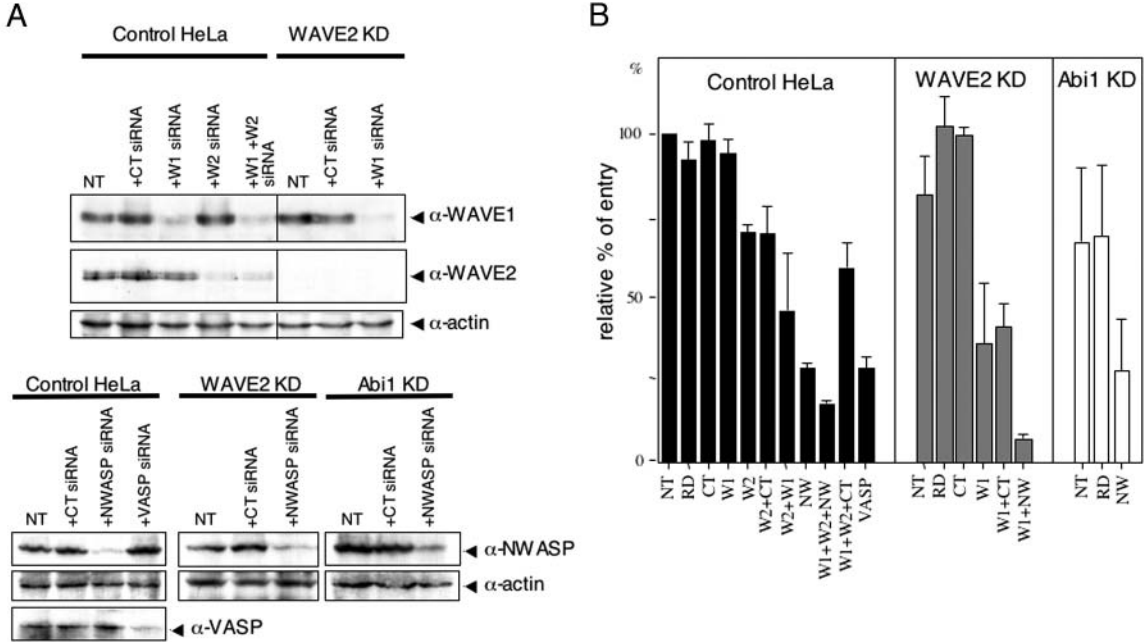**Bierne, H., Miki, H., Innocenti, M., Scita, G., Gertler, F. B., Takenawa, T. and Cossart, P.** (2005). WASP-related proteins, Abi1 and Ena/VASP are required for *Listeria* invasion induced by the Met receptor. *J. Cell Sci.* **118**, 1537-1547.

There was an error in the labelling of Fig. 5A (lanes 5 and 8) in both the online and print versions of this paper.

The correct figure is shown below. The authors apologise for this error.

WASP-related proteins, Abi1 and Ena/VASP are required for *Listeria* invasion induced by the Met receptor

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Summary

Internalisation of the pathogenic bacterium *Listeria monocytogenes* involves interactions between the invasion protein InlB and the hepatocyte growth factor receptor, Met. Using colocalisation studies, dominant-negative constructs and small interfering RNA (siRNA), we demonstrate a cell-type-dependent requirement for various WASP-related proteins in *Listeria* entry and InlB-induced membrane ruffling. The WAVE2 isoform is essential for InlB-induced cytoskeletal rearrangements in Vero cells. In HeLa cells, WAVE1, WAVE2 and N-WASP cooperate to promote these processes. Abi1, a key component of WAVE complexes, is recruited at the entry site in both cell types and its inactivation by RNA interference impairs InlB-mediated processes. Ena/VASP proteins also play a role in *Listeria* internalization, and their deregulation by sequestration or overexpression, modifies actin cups beneath entering particles. Taken together, these results identify the WAVE complex, N-WASP and Ena/VASP as key effectors of the Met signalling pathway and of *Listeria* entry and highlight the existence of redundant and/or cooperative functions among WASP-family members.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/118/7/1537/DC1

Key words: *Listeria monocytogenes*, Cytoskeleton, HGF, Actin, Rac, Cdc42, Phagocytosis

Introduction

The protein InlB promotes internalisation of the pathogenic bacterium *Listeria monocytogenes* in a wide variety of cells (reviewed by Bierne and Cossart, 2002), principally through interactions with Met, the hepatocyte growth factor (HGF) receptor (Shen et al., 2000). InlB-induced phagocytosis involves extension of membranes around the particle and formation of an F-actin cup, leading to the progressive engulfment of the bacterium into the target cell (Supplementary material, Movie 1). Interestingly, similar to HGF, purified InlB induces membrane ruffling when added to cells as a soluble factor. Entry of bacteria and InlB-induced ruffling share the same regulatory proteins (Bierne et al., 2001). Thus, InlB can be used to investigate the cascade of events leading to two types of actin-dependent processes: phagocytosis and ruffling.

We previously identified Arp2/3, the Rho GTPases Rac1 and Cdc42, cofilin and LIM-kinase as key regulators of InlB-Met induced actin rearrangements (Bierne et al., 2001). The Arp2/3 complex promotes nucleation of actin filaments and stimulates the formation of branched actin networks (Machesky and Gould, 1999; Welch, 1999; Pollard and Beltzner, 2002), a crucial event in the formation of phagocytic cups. Additionally, the disruption of the actin network during bacterial engulfment requires depolymerisation of actin at pointed ends by cofilin (Bierne et al., 2001). However, the factor(s) recruiting and activating Arp2/3 and how elongation of filament barbed ends is regulated during *Listeria* internalisation have not been investigated.

The Arp2/3 complex is recruited and activated by proteins of the WASP/WAVE (Wiskott-Aldrich syndrome protein/ WASP family Verprolin-homologous protein) family (reviewed by Takenawa and Miki, 2001; Stradal et al., 2004). Hematopoietic WASP and the ubiquitously expressed N-WASP are activated downstream of Cdc42, by directly interacting with this Rho-GTPase through their CRIB (Cdc42/Rac-interactive-binding) domain (Rohatgi et al., 1999; Takenawa and Miki, 2001). In contrast, WAVE proteins function downstream of Rac in mediating membrane protrusion (Takenawa and Miki, 2001). For instance, inactivation of WAVE2, by dominant-negative approaches (Miki et al., 1998) or by gene disruption in mice (Yamazaki et al., 2003; Yan et al., 2003), results in impairment of cell motility and Rac-dependent lamellipodia. Three WAVE

isoforms have been described, WAVE1, -2 and -3. Although WAVE2 is ubiquitous, WAVE1 is mainly found in the brain and WAVE3 is strictly located in the brain (Suetsugu et al., 1999; Sossey-Alaoui et al., 2003). Functionally, WAVE1 and WAVE2 have been proposed to play differential roles, being required for the formation of PDGF-induced ruffles at the dorsal surface and at peripheral regions of cells, respectively (Suetsugu et al., 2003).

WAVEs do not contain a CRIB motif. Consistently, no direct association of WAVEs with Rac has ever been detected. Two pathways may regulate WAVE proteins. First, WAVE2, among the three WAVEs, was shown to bind activated Rac via the insulin receptor tyrosine kinase substrate p53, IRSp53, which in turn, can stimulate WAVE2 nucleating activity (Miki et al., 2000). Alternatively, WAVE proteins can assemble into a multi-molecular complex, containing Nap1, PIR121/Sra1, HSPC300 and the Abl-kinase-interacting proteins Abi-1 or Abi-2 (Eden et al., 2002; Soderling et al., 2002; Kunda et al., 2003; Innocenti et al., 2004; Steffen et al., 2004). This complex serves as a link to the incoming signal from activated Rac and ensures that the WAVE-mediated actin nucleation is spatially restricted to the cellular leading edge, where actin polymerisation is needed for protrusion. Additionally, the integrity of the WAVE complex is required to prevent degradation of each single component (Kunda et al., 2003; Innocenti et al., 2004; Steffen et al., 2004). To date, the role of WAVE during the bacterial invasion process has not been explored, which prompted us to investigate this issue during both *Listeria* entry and InlB-induced membrane ruffling.

Following Arp2/3 activation and initiation of actin polymerisation, elongation of actin filaments is required to support the extension of membranous structures around entering bacteria. Probable candidates to stimulate this process are the proteins of the Ena/VASP family. Ena/VASP proteins are implicated in cytoskeletal reorganisation during actin-dependent motility processes (for a review, see Krause et al., 2003; Kwiatkowski et al., 2003). Recruitment of Ena/VASP proteins to sites of actin polymerisation is mediated by their conserved N-terminal EVH1 domain, which interacts with target proteins containing the consensus proline-rich motif FPPPP (Niebuhr et al., 1997). In fibroblasts, Ena/VASP proteins localize to dynamic actin-rich structures, such as the leading edge of lamellipodia, where they promote filament elongation. Ena/VASP stimulation of actin-based motility occurs by binding to and protecting the barbed ends of actin filaments from capping proteins, (Bear et al., 2002) or by increasing branch spacing of filaments (Skoble et al., 2001; Samarin et al., 2003).

Here we show that InlB-mediated phagocytosis and ruffling are dependent on WAVE2 in Vero cells and on WAVE1, WAVE2 and N-WASP in HeLa cells. Moreover, InlB-dependent processes are inhibited upon Abi1 inactivation, demonstrating a role of the WAVE complex in bacterial internalisation. We also show that Ena/VASP are essential for InlB-mediated cytoskeleton rearrangements.

Materials and Methods
Bacteria, cells and reagents
The *L. monocytogenes* wild-type EGD strain (Mackaness, 1962) and hyperinvasive strain BUG1641 (Bierne et al., 2001), were grown at

37°C in brain-heart infusion agar (BHI, Difco), supplemented with 8 µg/ml erythromycin for BUG 1641. Vero and HeLa cells were cultured in MEM (Gibco) supplemented with 10% FCS (Sera-Lab), 2 M glutamine, 1 mM sodium pyruvate and 1% non-essential amino acids at 37°C in 10% CO_2. HeLa WAVE2-knockdown and Abi-knockdown cells (Innocenti et al., 2004) were supplemented with 2.5 µg/ml puromycin. Inactivation of Abi1 in Abi1-knockdown cells was verified by immunofluorescence. Approximately 50-70% of cells were knocked down and the remainder re-expressed Abi1.

Transient transfections
Cells (2.5×10^4/ml) were plated on coverslips, transfected 18 hours or 36 hours later using Lipofectamine-plus (Gibco) or Effecten (Qiagen) and used in invasion, ruffling or immunoprecipitation assays 24-48 hours after transfection. The plasmids used were pEGFP-N3-WAVE2 (Suetsugu et al., 2003), pEGFP-N1-NWASP and FLAG-tagged ΔVPH-WAVE2 (Suetsugu et al., 1999), EGFP-Mena, EGFP-FPPPP-mito and EGFP-APPPPmito (Bear et al., 2000). The pEGFP-NWASP-ΔV was generated by cloning a *Bgl*II PCR fragment into *Bam*HI and *Eco*RI sites in pEGFP.

SiRNA assays
Cells were plated 1 day before transfection at 7×10^4 to 1×10^5 cells/well in six- and 24-well plates. They were then transfected using Oligofectamine (Invitrogen) according to the manufacturer's instructions and used for invasion, ruffling and immunoblotting assays, 72 hours later, as described below. The siRNA used were as follows: WAVE1 (Ambion; NM_003931); WAVE2 (Ambion; NM_006990); N-WASP (Santa Cruz; Sc-36006) and VASP (Santa Cruz; sc-29516). The control siRNA was a sequence in WAVE3 (Ambion; NM_006646), as WAVE3 expression is restricted to the brain (Sossey-Alaoui et al., 2003) and a random siRNA from Eurogentec (sense, 5′-CGGGUAGAGCUCUACGCGATT-3′; antisense, 5′-UCGCUAGAGCUCUACCCGTT-3′).

Quantification of phagocytosis
Quantification of invasion efficiency in transfection experiments using dominant-negative constructs were done by directly counting extracellular and intracellular particles by microscopy, as described previously (Bierne et al., 2001). In that case, a hyperinvasive variant of *L. monocytogenes* (BUG 1641) (Bierne et al., 2001) and/or InlB-coated latex beads was used, as entry of the wild-type strain into cells is relatively inefficient. To quantify the entry in siRNA assays, exponentially growing wild-type EGD bacteria were added at a multiplicity of infection of 50 to cells cultivated in 24-well plates, for invasion assays, and in six-well plates to study protein inactivation in cell lysates by immunoblot, and treated with oligofectamine alone or with the indicated siRNA. After 1 hour of infection in MEM, cells were washed several times and MEM-containing gentamicin (10 µg/ml) was added for 2 hours to kill extracellular bacteria. Cells were then washed and lysed in PBS containing Triton X-100 (0.2%) and lysates were plated on brain heart infusion plates for bacterial counting. In each experiment, four wells were treated with the indicated siRNA, one well being used to quantify the number of cells per well and the three other wells for counting intracellular bacteria. Three to six independent experiments were carried out. The score obtained in non-treated cells was arbitrarily set to 100, and the modification of the internalisation index in the treated cells is a relative value. Results were analyzed for statistic significance using the χ^2 goodness-of-fit test ($P<0.0001$).

Antibodies and immunofluorescence analysis
The primary antibodies used were polyclonal antibodies raised against

L. monocytogenes : R11 (Gouin et al., 1995), InlB (Braun et al., 1997), WAVEs (Miki et al., 1998), WAVE1 and WAVE2 (Suetsugu et al., 2003) and VASP (generously provided by U. Walter, Institute of Clinical Biochemistry and Pathobiochemistry, University of Würzburg, Germany) (Reinhard et al., 1992) and monoclonal antibodies against Abi1 (Innocenti et al., 2004), actin (Sigma), InlA (Mengaud et al., 1996), Myc (9E10, SC) and FLAG (Sigma). The secondary antibodies used were Cy3-conjugated, Cy5-conjugated (Jackson IR) or Alexa 546- or Alexa 488-conjugated (Molecular Probes) goat anti-mouse or anti-rabbit IgG antibodies. Total bacteria were visualized in phase contrast and total InlB-beads were detected by their intrinsic fluorescence in the 650-700 nm range. F-actin was labelled with phalloidin-488 or -546 (Molecular probes). Preparations were observed with a Zeiss Axiovert 135 microscope, with the use of plan apochromat 63× and 100× NA 1.4 objective lenses. Image acquisition from the Zeiss was made with cooled CCD camera (Princeton) and the images processed with Metamorph software (Universal imaging corporation).

Ruffle formation assays

Cells were stimulated with 4.5 nM or 6 nM InlB, or with 0.6 nM HGF, as indicated, for 5 minutes and fixed in 3% paraformaldehyde in PBS. Immunolabelling and ruffling quantification were performed as described (Bierne et al., 2001).

Immunoblotting

Cells in six-well plates were lysed in 200 μl RIPA buffer as described (Ireton et al., 1999). Protein concentrations of lysates were determined using a BCA kit (Pierce), and equal quantities of total protein were loaded and separated on 8% SDS-polyacrylamide gels. Transfer of proteins to nitrocellulose membranes, incubation of membranes with antibodies, detection with ECL Plus chemiluminescent systems (Amersham Pharmacia Biotech) and exposure to film were all as described (Ireton et al., 1999). Experiments were performed in duplicate with similar results.

Results

WAVE2 is involved in Met-induced membrane ruffles and phagocytosis in response to InlB stimulation in Vero cells

Vero cells are kidney epithelial cells, in which *Listeria* entry occurs by an InlB/Rac-dependent pathway (Bierne et al., 2001). WAVE and N-WASP intracellular distribution during InlB-induced internalisation and membrane ruffling was examined in these cells, using specific antibodies against N-WASP (anti-N-WASP) and the three isoforms of WAVE (anti-pan WAVE). These antibodies detected N-WASP and WAVEs in Vero cell lysates (Fig. 1A). Notably, Vero cells expressed only the WAVE2 ubiquitous isoform, as detected with specific anti-WAVE1 and WAVE2 antibodies (Suetsugu et al., 1999). The anti-pan WAVE antibody, as it was more sensitive than the WAVE2 antibody in immunofluorescence experiments, was then used to localize WAVE2. In response to soluble or bacterial-bound InlB stimulation, WAVE2 but not N-WASP was recruited to F-actin-rich ruffles (Fig. 1B) or F-actin cups (Fig. 1C) around entering bacteria.

Similar results were obtained using a GFP-WAVE2 construct (Suetsugu et al., 2003). WAVE2-GFP colocalized with F-actin at InlB-induced membrane ruffles (Fig. 2A) and was recruited to phagocytic cups of entering bacteria (Fig. 2B). WAVE2-GFP was not detected underneath extracellular bacteria that were

only adherent (Fig. 2B, bacteria a) but colocalized with F-actin rings around bacteria being internalized, whether still extracellular (Fig. 2B, bacteria c) or intracellular (Fig. 2B, bacteria d). Interestingly, WAVE recruitment could sometimes be detected beneath extracellular bacteria that were not associated with actin cups, suggesting that WAVE translocated to the entry site early in the process (Fig. 2B, bacteria b). Conversely, N-WASP-GFP (Suetsugu et al., 1999) was never localized to entering bacteria (Fig. 2B).

To address the role of WAVE2 in InlB-induced actin rearrangements, we used a WAVE2-ΔV construct, which acts as a dominant-negative mutant owing to its deficiency in actin-binding activity (Miki et al., 1998). WAVE2-ΔV inhibited the formation of InlB-mediated ruffles and entry of InlB-beads or bacteria by 70% and 60%, respectively (Fig. 3). In contrast, expression of an N-WASP-ΔV construct in cells did not significantly modify the invasion efficiency. Thus, these results support a role of WAVE2 in the InlB-mediated actin-based process occurring in Vero cells.
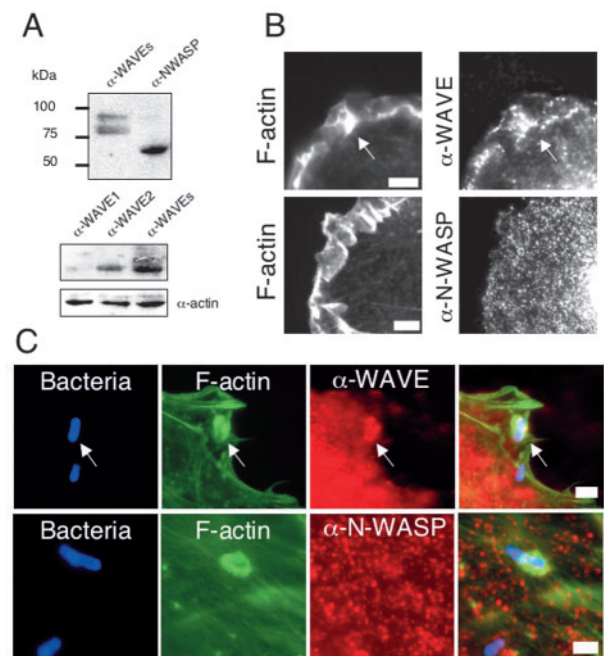

Fig. 1. WAVE2 is localized at membrane ruffles and phagocytic cups in Vero cells. (A) Immunoblots of Vero cell lysates using anti-pan WAVE and anti-N-WASP antibodies (upper panel), and anti-WAVE1, anti-WAVE2 and anti-pan-WAVE antibodies (lower panel). N-WASP is detected as a protein of ~65 kDa and WAVE at ~80 kDa and 90 kDa, the latter reflecting phosphorylated WAVE. Actin was detected as a loading control. (B) Colocalisation of WAVE and actin filaments in areas of ruffling (indicated by arrows) in Vero cells stimulated with 4.5 nM InlB. N-WASP is not localized at InlB-induced membrane ruffles. Cells were stained with anti-pan WAVE or anti-N-WASP antibodies and phalloidin-488. (C) Recruitment of WAVE but not N-WASP to bacteria-induced phagocytic cups in Vero cells. The accumulation of WAVE and actin filaments is indicated by arrows. Cells were stained with anti-pan WAVE or anti-N-WASP antibody (red), anti-InlA to detect bacteria (blue) and phalloidin-488 (green). Bar, 5 μm (B) and 2 μm (C).
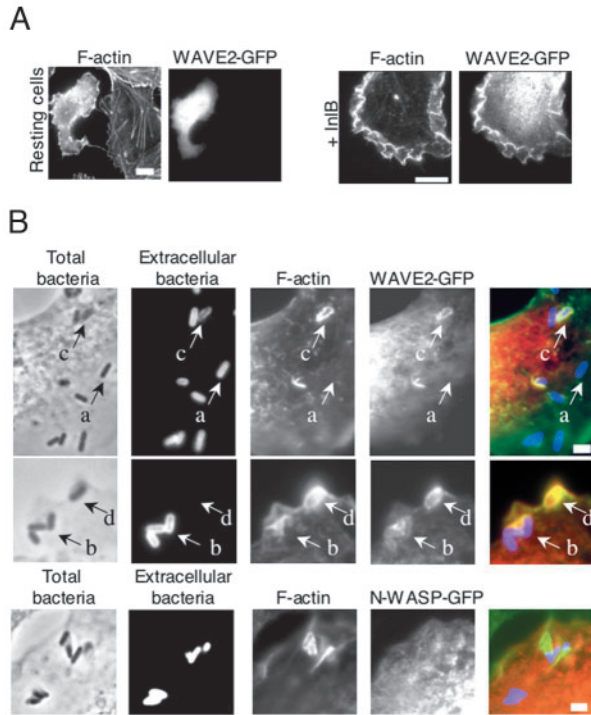
Fig. 2. WAVE2-GFP recruitment during InlB-mediated internalisation and ruffling in Vero cells. (A) Recruitment of WAVE2-GFP at InlB-induced ruffles. Vero cells, transiently transfected with WAVE2-GFP, untreated (resting cells) or stimulated with 4.5 nM InlB (+InlB) and stained with phalloidin-546 to detect F-actin. (B) Recruitment of WAVE2-GFP at bacteria-induced phagocytic cups at different stages of the internalisation process. Vero cells were transiently transfected with WAVE2-GFP or N-WASP-GFP. Total bacteria associated with cells were visualized in phase contrast and bacteria that are fully or partly extracellular were identified by labelling with anti-*Listeria* antibodies prior to cell permeabilisation. F-actin was detected with phalloidin-546. In the merged images (right-hand panels), extracellular bacteria are blue, F-actin is green and the GFP-tagged proteins are red. a, an extracellular bacterium not associated with WAVE-GFP; b, an extracellular bacterium associated with WAVE2-GFP and low amounts of actin filaments; c, an extracellular bacterium; d, an intracellular bacterium in an F-actin-rich phagocytic cup to which WAVE2-GFP localizes. N-WASP-GFP is not recruited to bacteria in F-actin phagocytic cups. Bar, 10 μm (A) and 2 μm (B).

WAVEs and N-WASP are involved in *Listeria*-induced phagocytosis in HeLa cells

Listeria entry is Rac-dependent in Vero cells but requires both Rac and Cdc42 in Ref52 fibroblasts (Bierne et al., 2001). In HeLa cells, in which entry is also mediated exclusively by the InlB protein, a Cdc42-N17 dominant-negative construct is more efficient in inhibiting *Listeria* entry than Rac-N17 (Fig. 3). This puzzling observation prompted us to also address the role of WASP-related proteins in HeLa cells, leading to unexpected findings. First, HeLa cells express not only WAVE2 but also WAVE1 (Fig. 4A), which could not be detected previously using a pan-WAVE antibody (Innocenti et al., 2004). Second, both N-WASP and WAVE proteins (Fig. 4B and data not shown) localized at InlB-mediated phagocytic cups in HeLa cells. N-WASP was recruited at actin-rich phagocytic
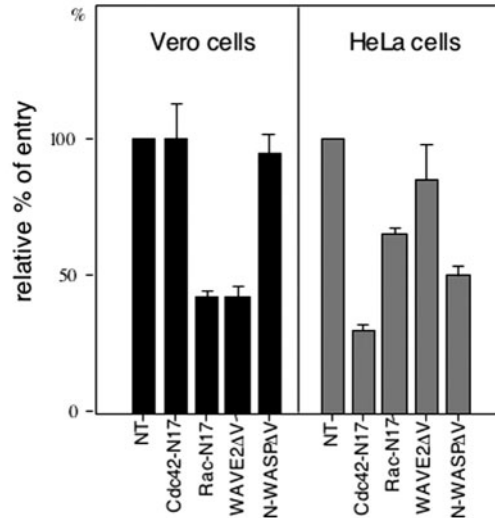

Fig. 3. Inhibition of Rho-GTPases and WASP-related proteins by dominant-negative constructs differentially affects invasion in Vero and HeLa cells. The percentage of intracellular bacteria was quantified in cells, non transfected (NT) or transiently transfected with the indicated constructs. The percentage in non-transfected cells has been assigned a value of 100. The level of entry in transfected cells is given as a relative value. Values are the mean±s.d. of at least three independent experiments.

cups (Fig. 4B, bacteria b), as well as beneath some adherent bacteria (Fig. 4B, bacterium c), suggesting that it translocates to activated receptors at an early step of the process. Moreover, in contrast to that observed in Vero cells, N-WASP-GFP was recruited at the entry site of bacteria (Fig. 4C). Finally, the expression of the dominant-negative construct WAVE2-ΔV only decreased bacterial entry by 15%, whereas expression of the N-WASP-ΔV decreased it by 50% (Fig. 3). Thus, these results supported a role of N-WASP in the InlB-mediated actin-based process occurring in HeLa cells, whereas the role of WAVE proteins remained to be clarified.

To address this point we employed siRNA to knockdown expression of each or both WAVE isoforms in transient transfection assays. The successful inhibition of WAVE1 and/or WAVE2 expression was demonstrated by immunoblotting lysates from HeLa cells transfected with single or double siRNAs (Fig. 5A). In parallel, cells were incubated with *L. monocytogenes* and internalisation was quantified by classical gentamicin bacterial survival assays. WAVE1 knockdown did not affect *Listeria* invasion, whereas WAVE2 knockdown moderately, but significantly, reduced it by 20%. More importantly, the double WAVE1 and WAVE2 knockdowns reduced entry by 50%, suggesting functional redundancy between the two WAVEs (Fig. 5B). To confirm this result, we inactivated WAVE1 in cells where WAVE2 had been stably suppressed (Fig. 4A) (Innocenti et al., 2004). *Listeria* entry into WAVE2-knockdown cells was not significantly decreased as compared to that in the control HeLa cells. This is probably due to the fact that WAVE1 expression was increased in these cells (Fig. 4A), suggesting a compensatory effect. Consistent with this, abrogation of WAVE1 expression by siRNA in WAVE2-knockdown cells resulted in ~65% decrease of *Listeria* entry. Taken together, these results
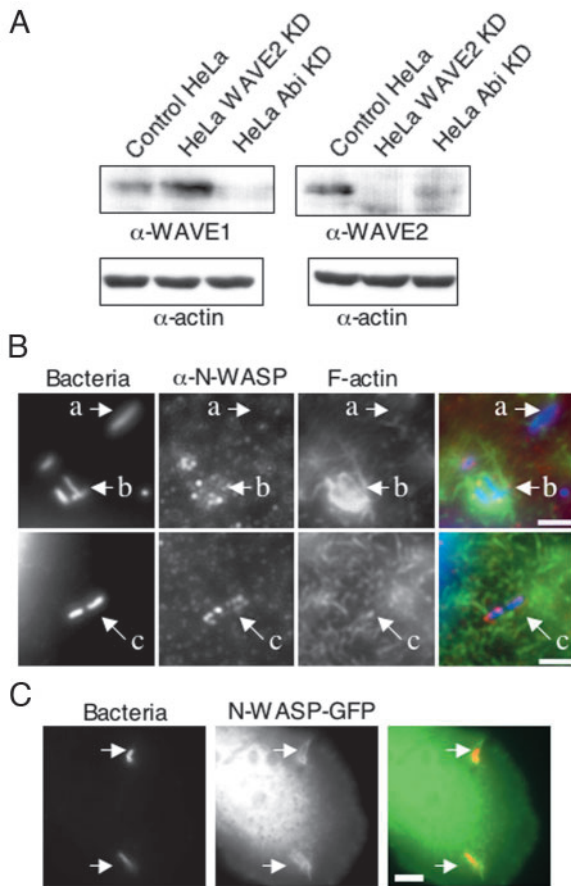
HeLa cells, we investigated whether the actin regulatory pathways involved in membrane ruffling were similar to those promoting internalisation. The dominant-negative constructs Rac-N17, Cdc42-N17 and NWASP-ΔV, but not WAVE2ΔV, impaired ruffling in HeLa cells (data not shown). Notably, purified InlB or HGF induced peripheral ruffles, as in Vero cells, but also dorsal ruffles (Fig. 6A). This might be due to the expression of WAVE1, which has been shown to be required for ruffles forming on the dorsal surface of cells (Suetsugu et al., 2003).

To address the role of WAVEs and N-WASP in ruffling, co-inactivation of the three proteins (WAVE1, WAVE2 and N-WASP) was required. We therefore studied InlB and HGF-mediated ruffle formation in WAVE2-knockdown cells, in which the co-inactivation of WASP-related proteins would be the most efficient. WAVE2-knockdown cells were still permissive for InlB- or HGF-induced ruffle formation (Supplementary material, Fig. S1 and Fig. 6B), showing that WAVE2 inactivation was not sufficient to abrogate this process. Indeed, most stimulated WAVE2-knockdown cells displayed both peripheral and dorsal ruffles. Interestingly, dorsal ruffles were larger and often circular, when compared to those forming in control HeLa cells (Supplementary material, Fig. S1). This result is consistent with WAVE1 overexpression in WAVE2-knockdown cells and a role of WAVE1 in formation of dorsal ruffles (Suetsugu et al., 2003). Notably, both WAVE1 and N-WASP were localized to ruffles in WAVE2-knockdown cells (Supplementary material, Fig. S1).

Inactivation of WAVE1 with siRNA in resting WAVE2-knockdown cells did not modify their morphology (Fig. 6B). Strikingly, upon HGF (Fig. 6B) or InlB stimulation (data not shown), the ability to form characteristic membrane ruffles, with projection of actin-rich membrane lamella, seemed to be abrogated in most cells, whereas cells displayed numerous hair-like structures reminiscent of filopodia and/or microspikes. These structures were formed both at the peripheral and dorsal parts of cells. This result suggested that ablation of both WAVE1 and WAVE2 proteins impairs ruffling and that the remaining actin polymerisation induced upon Met receptors activation was due to N-WASP activity.

To test this, WAVE1 and N-WASP were knocked down by siRNA in WAVE2-knockdown cells. In resting cells, this treatment did not affect the morphology of the cells. In HGF- or InlB- stimulated cells, we could not detect any membrane ruffle structures. Notably, these triple knockdown cells exhibited alterations in the surface membrane, highly reminiscent of those occurring upon cell contraction (Fig. 6B). These results suggested that the triple inactivation of WAVE1, WAVE2 and N-WASP might specifically abolish the capacity of HeLa cells to form actin-rich membrane protrusions in response to InlB or HGF, leaving the machinery promoting cell retraction unaffected.

Abi1 is required for InlB-induced membrane ruffling and internalisation

Abi1 plays a crucial role in the formation and activation of the WAVE signalling complex at the leading edge of fibroblast cells (Kunda et al., 2003; Innocenti et al., 2004). This prompted us to investigate a role of Abi1 in Met-induced cytoskeletal rearrangements. We first showed that endogenous Abi1 was
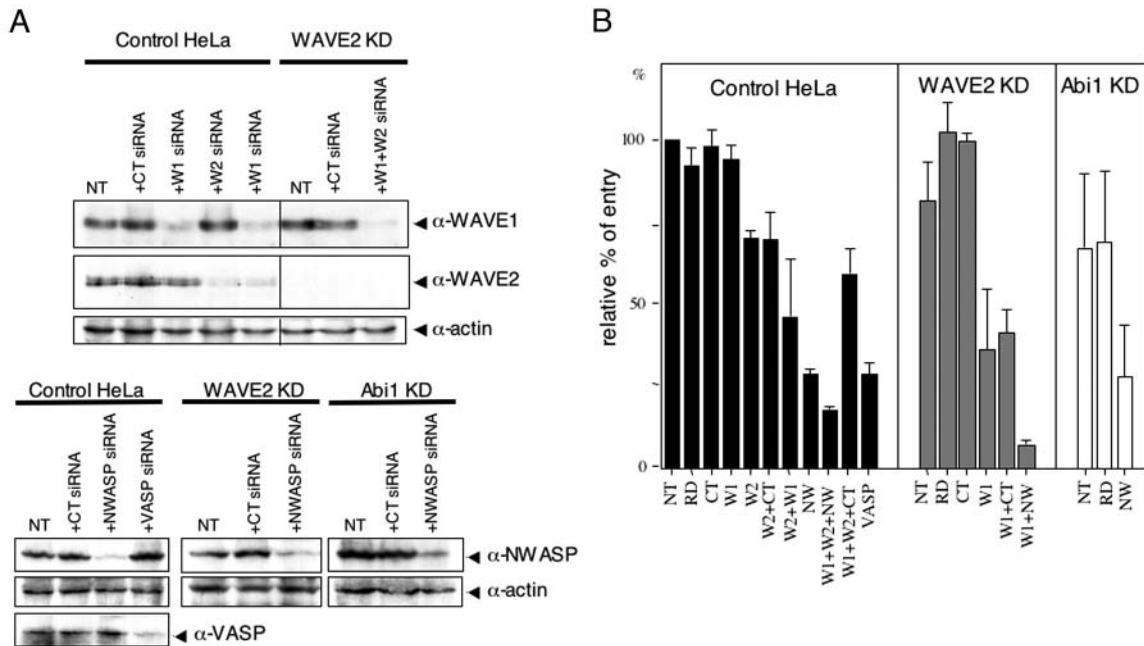

Fig. 4. Expression of WASP-related proteins in HeLa cells and recruitment of N-WASP at phagocytic cups. (A) HeLa, WAVE2-knockdown (KD) and Abi1-knockdown cell lysates were subjected to western blotting with anti-WAVE1, anti-WAVE2 and anti-actin. WAVE1 is overexpressed in WAVE2-knockdown cells. WAVE1 and WAVE2 protein amounts are strongly reduced in Abi1-knockdown cells. (B) N-WASP is localized at phagocytic cups in HeLa cells. Cells were stained with anti-N-WASP antibodies, DAPI to detect bacteria, and phalloidin-488 (red, blue and green, respectively, in the merged images in the right-hand panels). a, a bacterium not associated with N-WASP; b, a bacterium associated with N-WASP and F-actin-rich phagocytic cups; c, a bacterium associated with N-WASP and no actin filaments. (C) NWASP-GFP is recruited at bacterial entry site (arrows). Bacteria are labelled with DAPI. Bar, 2 μm.

establish the involvement of WAVE proteins in *Listeria* entry and demonstrate that WAVE1 and WAVE2 act redundantly.

Next, we studied the effect of N-WASP inactivation by siRNA (Fig. 5A). N-WASP knockdown reduced entry by 70%, strengthening the role of N-WASP in *Listeria* invasion of HeLa cells (Fig. 5B). More importantly, silencing of both WAVE isoforms together with N-WASP led to additive inhibitory effects and strongly decreased bacterial entry (Fig. 5B), thus supporting the notion that WAVE and N-WASP cooperate in promoting actin assembly at a phagocytic cup.

WAVE proteins and N-WASP cooperate to promote InlB- or HGF-induced membrane ruffling in HeLa cells

To generalize our findings to another Met-induced process in

Fig. 5. WAVE proteins, N-WASP, Abi-1 and VASP siRNA knockdown block *Listeria* entry into HeLa cells. (A) Silencing of WASP-related proteins and VASP by siRNA. Suppression of gene expression was analysed in control HeLa or in WAVE2-knockdown or Abi1-knockdown cells, by immunoblotting with antibodies against WAVEs isoforms, N-WASP, VASP or actin. (B) Invasion assays in siRNA-transfected cells. The percentage of intracellular bacteria were quantified by gentamicin assays in cells, non-transfected (NT) or transiently transfected, or co-transfected, with the indicated siRNA. The percentage in non-transfected cells was assigned a value of 100. The level of entry in transfected cells is given as a relative value. Values are the mean±s.d. of at least three independent experiments. CT, control siRNA; NW, N-WASP; Rd, random siRNA; W1, WAVE1; W2, WAVE2.
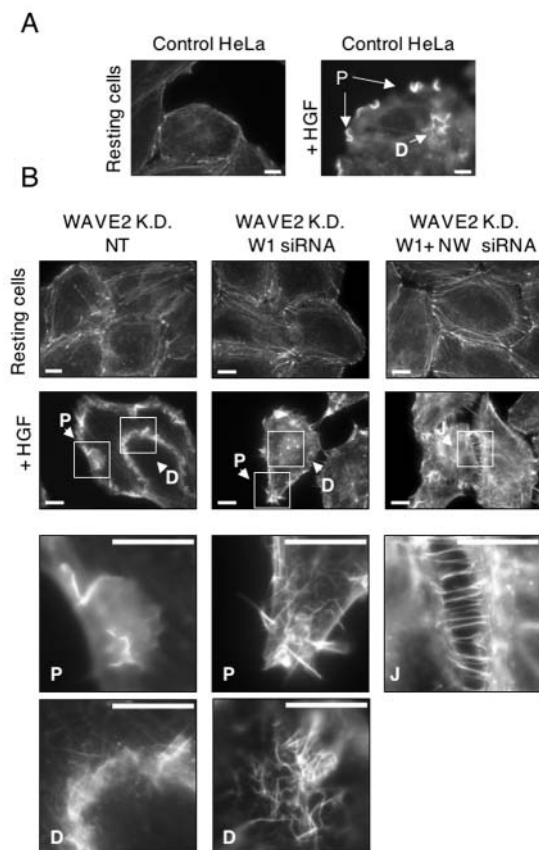
recruited at InlB-membrane ruffles and at phagocytic cups in both Vero cells and HeLa cells (Fig. 7A and data not shown). Then, to investigate a potential role of Abi1 in Rac-dependent actin remodelling, we used Abi1-knockdown HeLa cells, in which Abi1 protein expression had been stably suppressed (Innocenti et al., 2004). In these cells, ablation of Abi1 leads to the destabilisation of the WAVE complex and degradation of WAVE (Innocenti et al., 2004). In agreement with these previous reports, blockade of Abi1 resulted in a reduction of the amount of WAVE1 and WAVE2 protein (Fig. 4A).

In Abi1-knockdown cells, the percentage of cells displaying at least one ruffle upon InlB stimulation was decreased (20±4%; Fig. 7B) when compared to that in HeLa control cells (68±15%). InlB-mediated internalisation of bacteria was also

Fig. 6. Inactivation of WAVE1 or co-inactivation of WAVE1 together with N-WASP in WAVE2-knockdown cells profoundly affects HGF-induced actin cytoskeleton rearrangements. (A) HGF (0.6 nM) stimulation induces formation of peripheral (P) and dorsal (D) ruffles in HeLa cells. (B) WAVE2 knockdown (K.D.) HeLa cells were non-transfected (NT) or transfected with WAVE1 siRNA (W1) or co-transfected with WAVE1 and N-WASP siRNA (W1+NW), and stimulated or not with HGF, fixed and stained with phalloidin-488 to detect F-actin. Resting cells display no apparent morphological alterations. Ruffles are mainly formed in WAVE2- knockdown cells. Filopodia are mainly formed in WAVE2- knockdown cells treated with WAVE1 siRNA. Cells detach from each other in WAVE2-knockdown cells treated with WASP1 + NWASP siRNA. Squared regions indicate the position of the fields magnified below. D, dorsal; J, cell junctions; P, peripheral. Bar, 10 μm.
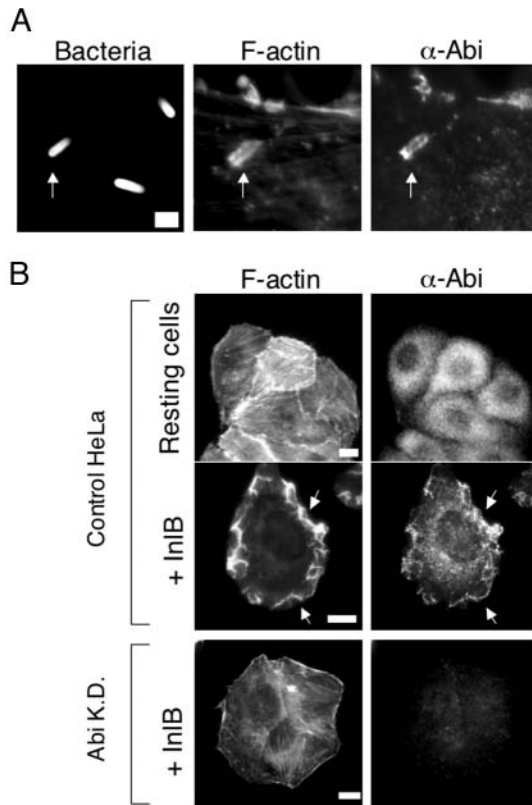
Fig. 7. A role for Abi1 in InlB-induced processes. (A) Colocalisation of Abi1 with F-actin at phagocytic cups. Vero cells were stained with Abi1 and anti-*Listeria* antibodies and phalloidin-488. Arrows show recruitment of endogenous Abi1 at an F-actin-rich phagocytic cup. (B) Abi1 gene silencing impairs the formation of InlB-induced membrane ruffles. Control HeLa or Abi-knockdown cells were untreated (resting cells) or stimulated with 4.5 nM InlB (+InlB) and stained with Abi1 antibody and Phalloidin-488 to detect F-actin. Abi1 colocalizes with F-actin at membrane ruffles (arrows). Ruffles are inhibited in Abi-knockdown cells. Bar, 2 μm (A) and 10 μm (B).

Fig. 8. VASP and Mena-GFP recruitment at bacteria-induced phagocytic cups. (A) Cells were stained with anti-InlA antibody to detect bacteria (blue), Phalloidin-488 to detect F-actin (green) and anti-VASP antibody (red). (B) Overexpression of Mena increases the F-actin content of phagocytic cups. Vero cells transiently transfected with Mena-GFP were stained with anti-*Listeria* antibody (blue) and phalloidin-546 to detect F-actin (green). Mena-GFP is shown in red. Phagocytic cups at the site of entry present a thicker actin network (arrows), magnified in the boxed region. Merged images are shown in bottom right-hand panels. Bar, 1 μm (A) and 2 μm (B).

significantly decreased upon siRNA-mediated ablation of Abi1, albeit to a lesser degree than in cells inactivated for both WAVE1 and WAVE2 protein expression by siRNA (Fig. 5B). This difference may be due to a higher residual expression of WAVE proteins in Abi1-knockdown cells. Indeed, while WAVE2 was not detectable in WAVE2-knockdown cell extracts, a faint band remained in Abi1-knockdown cell extracts (Fig. 4A and Fig. 5A). As expected from our previous results, silencing N-WASP with siRNA in Abi1-knockdown cells (Fig. 5A) led to an additive inhibitory effect on *Listeria* entry (Fig. 5B). Taken together, these results show that Abi1 is involved in Met-induced membrane ruffles and phagocytic cup formation.

Ena/VASP proteins are required for formation of InlB-induced membrane ruffling and internalisation

VASP has been localized to lamellipodia (Reinhard et al., 1992; Rottner et al., 1999; Nakagawa et al., 2001) and in FcγR-dependent phagocytic cups in macrophages (Coppolino et al., 2001). We therefore examined whether it was recruited at InlB-
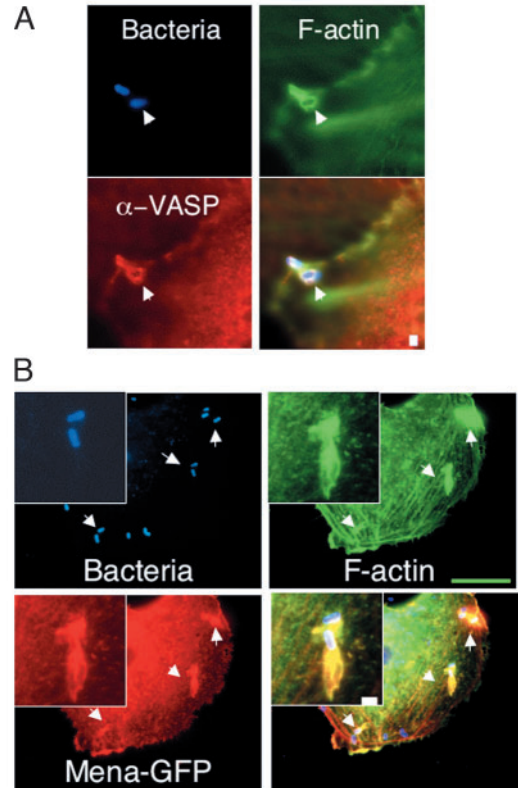
induced ruffles and during the InlB-mediated entry process. VASP localized at InlB-induced actin rich membrane ruffles (Supplementary material, Fig. S2) and at bacterial phagocytic cups (Fig. 8A), both in Vero and HeLa cells.

Upon expression in Vero cells, Mena-GFP was also recruited at InlB-induced ruffles (Supplementary material, Fig. S2) or at phagocytic cups (Fig. 8B). Strikingly, overexpressing Mena-GFP induced a dramatic change in the shape of the F-actin meshwork at the entry site of bacteria, with the appearance of large and abnormal actin foci beneath bacterial bodies. These observations are consistent with a stimulation of the actin filament elongation, as previously proposed (Bear et al., 2002; Samarin et al., 2003).

To determine whether Ena/VASP proteins were involved in InlB-induced entry and ruffling, we inactivated Ena/VASP by two approaches, sequestration of Ena/VASP proteins at the surface of mitochondria (Bear et al., 2000) and RNAi. The EVH1 domain of Ena/VASP efficiently binds FPPPP motifs, which are found in the central part of the *Listeria* protein ActA. When expressed in cells together with the C-terminal domain of ActA, which acts as a mitochondrial targeting domain, the FPPPP motifs of ActA protein sequester Ena/VASP to
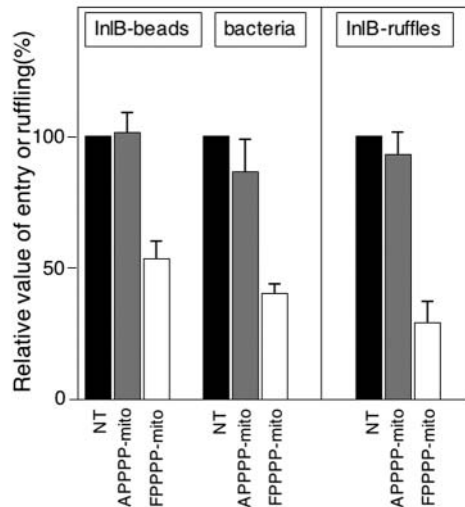
Fig. 9. Inhibition of invasion and ruffling upon Ena/VASP sequestration. The percentage of intracellular InlB-coated latex beads or bacteria, as well as the percentage of cells forming ruffles upon InlB stimulation, was determined in Vero cells, non transfected (NT) or transiently transfected with APPPP-Mito or FPPPP-mito. The percentage in non-transfected cells has been assigned a value of 100. The level of entry or of ruffling in transfected cells is given as a relative value. Values are the mean±s.d. of at least three independent experiments.

Fig. 10. Modulation of Ena/VASP activity affects the structure of InlB-mediated phagocytic cups. Vero cells, non transfected (NT) or transiently transfected with Mena-GFP, FPPPP-mito-GFP or APPPP-mito-GFP, were incubated with InlB-coated beads. In the merged images, F-actin is green, GFP is red, and InlB-beads or bacteria are blue. In cells overexpressing Mena (column 2), phagocytic cups around InlB-coated beads (in blue) present a thicker actin network (large arrow) compared to controls (actin cups in non-transfected cells; column 1). Conversely, in cells in which Ena/VASP is sequestered at the mitochondria (FPPPP-Mito, column 3), phagocytic cups seem to contain a less dense actin meshwork (thin arrows) when compared to controls (non-transfected cells, NT, column 1 and APPPP-Mito, column 4). Bar, 2 μm.

mitochondria. A mutation in the FPPPP motif (F→A) abolishes this effect. Sequestration of Ena/VASP to the mitochondrial surface inhibited ruffling and entry of bacteria, as well as that of InlB-coated latex beads, which are efficiently internalized in the absence of any other listerial factor (Bierne and Cossart, 2002) (Fig. 9). Similar results were obtained in HeLa cells, following inactivation of VASP expression by RNAi (Fig. 5A). Strikingly, VASP knockdown resulted in inhibition of *L. monocytogenes* entry by 70% (Fig. 5B). Thus, Ena/VASP proteins are important in Met receptor-mediated actin remodelling.

We compared the effect of overexpressing or sequestrating Ena/VASP proteins on the shape of actin phagocytic cups during internalisation of InlB beads. Expression of Mena-GFP stimulated actin polymerisation at the entry site of InlB beads, often with the appearance of large actin rings beneath particles, similar to those observed with bacteria. Conversely, in cells expressing FPPPP-Mito, phagocytic cups apparently contain less or shorter actin filaments when compared to non-transfected or APPPP-Mito-expressing cells (Fig. 10). These results are consistent with the reported role of Ena/VASP in enhancing actin-based motility (Bear et al., 2002; Samarin et al., 2003) and further support the notion that Ena/VASP proteins are recruited to Met signalling complexes.

Discussion

In this study we report the role of the WAVE complex and of Ena/VASP proteins in a pathogen-induced phagocytosis process and in the HGF-receptor signalling pathway. Moreover, we demonstrate that InlB-mediated signalling to the actin cytosk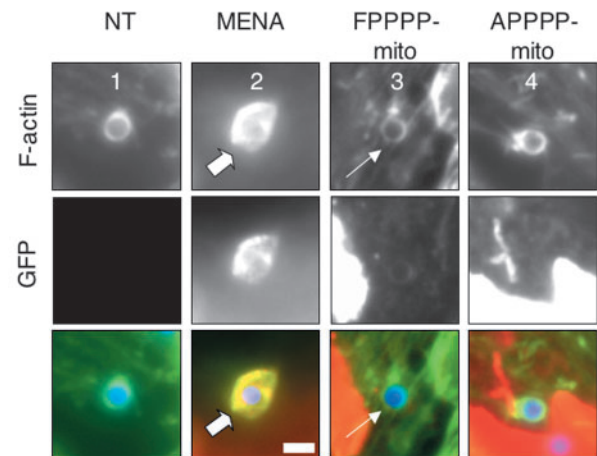eleton leading to *Listeria*-induced phagocytosis is cell-type dependent and may also involve N-WASP. Using the purified *Listeria* invasion protein InlB we establish that Met-induced membrane ruffling and internalisation use the same components of the actin cytoskeleton machinery.

Requirement for WASP-related proteins in *Listeria* internalisation is cell-type dependent

Listeria entry into kidney epithelial Vero cells is dependent on the small GTPase Rac and not on Cdc42 (Bierne et al., 2001). Conversely, it relies on both Rac and Cdc42 in the cervical carcinoma cell line HeLa. We thus used these two epithelial cell lines as model systems to explore cell type differences in signalling to the actin cytoskeleton. Several lines of evidence suggest that WAVE2 is necessary and sufficient to activate Arp2/3-dependent actin polymerisation required for *Listeria*-induced phagocytosis in Vero cells. Consistently, WAVE2 is specifically recruited to *Listeria* phagocytic cups and a WAVE2 deletion mutant, deficient in actin binding, acts as a dominant-negative inhibitor of internalisation. In HeLa cells, ablation of WAVE2 expression by RNAi approaches also impaired entry, albeit only moderately. However, in contrast to Vero cells, HeLa cells express not only WAVE2 but also substantial amounts of WAVE1 that may compensate for WAVE2 ablation. Accordingly, WAVE1 expression is augmented in WAVE2-knockdown cells, where WAVE2 expression had been stably suppressed. Ablation of WAVE1 and WAVE2 expression in HeLa cells by RNAi impairs *Listeria* entry by 50%, indicating that efficient phagocytosis is a WAVE-dependent process and that WAVE1 and WAVE2 exert redundant functions in

promoting entry. N-WASP appears to play no role in internalisation in Vero cells. Conversely, N-WASP is recruited at phagocytic cups and its ablation by RNAi or by a dominant-negative construct impairs entry in HeLa cells. Together these results indicate that the actin machinery required to initiate membrane protrusion is cell-type dependent.

Cooperation between Rac/WAVE and Cdc42/N-WASP pathways is required for ruffles and phagocytic cup formation in HeLa cells

It is now well established that WASP and N-WASP activities lead to formation of filopodia downstream of Cdc42 and WAVEs are involved in lamellipodia and ruffle formation downstream of Rac (for a review, see Hall, 1998; Takenawa and Miki, 2001; Stradal et al., 2004). Few reports describe a role of Cdc42 or of N-WASP activity in ruffles (Miki et al., 1996; Nakagawa et al., 2001; Ward et al., 2004). By using multiple knockdown experiments with RNAi, we show here that InlB-induced membrane ruffles and phagocytosis require both Rac/WAVE and Cdc42/N-WASP pathways in HeLa cells. Membrane ruffles are complex structures, containing both WAVE-dependent membrane lamella and N-WASP-dependent filopodia. Co-inactivation of WAVE1, WAVE2 and N-WASP leads to additive inhibitory effects on *Listeria* entry in HeLa cells, suggesting that as for ruffling, membrane extension at phagocytic cups is driven by WAVE-dependent lamella coupled to N-WASP-dependent filopodia, resulting in a structure related to Fc receptor-mediated pseudopodia (Swanson and Baer, 1995; Bierne and Cossart, 2002).

Cross-talk between Cdc42 and Rac has been clearly established, indicating a hierarchical link between these two GTPases (Nobes and Hall, 1999). It is likely that in HeLa Cdc42/N-WASP and Rac/WAVE pathways are coordinately required to mediate a number of actin remodelling events mediating *Listeria* invasion. In contrast, Cdc42 and N-WASP are dispensable for *Listeria* entry in Vero cells. The molecular mechanisms underlying these differences are not clear at the moment. One possibility is that a different set of signalling molecules may result in differential amplitude and duration of Rac and Cdc42 activation in different cell lines. For instance, InlB activates PI 3-kinase (Ireton et al., 1999), which acts upstream of Rac (Welch et al., 2003). Interestingly, PI 3-kinase inhibitors are more efficient in inhibiting *Listeria* entry in Vero cells than in HeLa cells (H.B. and P.C., unpublished results), suggesting that InlB-induced activation of PI 3-kinase, and therefore of Rac, might be critical in Vero cells. In that case, sustained Rac activation may overcome a requirement for Cdc42. This hypothesis deserves further investigation.

Differences may also result from the nature of the guanine-nucleotide-exchange factors (GEF) that activate Rho-GTPases upon InlB/HGF stimulation, and which are still unknown. A role for Vav2 is unlikely, as a dominant-negative mutant does not block InlB-mediated entry or ruffling (H.B., P. Mandin and P.C., unpublished).

A role for Abi1 and the WAVE complex in *Listeria* internalisation

In this study we have searched for a link between Rac and WAVE activation downstream of Met. A role for IRSp53 in Rac-induced cytoskeleton rearrangements upon InlB stimulation is unlikely, as a IRSp53ΔSH3 dominant-negative mutant, which cannot bind WAVE2, does not block InlB-induced membrane ruffles or phagocytosis either in Vero or HeLa cells (H.B. and P.C., unpublished results). Moreover, although IRSp53 binds only to WAVE2, InlB-induced rearrangements still occur in WAVE2-deficient HeLa cells. In contrast, our study supports a role for Abi1 in the formation and activation of the WAVE complex following Met activation. Abi1 localizes at InlB-induced membrane ruffles and at *Listeria*-induced phagocytic cups both in Vero and HeLa cells. Moreover Abi1 ablation in HeLa cells by RNAi induces the degradation of WAVE1 and WAVE2, indicating that Abi1 is essential for the stability of WAVE complexes, as previously reported (Innocenti et al., 2004). Finally removal of Abi1 impairs *Listeria* entry and ruffling. Recently, two other components of the WAVE complex, NAP1 and Sra1/PIR121, were shown to participate in WAVE recruitment to activated Rac (Steffen et al., 2004). Thus, a role for these proteins in InlB-mediated entry is expected.

The link between Met and Cdc42/N-WASP activation remains unknown. Interestingly, a recent work has revealed the critical role of a newly identified protein, Toca-1, in the Cdc42/N-WASP pathway (Ho et al., 2004). It is proposed that activated Cdc42 interacts with both Toca-1 and the N-WASP-WIP complex and that these interactions activate N-WASP and Arp2/3-dependent actin nucleation. It will be very interesting to address the role of Toca-1 and WIP in Cdc42-dependent *Listeria* phagocytosis.

A role for Ena/VASP in *Listeria* internalisation

We report that activity of Ena/VASP proteins is crucial for efficient internalisation. VASP is recruited at InlB-induced phagocytic cups and its inactivation by RNAi dramatically decreases entry. Moreover, both down- and upregulation of Ena/VASP activity affect the structure of the phagocytic cup. In cells overexpressing MENA-GFP, phagocytic cups present an extended thick actin network, whereas in cells in which Ena/VASP are sequestered at the mitochondrial surface, the actin meshwork at the cups seems to be less dense, as compared to cups in non-transfected cells. These results fit with a model in which inhibition of endogenous Ena/VASP would result in increased filament capping (Bear et al., 2002; Krause et al., 2003) and impairment of the actin cup formation. Conversely, overexpressing Ena/VASP would compete with capping proteins at barbed ends or increase branching, stimulating actin filament elongation beneath the entering particles. Interestingly, we observed similar modifications of the actin network at phagocytic cups, when modulating the extension of actin filaments at pointed ends by deregulation of the cofilin phosphocycle (Bierne et al., 2001). Therefore, the structure of actin cups depends on Ena/VASP and cofilin activity at opposite ends of actin filaments. An intriguing question is how Ena/VASP proteins are spatially and temporally targeted to the site of Met receptor activation. A recent two-hybrid screen has revealed a direct interaction between Mena and Abi1 (Tani et al., 2003). Therefore, Abi1 may favour a co-recruitment of both WAVE and Ena/VASP to Met signalling complexes, leading to actin polymerisation and formation of actin-based structures.

Signalling to the actin cytoskeleton for cell invasion by other pathogens

WASP (Zhang et al., 1999; Lorenzi et al., 2000; Leverrier et al., 2001; Seastone et al., 2001; Pearson et al., 2003) and Ena/VASP proteins (Coppolino et al., 2001) are known to play a role in phagocytosis in phagocytic cells, but they have not yet been clearly associated with induced phagocytic processes triggered by invasive bacteria, such as *Listeria*. Other invasive bacteria also modulate their uptake into cells by controlling actin rearrangements (Cossart and Sansonetti, 2004). *Yersinia pseudotuberculosis*-induced phagocytosis occurs through interaction of the bacterial protein invasin with β1-integrins (for a review, see Isberg et al., 2000). WASP or N-WASP plays no role in internalisation of *Y. pseudotuberculosis* in cells derived from WASP and N-WASP knockout mice (Alrutz et al., 2001). However, by the use of dominant-negative approaches another report indicates that invasin-mediated phagocytosis of *Y. pseudotuberculosis* into HeLa cells involves N-WASP and not WAVE2 (McGee et al., 2001). As shown here, the use of RNAi strategies should clarify this issue. *Shigella* and *Salmonella* enter epithelial cells by a 'trigger' mechanism achieved by delivery of bacterial proteins into the host cytosol and associated with formation of cellular projections and cytoskeleton reorganisation (for a review, see Tran Van Nhieu et al., 2000; Zhou and Galan, 2001). In these cases, a role for a WASP family-dependent mechanism has not been reported. However, cortactin is recruited at the entry site of *Shigella* and is proposed to activate the Arp2/3 complex (Bougneres et al., 2004), as may also be the case for the internalin/E-cadherin pathway (S. Soussa and P.C., unpublished), the other pathway of entry of *L. monocytogenes* (Cossart et al., 2003). Finally, pathogen-induced invasion processes also display a different requirement for Rho-GTPases (Cossart and Sansonetti, 2004). For instance, in contrast to *L. monocytogenes*, *Rickettsia conorii* internalisation into Vero cells is Cdc42- and not Rac-dependent (Martinez and Cossart, 2004). More subtle differences can occur between different species inside a genus. For example, *Chlamydia trachomatis* invasion of HeLa cells requires only Rac (Carabeo et al., 2004), whereas *Chlamydia caviae* invade these cells by Rac- and Cdc42-dependent pathways (Subtil et al., 2004). The reasons for such differences are unknown.

All these studies point to the existence of controlled exploitations of the cell machinery by bacterial pathogens. Variation in signalling pathways induced by pathogens in different cell lines might have biological consequences. The challenge will be to break away from established cell culture systems to directly dissect these events in infected tissues explants and thereby improve our knowledge of signalling as it relates to pathogenesis.

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