellular proteins required for *Listeria* entry are recruited at the entry site. We thus investigated the localization of endogenous myosin VIIa during *Listeria* entry into cells. Caco-2 cells infected with *L. innocua* expressing InlA were fixed and incubated with an antibody raised against myosin VIIa (anti-SSI) (Kussel-Andermann et al., 2000) and phalloidin/Alexa-488 to label F-actin, which is known to be recruited at the entry site of *Listeria* (Lecuit et al., 2000). As shown in Fig. 5A, endogenous myosin VIIa is recruited at the site of entry of *Listeria*, where it localizes with actin. Because myosin VIIa and its ligand vezatin localize with E-cadherin and catenins at adherens junction (Kussel-Andermann et al., 2000), we also investigated whether vezatin was recruited to the *Listeria* entry site. It is indeed the case (Fig. 5B). The recruitment of myosin VIIa, vezatin and actin was still observed in Caco-2 cells treated with BDM (Fig. 5A,B), showing that the effect of BDM on InlA-mediated uptake occurs downstream the recruitment of myosin VIIa. Taken together, these observations support a role for myosin VIIa in InlA-dependent entry and suggest that vezatin might be implicated in the recruitment of myosin VIIa to the site of bacterial entry. In addition, they also strongly indicate that myosin activity is not required in the early stages of internalization.

Distal C-terminal part of α-catenin recruits vezatin during InlA-dependent entry and adherens junction formation

A protein chimera (hEcad-Cαcat) fusing the extracellular

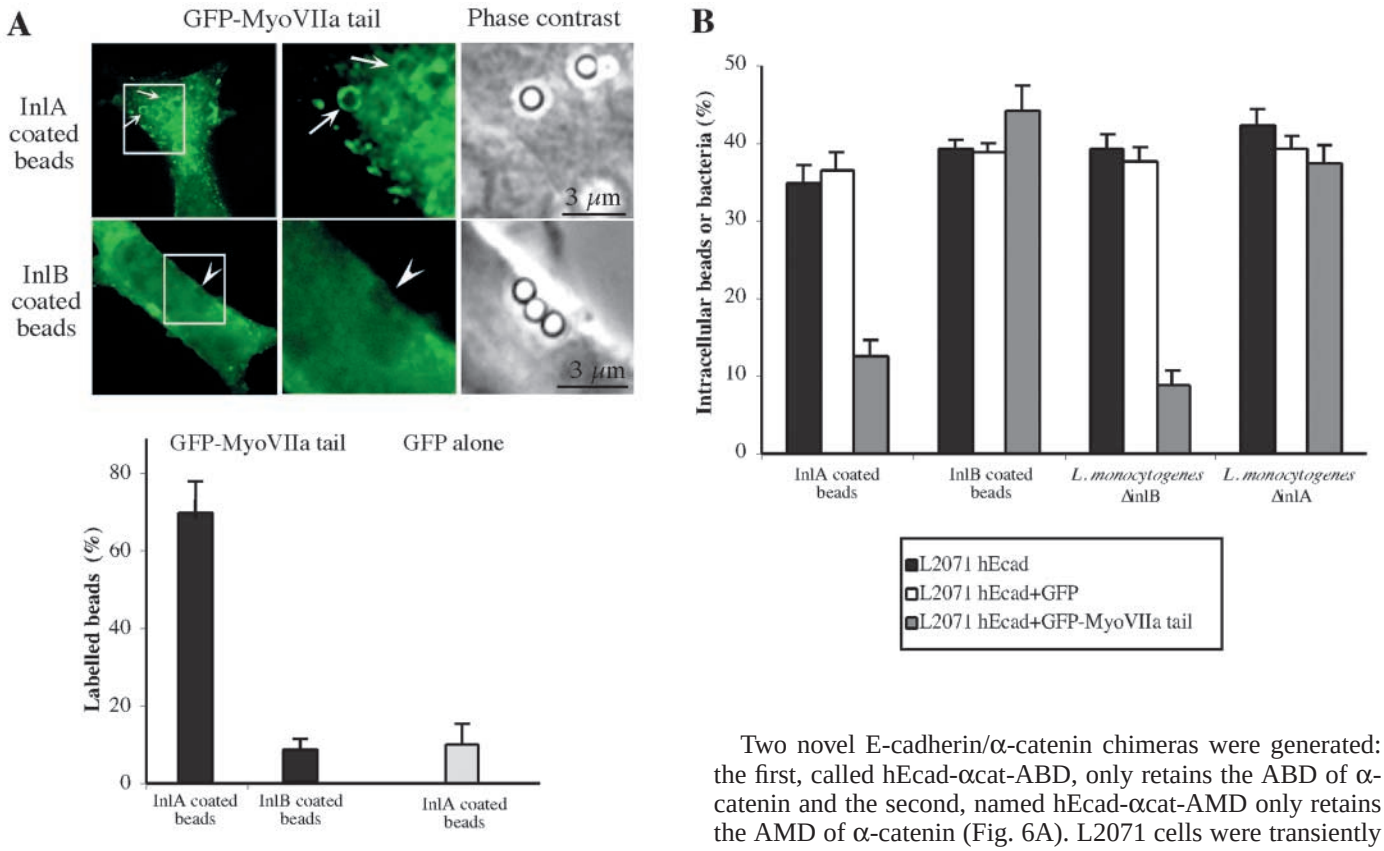

Fig. 4. Effect of myosin VIIa tail overexpression on the entry of InlA- and InlB-coated beads, *L. monocytogenes* Δ*inlA* and Δ*inlB*. (A) L2071 hEcad overexpressing the GFP/myosin-VIIa-tail were incubated with InlA- or InlB-coated beads. Arrows show sites where the GFP/myosin-VIIa-tail is recruited around entering InlA-coated beads. No recruitment was observed around InlB-coated beads. Boxed regions (left) are enlarged in the middle and on the right. The proportion of InlA- and InlB-coated beads associated with cells that recruit GFP/myosin-VIIa-tail was evaluated and compared with that of InlA-coated beads recruiting GFP alone. (B) L2071 hEcad were transfected with a plasmid expressing GFP alone (L2071 hEcad+GFP) or the tail of myosin VIIa fused to GFP (L2071 hEcad+ GFP-MyoVIIa tail). Entry values are expressed as proportion of intracellular beads or bacteria compared with the total number of beads or bacteria associated with the cells. Results are means±SD, from three independent experiments, each done in triplicate.

domain of human E-cadherin to the C-terminal domain of α-catenin has previously been used to show the role of the C-terminal domain of α-catenin in the establishment of adherens junctions formation (Nagafuchi et al., 1994), in *Listeria* internalization (Lecuit et al., 2000) and in vezatin recruitment to cell-cell contacts (Kussel-Andermann et al., 2000). The C-terminus of α-catenin contains two functional domains (Imamura et al., 1999): the large distal actin-binding domain (ABD) (amino acids 631-906), which is sufficient for the formation of weak adherens junctions, and the smaller adhesion modulation domain (AMD) (amino acids 509-643), which is required for strong adherens junctions establishment. We thus investigated whether these two subdomains are necessary for vezatin recruitment during adherens junction formation and InlA-dependent entry.

Two novel E-cadherin/α-catenin chimeras were generated: the first, called hEcad-αcat-ABD, only retains the ABD of α-catenin and the second, named hEcad-αcat-AMD only retains the AMD of α-catenin (Fig. 6A). L2071 cells were transiently transfected with plasmids expressing hEcad-Cαcat or the two novel chimeras. We first assessed the level of expression of each chimera in L2071 cells using an antibody that recognizes the ectodomain of E-cadherin. As shown in Fig. 6B, the three chimera were expressed at similar levels. Transiently transfected L2071 cells expressing either of the chimeras were immunolabelled for vezatin. Vezatin localized with E-cadherin at cell-cell contacts in L2071 hEcad-Cαcat and L2071 hEcad-αcat-ABD, but did not in L2071 hEcad-αcat-AMD and L2071 hEcad-Δcyto (Fig. 6C). This indicates that the distal ABD of α-catenin is required for the recruitment of vezatin at cell-cell contacts, whereas the AMD is not.

We then compared the capacity of InlA-coated beads and *L. monocytogenes* Δ*inlB* to adhere to and enter into cells expressing either one of the two novel chimeras with the capacity of cells expressing hEcad, hEcad-Cαcat or hEcad-Δcyto (Fig. 6). The level of adhesion of InlA-coated beads and bacteria to cells was similar for the five constructs (data not shown), which all express the ectodomain of hEcad at a similar level. By contrast, the level of entry in L2071 hEcad-αcat-AMD was as low as that observed in cells expressing hEcad-Δcyto (Fig. 6D). Thus, the reduced level of uptake of InlA-coated beads and bacteria in L2071 hEcad-αcat-AMD is due not to a reduced level of adhesion or expression of this chimera but to the absence of the ABD. The hEcad-αcat-ABD construct allowed a level of entry slightly lower than that of cells expressing hEcad or hEcad-Cαcat but significantly higher than the entry level in L2071 hEcad-αcat-AMD or hEcad-Δcyto (Fig. 6D).

In summary, the C-terminal ABD is crucial for bacterial uptake and the AMD seems to act only as a modulator required for optimal bacterial uptake. This situation is similar to that

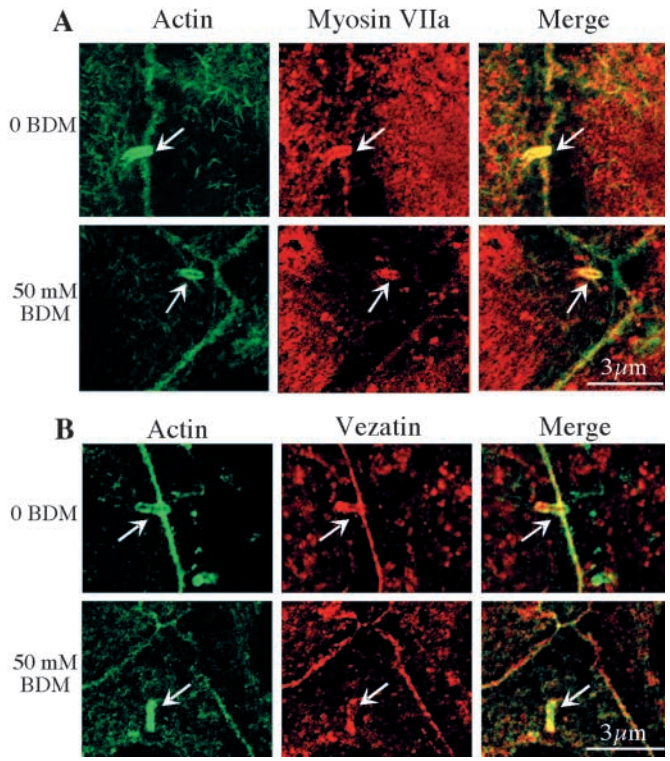

Fig. 5. Recruitment of myosin VIIa and vezatin during entry of *L. innocua* expressing InlA into Caco-2 cells. (A) Actin and myosin VIIa labelling. Myosin VIIa is recruited and colocalizes with actin at the entry site of *L. innocua* expressing InlA and at the adherens junctions in cells not treated (0 BDM) or treated with 50 mM BDM. (B) Actin and vezatin labelling. Actin and vezatin colocalize at the entry site of *Listeria* and at the adherens junctions in untreated cells (0 BDM) or cells treated with 50 mM BDM. Scale bar, 3 μm.

observed for E-cadherin-based cell-cell junction formation (Imamura et al., 1999), further extending the similarities between the molecular mechanisms of E-cadherin-dependent *Listeria* entry and cell-cell adhesion.

Vezatin is necessary for InlA-dependent uptake

To establish definitively the role of vezatin in the *Listeria* entry process, we generated three constructs encoding the full-length vezatin and two truncated versions fused to GFP (Fig. 7A). The myosin VIIa binding site on vezatin was previously shown to be its C-terminal domain (Fig. 7A) (Kussel-Andermann et al., 2000). L2071 hEcad cells were transiently transfected with the three constructs and the expression level of each vezatin-GFP fusion was assessed. As shown in Fig. 7B, they were expressed at similar levels. Transfected cells were incubated with InlA-coated beads. The localization of GFP and endogenous myosin VIIa (using the anti-myosin VIIa antibody anti-SSI) was then examined. In cells expressing the VezFL-GFP, GFP accumulates around the entering beads (Fig. 7C), showing that full-length vezatin is recruited at the entry site. Moreover, in these cells as in non-transfected L2071 hEcad cells (Fig. 7C), myosin VIIa was recruited to the entry site of InlA-coated beads. By contrast, we did not observe any recruitment of GFP or myosin VIIa around the beads entering cells overexpressing the VezC-ter/GFP or VezC-terT/GFP constructs (Fig. 7C). Altogether, these results suggest that the overexpression of the C-terminal domain of vezatin, which contains the myosin VIIa binding site, prevents its recruitment to the entry site of *Listeria*, probably by sequestering the myosin VIIa in the cytoplasm. We then measured the uptake level. The entry level of *Listeria* or InlA-coated beads was threefold lower in cells overexpressing either of the two truncated versions of vezatin (VezC-ter and VezC-terT) (Fig. 7A,D) compared with cells expressing VezFL-GFP. These results demonstrate that vezatin is crucial for the InlA-dependent entry, a role that correlates with its capacity to recruit myosin VIIa to the entry site.

Discussion

We report here the involvement of two novel proteins, an unconventional myosin (myosin VIIa) and its ligand (vezatin) in *Listeria*-induced phagocytosis. We show that myosin VIIa is required for the InlA/E-cadherin-mediated internalization of *Listeria* but not for entry mediated by InlB, another invasion protein of *Listeria* (Bierne and Cossart, 2002). Vezatin is a transmembrane protein previously shown to bind myosin VIIa and to recruit it to adherens junctions. Myosin VIIa and vezatin are recruited at adherens junctions and the entry site of *Listeria*, and localize with actin, suggesting that vezatin acts as the molecular link between myosin VIIa and the E-cadherin/catenins/actin complex.

Two major routes of entry of *Listeria* into mammalian cells have been identified: the InlA- and InlB-mediated pathways (Cossart et al., 2003). Both pathways have been shown to require actin polymerization and membrane rearrangements. InlB is a potent signalling protein that, following interaction with its co-receptors Met (hepatocyte growth factor receptor or HGF-R), gC1q-R and proteoglycans, activates signalling cascades that lead to the actin rearrangements necessary for internalization (Bierne and Cossart, 2002). Key components include phophoinositide-3-kinase (PI 3-kinase), Rac, the Arp2/3 complex and cofilin. Our knowledge of the signals transduced downstream from the InlA/E-cadherin interaction is less advanced. It has been previously shown to involve α- and β-catenins, actin and PI 3-kinase. Here, we show that, in addition to E-cadherin and catenins (Lecuit et al., 2000), two new cytoskeleton-associated proteins, myosin VIIa and vezatin, are required for the InlA-dependent entry of *Listeria*. Vezatin plays a role in the recruitment of myosin VIIa to the site of bacterial entry, by interacting (probably indirectly) with the distal C-terminal domain of α-catenin. Indeed, two-hybrid screens using α-catenin or vezatin as the bait did not detect preys corresponding to vezatin or α-catenin, suggesting that at least one other protein bridges vezatin to α-catenin (S.S., D.C., A.E.-A., C.P., M.L. and P.C., unpublished). We propose that the contractile force generated by myosin VIIa motor activity, recruited to the entry site by the transmembrane protein vezatin, is crucial for the tension necessary for internalization of the bacteria, which is coupled to an active actin polymerization process at the *Listeria* entry site. These new results shed light on our understanding of the exploitation of a cell adhesion molecule to mediate a phagocytic process. To our knowledge, this is the first demonstration of a role for an unconventional myosin in bacterium-induced phagocytosis.

Phagocytic processes are generally driven by rearrangement

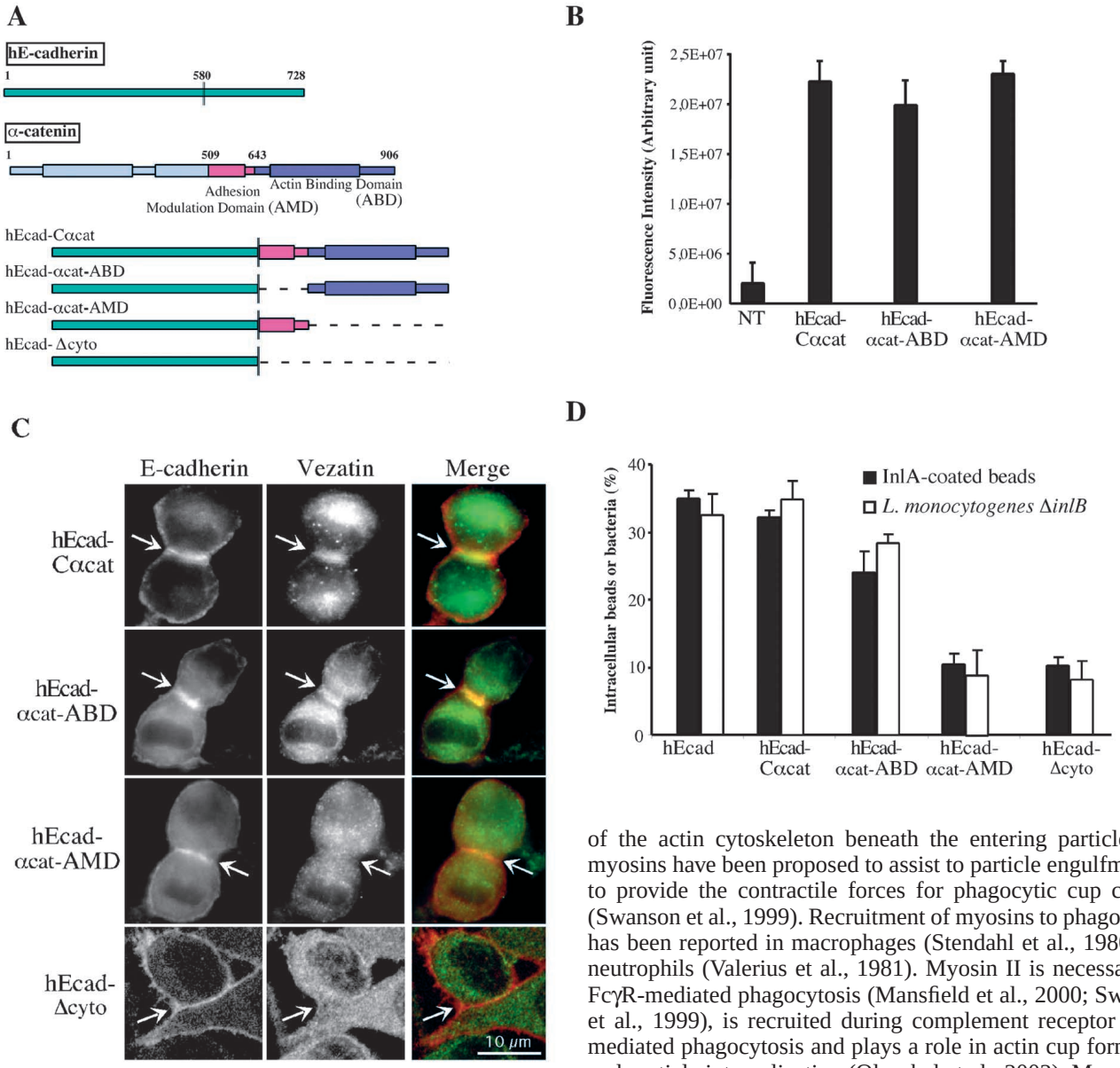

Fig. 6. Contribution of the C-terminus of α-catenin in the recruitment of vezatin at adherens junction and in InlA-mediated entry. (A) Schematic representations of E-cadherin, α-catenin and the chimeras fusing the extracellular domain of human E-cadherin (amino acids 1-580) to different regions of the C-terminal domain of α-catenin and of the human E-cadherin variant lacking its cytoplasmic tail. Dotted lines indicate the regions of α-catenin absent from each construct. (B) Expression level of each chimera in L2071 fibroblasts. Using the HECD1 antibody, the expression level of each chimera (defined by the fluorescence intensity of individual transfected cells) was measured for 150 transfected cells (50 transfected cells per coverslip) and compared with untransfected cells. Values are means±SD of three independent experiments. (C) Localization by fluorescence microscopy of vezatin and human E-cadherin in transfected L2071 cells expressing the different chimeras. (D) Proportion of intracellular beads and bacteria compared with total associated beads or bacteria in L2071 fibroblasts expressing the different chimeras. hEcad cells express the full-length human E-cadherin and hEcad-Δcyto cells express a variant of human E-cadherin lacking its cytoplasmic domain. Values are means±SD for three independent experiments, each done in triplicate.

of the actin cytoskeleton beneath the entering particle, and myosins have been proposed to assist to particle engulfment or to provide the contractile forces for phagocytic cup closure (Swanson et al., 1999). Recruitment of myosins to phagosomes has been reported in macrophages (Stendahl et al., 1980) and neutrophils (Valerius et al., 1981). Myosin II is necessary for FcγR-mediated phagocytosis (Mansfield et al., 2000; Swanson et al., 1999), is recruited during complement receptor (CR)-mediated phagocytosis and plays a role in actin cup formation and particle internalization (Olazabal et al., 2002). Myosin I is recruited to zymosan particle phagosomes (Allen and Aderem, 1995), and myosin IC, myosin V and myosin IXb are recruited to FcγR phagosomes (Swanson et al., 1999). Recently, myosin X (a vertebrate-specific myosin) was also shown to be required for FcγR-mediated phagocytosis (Cox et al., 2002). The class IX myosin myr5 has been suggested to be involved in regulating *Shigella flexneri* infection (Graf et al., 2000) but its major role in infection seems to be played by its GAP activity. In *Dictyostelium*, null mutants for the myosin VII gene showed defects in phagocytosis and cell adhesion (Titus, 1999; Tuxworth et al., 2001). Interestingly, the analysis of a myosin family tree shows that *Dictyostelium* myosin VII and human myosins VIIa, VIIb, X and XV are structurally related, with their tails sharing conserved domains such as MyTH4 and FERM domains (Berg et al., 2001; Hodge and Cope, 2000). FERM domains are involved in membrane attachment by binding to phospholipids or interactions with specific transmembrane proteins (Chishti et al., 1998). Myosin VIIa FERM domain interacts with the transmembrane protein

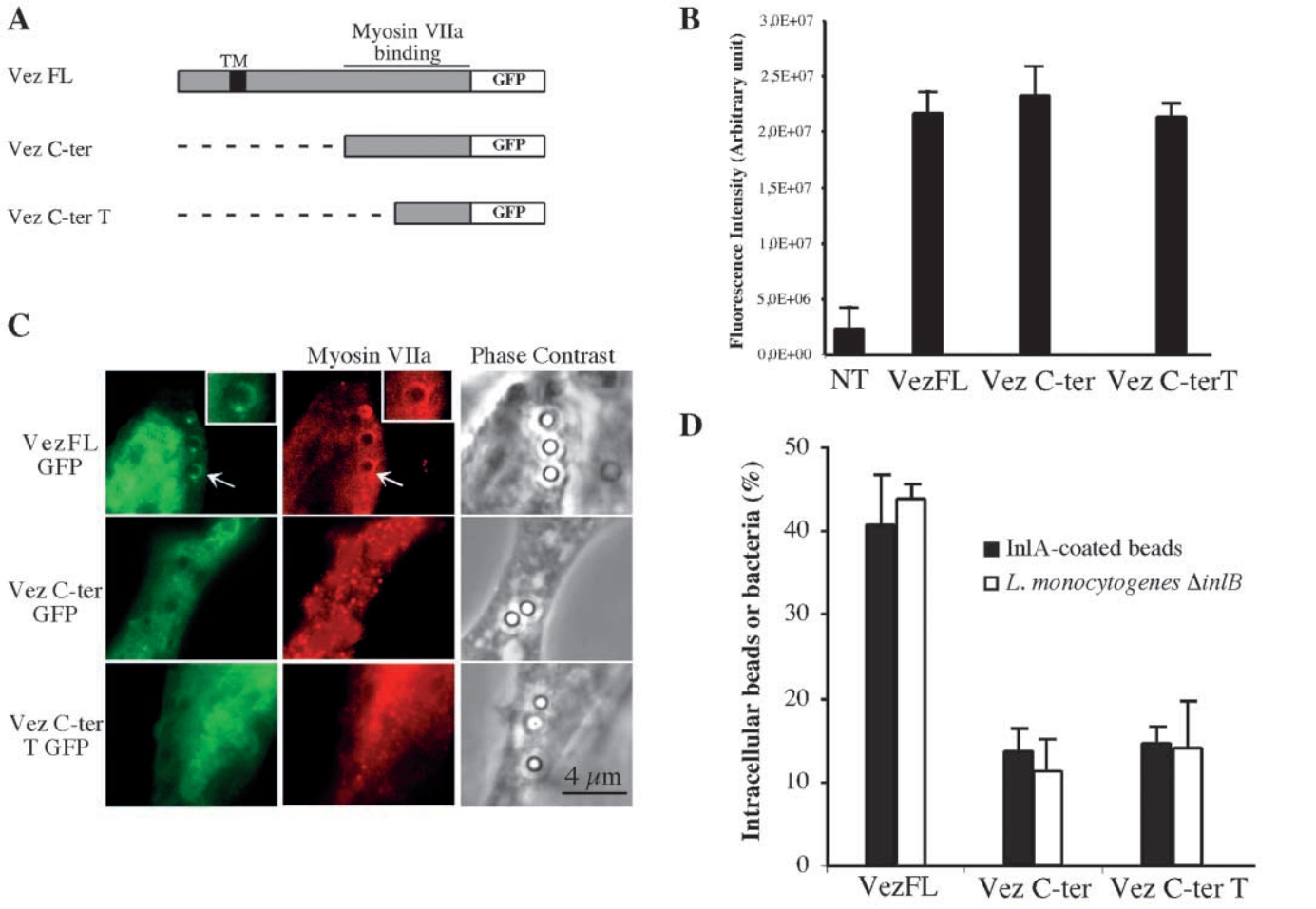

Fig. 7. Localization of myosin VIIa and uptake of InlA-coated beads and *Listeria* in cells expressing truncated forms of vezatin. (A) Schematic representation of vezatin variants fused to GFP. The transmembrane fragment (TM) of full-length vezatin (VezFL) is indicated in black. The region containing the myosin-VIIa-interacting site is indicated. Dotted lines indicate regions absent from each construct. (B) Expression level of each vezatin construct in L2071 hEcad fibroblasts. The fluorescence intensity of GFP was quantified in 150 transfected cells (50 cells per coverslip) for each vezatin-GFP construct and compared with that of untransfected cells. Values are means±SD of three experiments. (C) Localization by fluorescence microscopy of myosin VIIa and vezatin-GFP fusions in L2071 hEcad cells expressing the VezFL-GFP, VezC-ter and VezC-terT constructs. Arrows show sites where myosin VIIa and vezatin-GFP constructs are recruited around entering InlA-coated beads. (D) Proportion of intracellular beads and bacteria compared with the total number of associated beads or bacteria in L2071 hEcad cells expressing the different vezatin constructs. Values are means±SD of three experiments, each done in triplicate.

vezatin (Kussel-Andermann et al., 2000). Strikingly, tandem association of MyTH4 and FERM domains in myosin tails appears to be a common feature of all myosins so far known to be involved in phagocytosis. Myosin VIIa is a motor protein. It is a plus-end-oriented myosin (Inoue and Ikebe, 2002) and has been proposed to be required for protein (Boeda et al., 2002) and organelle (El-Amraoui et al., 2002) transport towards apical cell membranes. During *Listeria* uptake, although it is connected to the cadherin-catenins complex, it might move towards the barbed ends of actin filaments, providing the force necessary for the membrane remodelling around the entering bacteria (Fig. 8). Indeed, our BDM experiments suggest that myosin VIIa is probably not involved in clustering E-cadherin molecules at the entry site of *Listeria* and plays a role in later stages of the internalization process. Strikingly, myosin VIIa is highly expressed in enterocytes (Sahly et al., 1997), a cell type in which the InlA/E-cadherin interaction is crucial in vivo for the translocation of *Listeria* across the intestinal barrier (Lecuit et al., 2001). We thus anticipate an equivalently crucial role for myosin VIIa in *Listeria* internalization in enterocytes in vivo (i.e. in early steps of infection). In addition, our results concerning *Listeria* entry into cells are in line with a recent study that shows abnormal phagocytosis of photoreceptor outer segment disks membranes by retinal pigment epithelium that lacks myosin VIIa (Gibbs et al., 2003).

The involvement of a myosin in the formation and maintenance of adherens junction has long been suggested and, in agreement with this hypothesis, it has been shown that BDM disrupts the organization of cell-cell contacts (Gloushankova et al., 1998). The hypothesis that the unconventional myosin VIIa could play an active role in adherens junction formation has been proposed when its localization at the adherens junction was discovered (Kussel-Andermann et al., 2000). Recently, myosin VIIa was also found to bind harmonin, a protein that interacts with cadherin23, ensuring the cohesion of the

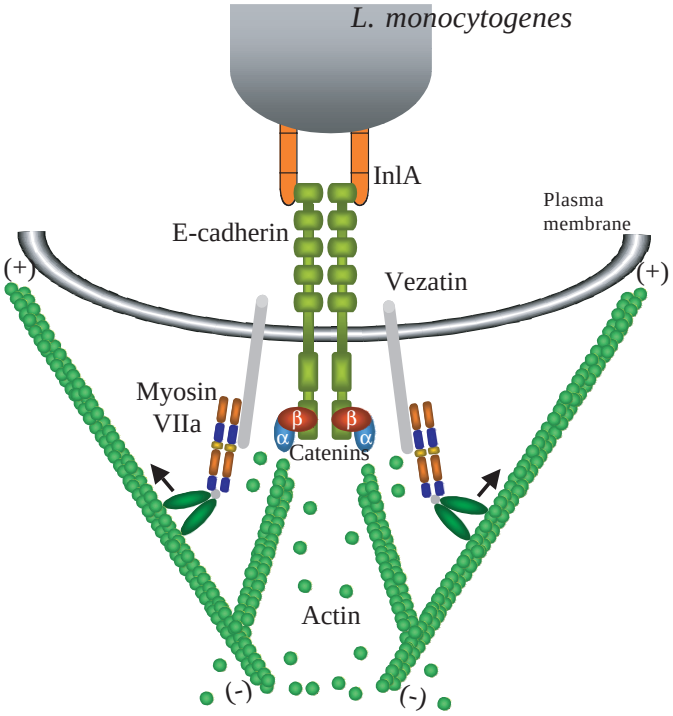

Fig. 8. Model for InlA-dependent entry of *Listeria* into epithelial cells. Proteins known to play a role in entry are indicated, including E-cadherin, α- and β-catenins, vezatin, myosin VIIa, and actin. This model highlights how myosin VIIa could help the membrane rearrangements during *Listeria* entry.

stereocilia of the sensory hair cells (Boeda et al., 2002). Because myosin VIIa tail localizes at the adherens junctions and has a dominant negative effect on InlA-dependent entry, it might also have dominant negative effect on the formation of adherens junctions. We have tested this hypothesis. However, under the conditions we used, no difference was detected between the abilities of control cells and of epithelial cells (MDCK type) overexpressing the myosin VIIa tail to form adherens junctions (S. Sousa et al., unpublished). Thus, despite similarities between *Listeria* phagocytosis and adherens junction formation, there are also crucial differences. First, the diameter of the InlA-coated particle to be phagocytosed – a bacterium or a bead – is ~1 μm, whereas the cell-cell interface during adherens junction formation is much larger. Moreover, the tension generated by myosin VIIa during InlA/E-cadherin-mediated phagocytosis is not counteracted by a similar opposite force on the particle side but is counteracted by the neighbouring cell during adherens junction formation. Thus, impairment of myosin function might not totally inhibit adherens junction formation but might compromise their functional integrity in a more subtle way, which is difficult to assess in our simplified in vitro assay. In the process of adherens junction formation, one might consider that each contacting cell tries to engulf the other in a way similar to what has been called 'frustrated phagocytosis' to describe adhesion of macrophages on immobilized immune complexes (Cannon and Swanson, 1992; Takemura et al., 1986). Finally, we cannot exclude the possibility that, during adherens junction formation, several myosins are involved, in agreement with an effect of BDM on adherens junction formation (Gloushankova et al., 1998).

In conclusion, we have identified two new cellular factors, myosin VIIa and vezatin, that play a crucial role in the dynamics of *Listeria* internalization. Future studies will address how actin polymerization and actin-based myosin activity are coupled during the InlA/E-cadherin entry.

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