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Contribution of Ena/VASP Proteins to Intracellular Motility of *Listeria* Requires Phosphorylation and Proline-rich Core but Not F-Actin Binding or Multimerization

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The *Listeria* model system has been essential for the identification and characterization of key regulators of the actin cytoskeleton such as the Arp2/3 complex and Ena/vasodilator-stimulated phosphoprotein (VASP) proteins. Although the role of Ena/VASP proteins in *Listeria* motility has been extensively studied, little is known about the contributions of their domains and phosphorylation state to bacterial motility. To address these issues, we have generated a panel of Ena/VASP mutants and, upon expression in Ena/VASP-deficient cells, evaluated their contribution to Ena/VASP function in *Listeria* motility. The proline-rich region, the putative G-actin binding site, and the Ser/Thr phosphorylation of Ena/VASP proteins are all required for efficient *Listeria* motility. Surprisingly, the interaction of Ena/VASP proteins with F-actin and their potential ability to form multimers are both dispensable for their involvement in this process. Our data suggest that Ena/VASP proteins contribute to *Listeria* motility by regulating both the nucleation and elongation of actin filaments at the bacterial surface.

INTRODUCTION

A crucial step in the life cycle of intracellular pathogens such as the Gram positive bacterium *Listeria monocytogenes* is their ability to recruit components from the host actin cytoskeleton to their surface. These components are then rearranged into phase-dense actin comet tails that are required for the intracellular motility of these parasites and confer on them the ability to directly invade neighboring cells. Because these pathogens use key cytoskeletal components that are essential for actin-based processes such as cell motility, they have inadvertently provided us with a powerful model system to study the molecular mechanisms that control the dynamics of the actin cytoskeleton (Cossart and Bierne, 2001; Frischknecht and Way, 2001).

L. monocytogenes (simply referred to as *Listeria* in the following sections of the text) subvert the host cell actin cytoskeleton through the expression of a single virulence factor, the ActA protein (Domann *et al.*, 1992; Kocks *et al.*, 1992). ActA harbors three major regions that are required for its interaction with cytoskeletal proteins: an amino-terminal actin monomer-binding site, an adjacent positively charged motif, and a central proline-rich region. The positively charged motif binds to, and activates the Arp2/3 complex, a cytoskeletal component that can nucleate actin filaments and is essential for bacterial motility (May *et al.*, 1999; Pistor *et al.*, 2000; Skoble *et al.*, 2000, 2001; Zalevsky *et al.*, 2001). The G-actin–binding site is not required for intracellular bacterial motility, although it plays a role in the Arp2/3-mediated actin filament nucleation in vitro (Pistor *et al.*, 2000; Skoble *et al.*, 2000, 2001). The central proline-rich domain, which includes three to four copies of the E/DFPPPPXD/E motif (Pistor *et al.*, 1995; Smith *et al.*, 1996; Niebuhr *et al.*, 1997), binds to proteins of the Ena/VASP family (Pistor *et al.*, 1995; Smith *et al.*, 1996; Niebuhr *et al.*, 1997; Machner *et al.*, 2001), which includes the mammalian proteins vasodilator-stimulated phosphoprotein (VASP), mammalian Enabled (Mena), Ena-VASP-like (EVL), and the *Drosophila* protein Ena (Gertler *et al.*, 1990, 1996; Halbrugge *et al.*, 1990).

Several lines of evidence support the notion that Ena/VASP proteins are key regulators of the dynamics of the

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actin cytoskeleton. They associate with the surface of motile *Listeria* in an asymmetric manner (Chakraborty *et al.*, 1995; Gertler *et al.*, 1996) and are necessary for efficient *Listeria* motility in both infected cells and cell-free extracts (Smith *et al.*, 1996; Niebuhr *et al.*, 1997; Laurent *et al.*, 1999; Loisel *et al.*, 1999). Ena/VASP proteins localize at subcellular regions where remodeling of the actin cytoskeleton takes place, such as the front of spreading lamellipodia in motile cells (Rottner *et al.*, 1999), tips of growth cone filopodia, focal adhesions, and epithelial cell-cell junctions (Reinhard *et al.*, 1992; Gertler *et al.*, 1996; Lanier *et al.*, 1999; Vasioukhin *et al.*, 2000). In hematopoietic systems, Ena/VASP proteins localize at the immunological synapse in Jurkat T cells and at phagocytic cups during Fcγ receptor-mediated phagocytosis (Krause *et al.*, 2000; Castellano *et al.*, 2001; Coppolino *et al.*, 2001), where they colocalize with the Ena/VASP-binding protein Fyb/SLAP (Krause *et al.*, 2000; Coppolino *et al.*, 2001). In both systems, the displacement of Ena/VASP proteins from these sites inhibits the remodeling of the actin cytoskeleton that accompanies both the formation of immunological synapses and phagocytic cups, suggesting that they are essential for these processes (Krause *et al.*, 2000; Coppolino *et al.*, 2001). Finally, experiments with Ena/VASP-deficient fibroblasts and Rat2 cells in which Ena/VASP proteins were neutralized indicate that these proteins negatively influence random cell motility (Bear *et al.*, 2000). In addition, in vitro they stimulate actin polymerization by shortening the lag phase of actin filament formation (Laurent *et al.*, 1999; Harbeck *et al.* 2000; Lambrechts *et al.*, 2000).

Ena/VASP proteins are characterized by a common tripartite structure. Their N-terminal region, the EVH1 domain, interacts with the motif E/DFPPPPXD/E, which is present in ActA and in the cytoskeletal proteins vinculin, zyxin, palladin, and Fyb/SLAP (Brindle *et al.*, 1996; Niebuhr *et al.*, 1997; Carl *et al.*, 1999; Drees *et al.*, 2000; Krause *et al.*, 2000; Mykkanen *et al.*, 2001). The central domain of the Ena/VASP proteins harbors a proline-rich region that binds to profilin and, in addition, to SH3 and WW domains (Reinhard *et al.*, 1995; Gertler *et al.*, 1996; Ermekova *et al.*, 1997). The C terminus of Ena/VASP proteins binds to F-actin in vitro and is thought to mediate the multimerization of these proteins (Bachmann *et al.*, 1999).

The EVH1 domain is required for targeting Ena/VASP proteins to *Listeria* surface as well as to focal adhesions (Gertler *et al.*, 1996; Niebuhr *et al.*, 1997; Carl *et al.*, 1999). In contrast, little is known about the functions of the other domains and whether the phosphorylation state of Ena/VASP proteins plays a role in bacterial motility. To address these points, we have generated several Mena and VASP mutants that either lack one of these domains or carry mutated phosphorylation sites. We expressed these mutants in Ena/VASP-deficient cells and analyzed their contribution to *Listeria* motility. In a parallel study, the ability of Mena mutants to rescue normal motile properties of this cell line was also evaluated (Loureiro *et al.*, 2002). Our results clearly indicate that the interaction of Ena/VASP proteins with F-actin and their potential ability to form multimers are both dispensable for their function in actin-based *Listeria* movement, whereas the proline-rich region, the putative G-actin binding site, and the Ser/Thr phosphorylation of Ena/VASP proteins are required for efficient *Listeria* motility.

MATERIALS AND METHODS

Cloning of VASP, Mena, and Profilin Constructs

The cloning of all Mena constructs is described in Loureiro *et al.* (2002). Enhanced green fluorescent protein (EGFP)-tagged full-length VASP (Carl *et al.*, 1999) was cloned into the pMSCV vector after introducing *Eco*RI and *Cla*I restriction sites by polymerase chain reaction (PCR) by using the following primers: forward, CGGAATTCGCCACCATGGTGAGCAAGGGC; and reverse, GCATCGATTCAGGGAGAACCCCGCTTCCTCAG.

Mutagenesis of the VASP phosphorylation sites S157, S239, and T278 was done using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The following primers were used (mutated codons are underlined): pMSCV+EGFP-VASP AST (S157A), forward, GCACATAGAGCGCCGGGTCGCCAATGCAGGAGGC; and reverse, GCCTCCTGCATTGGCGACCCGGCGCTCTATGTGC; pMSCV+EGFP-VASP DST (S157D), forward, CATAGAGCGCCGGGTCGACAATGCAGGAGGCC; and reverse, GGCCTCCTGCATTGTCGACCCGGCGCTCTATG; pMSCV+EGFP-VASP SAT (S239A), forward, CTCAGGAAAGTCGCCAAGCAGGAGGAGGCC; and reverse, GGCCTCCTCCTGCTTGGCGACTTTCCTGAG; pMSCV+EGFP-VASP SDT (S239D), forward, CTCAGGAAAGTCGACAAGCAGGAGGAGGCC; and reverse, GGCCTCCTCCTGCTTGTCGACTTTCCTGAG; pMSCV+EGFP-VASP SSA (T278A), forward, GGAGAAGGAAAGCCGCGCAAGTTGGGGAGAAAAC; and reverse, GTTTTCTCCCCAACTTGCGCGGCTTTCCTTCTCC; and pMSCV+EGFP-VASP SSD (T278D), forward, GGAGAAGGAAAGCCGACCAAGTTGGGGAGAAAAC; and reverse, GTTTTCTCCCCAACTTGGTCGGCTTTCCTTCTCC.

EGFP-VASP constructs harboring mutations of both S157 and S239 were generated as follows. EGFP-VASP constructs mutated at S157 or S239 were excised from the pMSCV vector, digested with *Pst*I, and recloned into the pMSCV vector to obtain the desired double mutations.

The triple phosphorylation mutants S157A/S239A/T278A and S157D/S239D/T278D were generated from the corresponding double phosphorylation mutants by site-directed mutagenesis of T278.

Generation of VASP deletion mutants was done using the overlap-extension PCR method using the following primers. First PCR reaction (internal primers; parts of the primers that do not hybridize are underlined): VASP ΔGP_5 (Δ162–186), forward, CTCCAATGCAGGAGGCGGTTTGCCCCCTTCG; and reverse, CGAAGGGGGCAAACCGCCTCCTGCATTGGAG; VASP ΔPRR (Δ 118–122, 162–186, 204–209), forward, GTTGGAAGGAGGTGGGGCACTTCCCACCTGG; and reverse, CCAGGTGGGAAGTGCCCCACCTCCTTCCAAC; forward, GGAGCAGGGGGAGGACTCCCGGCAGCACAG; and reverse, CTGTGCTGCCGGGAGTCCTCCCCCTGCTCC (to generate this mutant we used VASP ΔGP_5 as the template); VASP ΔFAB (Δ259–277), forward, GAGAGTGGTCGAAGCACGCAAGTTGGGGAG; and reverse, CTCCCCAACTTGCGTGCTTCGACCACTCTC; and VASP ΔCoco (Δ352–373), forward, GTGAAACAGGAGCTTCTGAGGAAGCGGGG; and reverse, CCCCGCTTCCTCAGAAGCTCCTGTTTCAC.

First PCR reaction (external primers; used for all deletion mutants) was as follows: forward, GCTGTACAAGTCCGGCCGGACTCAGATCTC; and reverse, GTGGGGTCTTTCATTCCCCCCTTTTTCTGG.

Second PCR reaction was as follows: forward, GCTCAAGCTTAGCAGCCATGAGCGAGACGG; and reverse, CTAAATAAAATCTTTTATTTTATCGATTCAGG.

All VASP deletion mutants were cloned into the pMSCV vector by using *Hin*dIII and *Cla*I. The correct molecular size of all mutants was verified by Western blotting after staining with a monoclonal antibody against green fluorescent protein (GFP).

Cyan fluorescent protein (CFP)-tagged wild-type VASP and VASP-ΔPRR and Profilin II-yellow fluorescent protein (YFP) were generated as follows. Enhanced cyan fluorescent protein (ECFP) was amplified using the following primers: forward, CGGAAT-

TCACCATGGTGAGCAAGGGCGAGG; and reverse, TTCGAAGCTTTGAGCTCGAGATCTGAGTCCG by using pECFP-C1 (CLONTECH, Palo Alto, CA) as the template. The PCR product was then digested with *Eco*RI and *Hin*dIII and cloned into the same restriction sites of pMSCV+EGFP-VASP and pMSCV+EGFP-VASP ΔPRR.

To generate profilin II-YFP, we first amplified profilin II-GFP by using the following primers: forward, ACGCGGCCGCCCTTCCATGGCCGGTTGGCAGAGCTACG; and reverse, CGCAAGCTTTACTTGTACAGCTCGTCCATGCC and profilin II-EGFP as the template. The PCR product was then digested with *Not*I and *Hin*dIII and cloned into the same restriction sites of pML2X. pML2X+profilin II-EYFP was obtained after amplification of EYFP (forward primer, CCGGGATCCACCGGTCGCCACCATGGTGAGC; and reverse primer, CGCGGAAGCTTTACTTGTACAGCTCGTCCATGC; template pEYFP-N1; CLONTECH), digestion with *Bam*HI and *Hin*dIII, and cloning into the same restriction sites of pML2X+profilin II-EGFP.

Bacterial Culture

The wild-type weakly hemolytic *L. monocytogenes* strain EGD (serotype 1/2) and its isogenic *Listeria* mutants ActA5 and ActA12 (Domann *et al.* 1992; Niebuhr *et al.*, 1997; Pistor *et al.*, 2000) were grown in brain heart infusion broth (Difco, Detroit, MI) at 37°C with agitation.

Cell Culture and Infection

MV^{D7} cells and G7 mouse fibroblasts were grown in DMEM supplemented with 15% fetal calf serum, 2 mM L-glutamine, and 50 U/ml mouse interferon-γ at 32°C in the presence of 5% CO_2. All media and supplements were obtained from Invitrogen (Carlsbad, CA). Infection of MV^{D7} cells with *L. monocytogenes* was done according to Sechi *et al.* (1997) by using a final bacterial concentration of 10^9–10^{10} colony-forming units/ml and an incubation time for bacterial entry of 90 min at 37°C.

Cell Transfection and Sorting

MV^{D7} cells were transfected using a retroviral transfection system. Briefly, pMSCV plasmids harboring the EGFP-VASP constructs and the helper plasmid pCL-Eco (Imgenex, San Diego, CA) were introduced into BOSC23 cells by using a calcium phosphate transfection procedure. Two days later, the cell medium containing the retroviral particles released by the BOSC23 cells was collected and used to transfect MV^{D7} cells. Afterward, MV^{D7} cells were sorted according to low, medium, and high levels of EGFP expression using a fluorescence-activated cell sorting (FACS) sorter (MoFlo; Cytomation, Ft. Collins, CO). After cell thawing, the correct expression levels of all GFP-tagged constructs were confirmed using an FACS Calibur device (BD Biosciences, San Jose, CA).

Immunofluorescence Microscopy

Four hours after the beginning of the infection, cells were fixed with 4% paraformaldehyde in cytoskeleton buffer (10 mM MES, 150 mM NaCl, 5 mM EGTA, 5 mM glucose, and 5 mM $MgCl_2$, pH 6.1) for 20 min at room temperature and then extracted with 0.1% Triton X-100 in cytoskeleton buffer for 1 min at room temperature. Bacteria were labeled with the polyclonal antibody K52 followed by Alexa 488-conjugated goat anti-rabbit IgG (Dianova, Hamburg, Germany). The actin cytoskeleton was labeled with Alexa 594-conjugated phalloidin (Molecular Probes, Eugene, OR). Coverslips were mounted in Prolong (Molecular Probes).

Fluorescence Video Microscopy

For fluorescence video microscopy, cells were plated onto 40-mm round coverslips. Four hours after beginning the infection, coverslips carrying infected cells were mounted in a Focht Chamber System (FCS2; Bioptechs, Butler, PA). An objective heater (Bioptechs) was used to eliminate the temperature gradient between chamber and objective. The cells were observed by phase contrast or epifluorescence with an Axiovert 135 TV microscope (Carl Zeiss, Thornwood, NY) equipped with a Plan-Apochromat 100×/1.40 numerical aperture oil immersion objective in combination with 1.6× or 2.5× optovar optics. Images were recorded with a cooled, back-illuminated charge-coupled device camera (TE/CCD-1000 TKB; Princeton Instruments, Trenton, NJ) driven by IPLab Spectrum software (Scanalytics, Fairfax, VA). Digital handling of the images was done using IPLab Spectrum and Adobe Photoshop 5.0 (Adobe Systems, Mountain View, CA).

Analysis of Bacterial Speed

All motile bacteria within a single cell were scored according to the following criteria: 1) they did not interact with other motile or stationary bacteria; 2) they never stopped or started to move during the observation period; and 3) they did not move within cellular extensions (pseudopodia). Paths of motile bacteria (observed in at least 20 infected cells; 3 independent experiments) were generated after marking the bacterial poles proximal to the actin tails using the Dynamic Imaging Analysis system (Solltech, Oakdale, IA). To smooth out sudden speed oscillations, the instantaneous speed of the bacteria was calculated according to the central difference method. Analysis of the bacterial speed was done using MiniTab 10.5 (MiniTab, State College, PA) and DeltaGraph 3.5 (Delta Point, SSPS Inc., Chicago, IL). Because the measured values of *Listeria* speed were not normally distributed as determined using the Anderson-Darling test, we analyzed differences in bacterial speed using the Mann-Whitney nonparametric *U* test and rejected the null hypothesis (the two groups have the same median value, i.e., they are not different) when $p < 0.05$.

RESULTS

Motility of Listeria monocytogenes Is Impaired in MV^{D7} Fibroblasts

An essential prerequisite for studying the function of Ena/VASP protein domains in *Listeria* motility is the availability of a cell line that does not express any of the known Ena/VASP proteins to avoid any interference with the function of the ectopically expressed Mena and VASP mutants. We have recently isolated one clonal cell line (MV^{D7} fibroblasts) from *mena/vasp*-null mouse embryos, which does not express detectable levels of EVL (Bear *et al.*, 2000).

To test whether MV^{D7} cells are suitable for studying the intracellular motility of *Listeria*, we analyzed these cells after infection with the wild-type strain EGD of *L. monocytogenes*. Immunofluorescence microscopy revealed that, in all cells analyzed, *Listeria* induced the formation of very short actin comet tails (Figure 1A). To verify whether the formation of such short tails was due to the lack of Ena/VASP proteins, we infected a fibroblast cell line (G7 cells; Lommel *et al.*, 2001), which, like MV^{D7} fibroblasts, was immortalized with a temperature-sensitive version of the simian virus 40 large-T antigen and grown under the same culture conditions (see MATERIALS AND METHODS). In G7 mouse fibroblasts, which express Ena/VASP proteins (our unpublished data), wild-type *Listeria* were associated with normal actin tails (Figure 1C). The same result was obtained using cell lines such as PtK2 and HeLa (our unpublished data). Overall, these observations suggest that the formation of

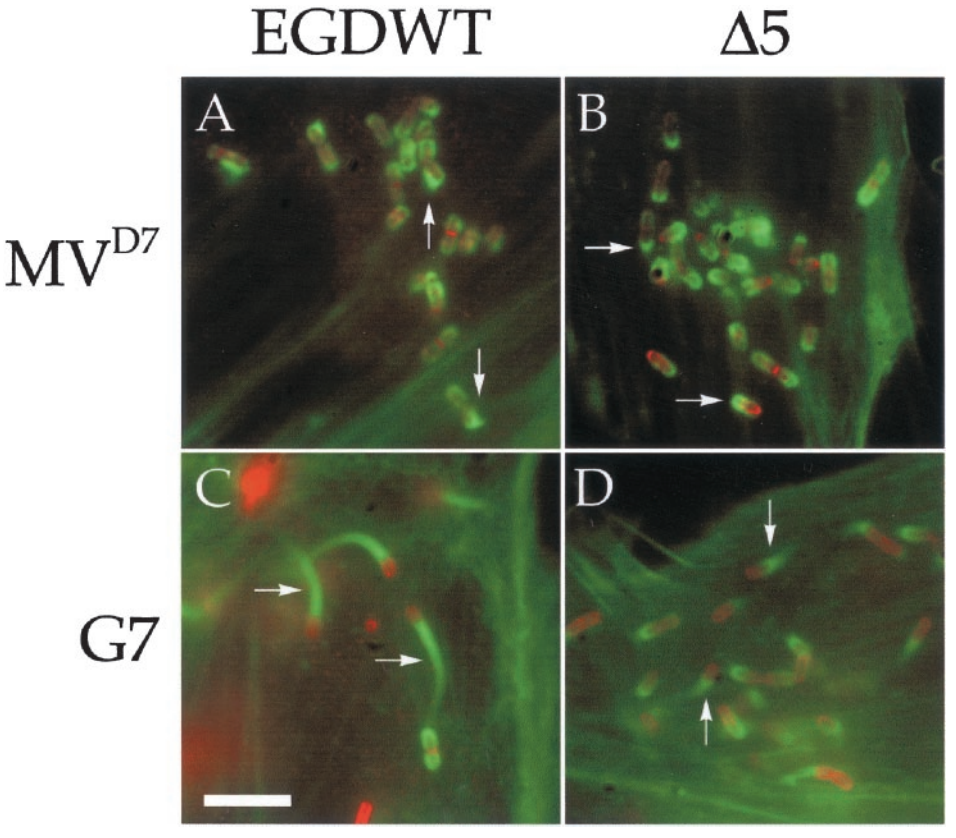

Figure 1. Motility of *L. monocytogenes* is impaired in MV^{D7} cells. MV^{D7} fibroblasts (A and B) and G7 mouse fibroblasts (C and D) were infected with wild-type *Listeria* (A and C) or the *Listeria* mutant Δ5 (B and D), fixed, and stained with antibodies against the bacteria (red) and fluorescent phalloidin (green). In G7 mouse fibroblasts wild-type *Listeria* induced the formation of long actin tails (arrows in C). In contrast, the actin tails induced by these bacteria in MV^{D7} cells were much shorter (arrows in A) and closely resembled those induced by the *Listeria* mutant Δ5 in both cell types (arrows in B and D). Bar, 2 μm.

short tails in MV^{D7} is due to the deficiency in Ena/VASP proteins.

To corroborate this result, we infected both MV^{D7} cells and G7 cells with the *Listeria* mutant Δ5. This mutant expresses on its surface a mutated version of ActA that lacks the central proline-rich region and is, as a consequence, unable to interact with Ena/VASP proteins and therefore leads to the formation of short actin tails (Niebuhr *et al*., 1997). As expected, *Listeria* Δ5 was associated with short actin tails in both normal G7 fibroblasts and Ena/VASP-deficient fibroblasts (Figure 1, B and D).

The visual impression that the intracellular motility of *Listeria* is impaired in MV^{D7} cells was further confirmed by video microscopy. In particular, the average speed of wild-type *Listeria* in MV^{D7} cells was comparable with that of the *Listeria* mutant Δ5 in the same cell line (Figure 2D) and was 5–10 times lower than their average speed measured in G7 fibroblasts and other cell lines (our unpublished data; Niebuhr *et al*., 1997). Thus, MV^{D7} cells represent a suitable system for analyzing the contribution of Ena/VASP protein domains to *Listeria* motility.

Efficient Motility of Listeria in MV^{D7} Cells Is Rescued by Full-Length Ena/VASP Proteins in a Concentration-dependent Manner

Because the impaired movement of *Listeria* in MV^{D7} cells seems to be due to the lack of Ena/VASP proteins, we reasoned that normal bacterial motility could be rescued upon expression of Ena/VASP proteins. We therefore infected MV^{D7} cells with a retrovirus that drives the expression of GFP-tagged full-length Mena or VASP and sorted them by FACS into three populations expressing low, medium, and high levels of the fusion proteins according to the intensity of GFP fluorescence signal (Figure 2A). The relative expression levels of GFP-Mena and GFP-VASP corresponded to 35, 71, and 100% for the low, medium, and high populations, respectively, as calculated after setting the average intensity of the high population to 100%.

These fusion proteins properly localized to subcellular regions in MV^{D7} cells (our unpublished data; Loureiro *et al*., 2002) and at the surface of both nonmotile and motile bacteria (Figure 2, B′ and C′). Moreover, in MV^{D7} fibroblasts expressing high levels of GFP-Mena or GFP-VASP *Listeria* induced the formation of normal actin tails that were indistinguishable from those induced by these bacteria in G7 cells (Figure 2, B and C; compare with Figure 1C).

We then analyzed the bacterial movement in MV^{D7} cells expressing low-to-high levels of the GFP fusion proteins by using video microscopy. As shown in Figure 2D, the enhancement of *Listeria* motility directly correlated with the increase in the cellular levels of the ectopically expressed Ena/VASP proteins (Figure 2D). The expression of high levels of GFP-Mena or GFP-VASP rescued *Listeria* motility equally well (Figure 2D), suggesting that Mena and VASP are interchangeable in this process. Moreover, the average speed of *Listeria* in MV^{D7} fibroblasts expressing high levels

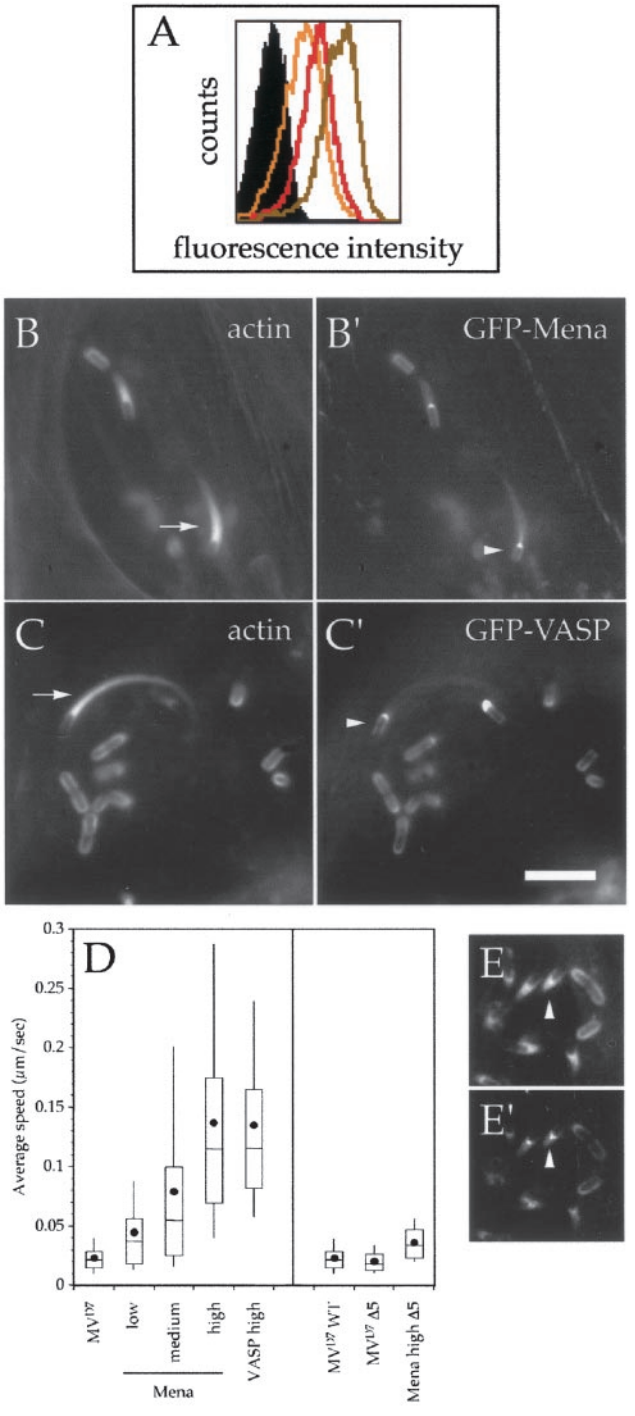

Figure 2. (A) FACS analysis of MV^{D7} cells expressing low (yellow line), medium (red line), and high (light brown line) levels of GFP-tagged Mena and VASP constructs. The black-filled area indicates the background fluorescence of untransfected MV^{D7} cells. (B–C′) The expression of wild-type GFP-Mena and GFP-VASP rescues *Listeria* motility in MV^{D7} cells in a concentration-dependent manner. MV^{D7} cells expressing high levels of wild-type GFP-Mena or GFP-VASP were infected with *Listeria*, fixed, and stained with fluorescent phalloidin. In these cells, *Listeria* recruited GFP-Mena and GFP-VASP at their surface (arrowheads in B′ and C′) and induced the formation of normal actin tails (arrows in B and C; compare with Figure 1). (D) Box and whiskers plots of bacterial speed. Dot indicates the mean, line in the middle of the box indicates the median, top of the box indicates the 75th quartile, whereas the bottom of the box indicates the 25th quartile, and whiskers indicates the 10th and 90th percentiles, respectively. (E–E′) GFP-tagged wild-type Mena and VASP localize at the actin tails induced by the *Listeria* mutant Δ5. MV^{D7} cells expressing high levels of wild-type GFP-Mena were infected with *Listeria* Δ5, fixed, and stained with fluorescent phalloidin. This *Listeria* mutant typically induced the formation of short actin tails (arrowhead in E), which were robustly stained with GFP-Mena (arrowhead in E′). Bar (for B–C′ and E–E′), 2 μm.

of GFP-tagged Mena and VASP was similar to that measured in G7 fibroblasts (our unpublished data).

The rescue of *Listeria* motility in MV^{D7} cells clearly depends on the expression of Ena/VASP proteins as indicated by the observation that the speed of the *Listeria* mutant Δ5 in MV^{D7} cells expressing high levels of GFP-Mena was comparable with its speed measured in G7 cells (Figure 2D; our unpublished data). However, despite the fact that this *Listeria* mutant is unable to recruit Ena/VASP proteins at its surface, its speed in MV^{D7} cells expressing high levels of GFP-Mena was significantly higher than the speed of wild-type *Listeria* in the parental Ena/VASP-deficient cells (Mann-Whitney *U* test; $p < 0.05$; wild type, $n = 31$; Δ5, $n = 28$). Because GFP-tagged Ena/VASP proteins localized at the short actin tails induced by the *Listeria* mutant Δ5 (Figure 2, E and E′), it may be that cytoplasmic Ena/VASP proteins can influence bacterial motility via an EVH1-independent recruitment to actin tails, perhaps mediated by their ability to interact with actin filaments.

Generation of Ena/VASP Mutants and Their Expression in MV^{D7} Cells

Next, we generated a panel of GFP-tagged fusion proteins in which various domains and phosphorylation sites of Ena/VASP proteins were deleted or mutated, respectively (Figure 3A). The introduction of the GFP moiety at the NH_2 terminus in all these fusion proteins did not affect the ability of the EVH1 domain to interact with ActA as indicated by their proper localization at the *Listeria* surface (Figure 2, B′ and C′; Carl *et al.*, 1999). In addition, Western blot analysis showed that all GFP-tagged constructs migrated at the expected molecular size and that they were not degraded when expressed in MV^{D7} cells (Figure 3B), suggesting that neither the presence of GFP nor the deletions or point mutations grossly affected the protein stability. We cannot exclude, however, that these deletions or point mutations may affect the overall folding of the proteins.

As noted above, normal intracellular *Listeria* motility can be rescued by expressing high levels of Ena/VASP proteins. We therefore evaluated the influence of these Ena/VASP mutants on *Listeria* motility in MV^{D7} cells that expressed high levels of these mutant proteins. To this end, MV^{D7} fibroblasts transfected with Ena/VASP mutants were sorted by FACS by using MV^{D7} cells expressing high levels of the corresponding wild-type Ena/VASP protein as reference. As shown in Figure 3C, the overlap between the different pairs of FACS scans clearly indicated that the expression levels of all Ena/VASP mutants were similar to those of the nonmutated counterparts.

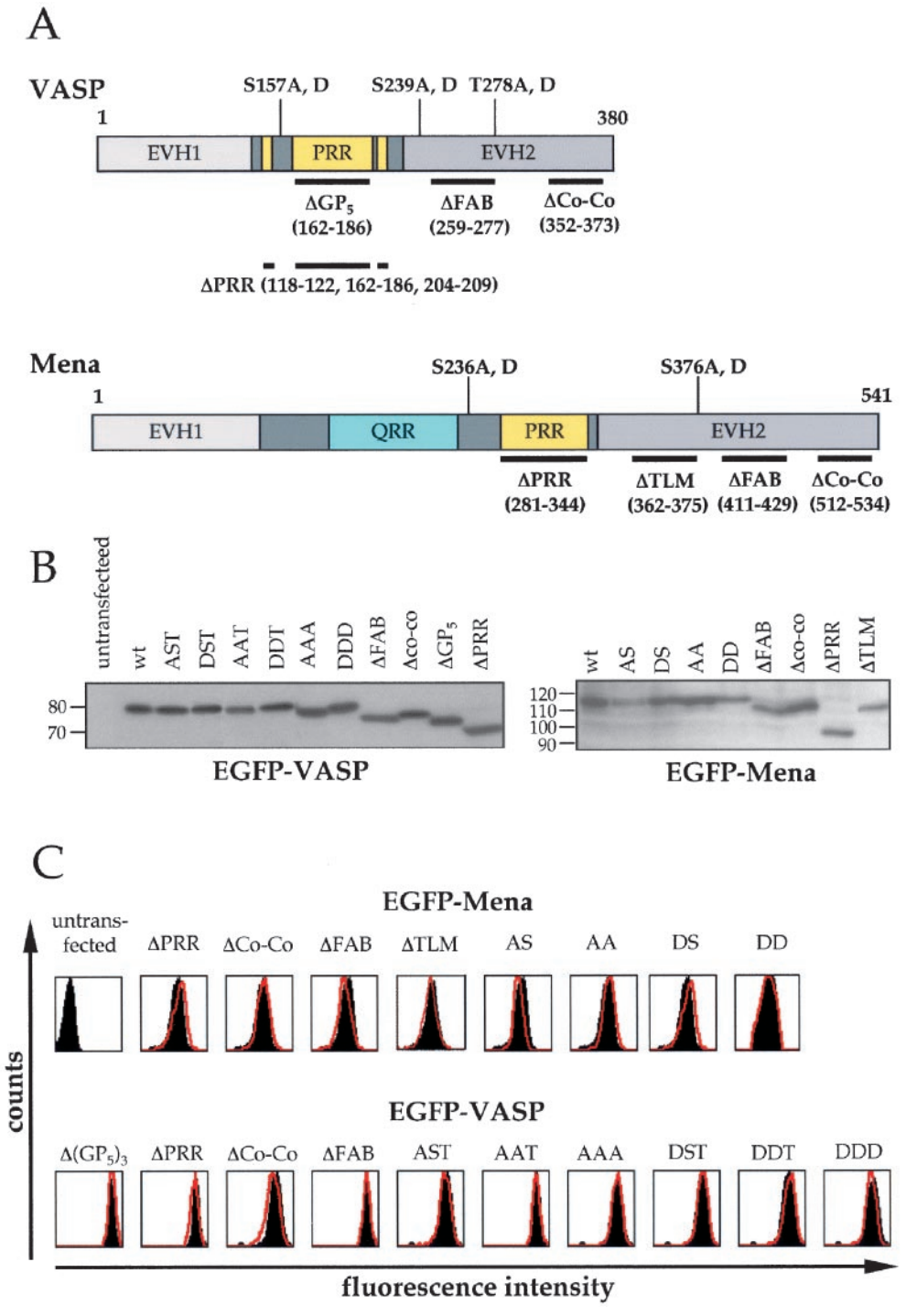

Figure 3. (A) Schematic diagram of Mena and VASP constructs. Deletions (thick lines) and phosphorylation sites are shown at the bottom and top of Mena and VASP cartoons, respectively. EVH1, Ena-VASP homology 1; PRR, proline-rich region; QRR, glutamine-rich region; EVH2, Ena-VASP homology 2. (B) Western blot analysis of GFP-tagged Mena and VASP fusion proteins. Cell lysates of MV^{D7} cells expressing high levels of all Mena, and VASP constructs were resolved by SDS-PAGE, blotted, and probed with an anti-GFP monoclonal antibody. Numbers on the left side of each blot represent molecular weight markers in kilodaltons. (C) Comparison between the expression levels of GFP-tagged wild-type Mena and VASP and their mutated fusion proteins. MV^{D7} cells expressing high levels of wild-type GFP-Mena and GFP-VASP (black-filled areas) were compared with MV^{D7} cells expressing high levels of each of the GFP-tagged Mena and VASP constructs (red lines) by FACS. The overlapping between the black-filled areas and the red lines in each FACS scan indicates that all cellular populations are equivalent with respect to the expression levels of all Ena/VASP constructs.

Proline-rich Region of Ena/VASP Proteins Is Essential for Efficient Listeria Motility

A combination of biochemical, genetic, and cell biological approaches suggests that the interaction between the proline-rich region of Ena/VASP proteins and profilin serves to recruit polymerization-competent actin monomers to sites of actin assembly (Reinhard *et al.*, 1995; Smith *et al.*, 1996; Lanier *et al.*, 1999; Geese *et al.*, 2000).

To study the contribution of this region to *Listeria* motility, we expressed GFP-Mena ΔPRR (for proline-rich region), GFP-VASP ΔGP_5, and GFP-VASP ΔPRR in MV^{D7} cells. The GFP-VASP ΔGP_5 construct lacks the central GP_5 motifs that

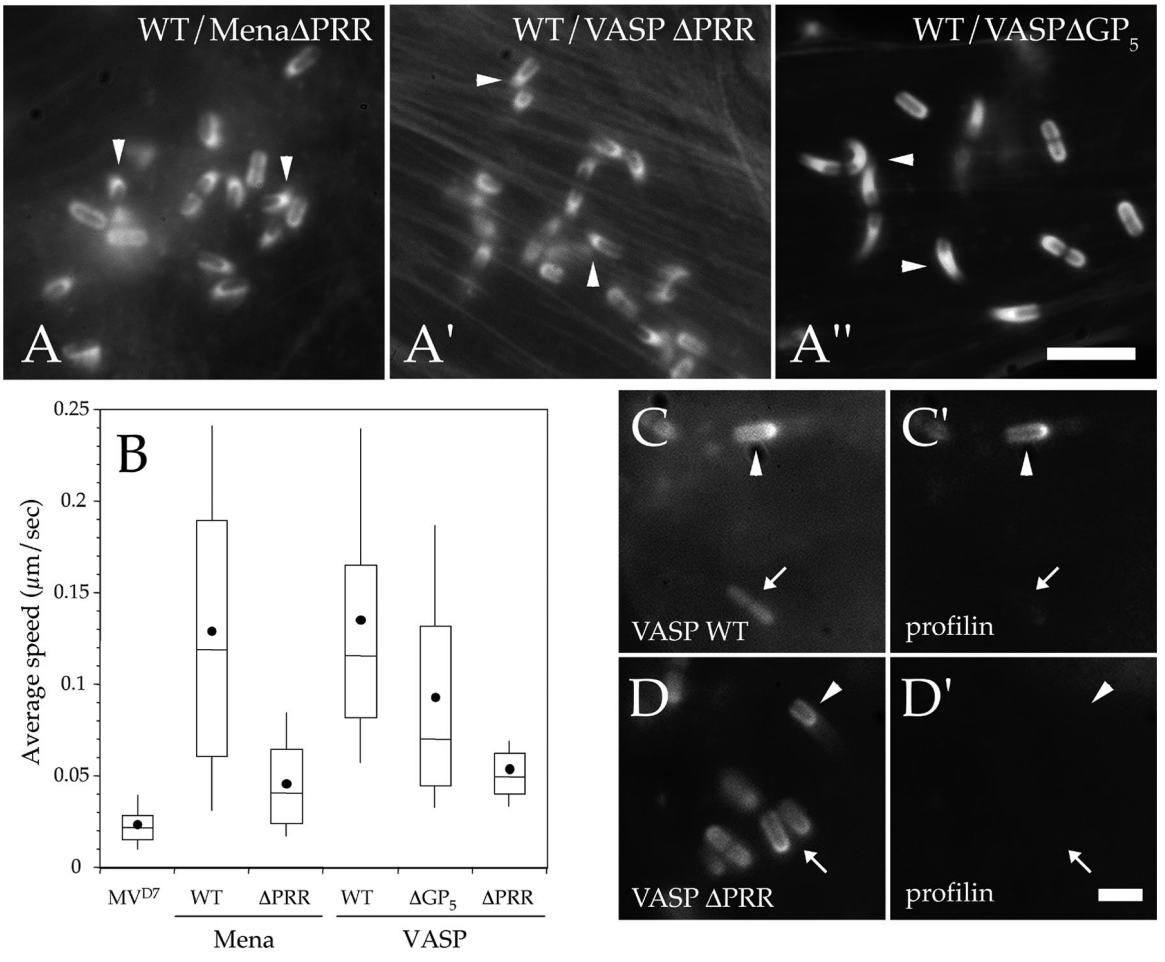

Figure 4. Proline-rich region of Ena/VASP proteins is essential for efficient *Listeria* motility. (A–A″) MV^{D7} cells expressing high levels of GFP-Mena ΔPRR, GFP-VASP ΔGP_5, or GFP-VASP ΔPRR were infected with wild-type *Listeria*, fixed, and stained with fluorescent phalloidin. In cell lines expressing GFP-Mena ΔPRR and GFP-VASP ΔPRR *Listeria* induced the formation of very short actin tails (arrowheads in A and A′), which were morphologically similar to those induced by this bacterium in the parental MV^{D7} cells (compare with Figure 1A), whereas *Listeria* actin tails were significantly longer in cells expressing GFP-VASP ΔGP_5 (arrowheads in A″). Bar, 2 μm. (A) Box and whiskers plots of bacterial speed. (C–D′) Deletion of the proline-rich region of Mena and VASP inhibits the targeting of profilin to the *Listeria* surface. MV^{D7} cells were transfected with CFP-tagged wild-type VASP or VASP ΔPRR and profilin II-YFP and then infected with wild-type *Listeria*. In cells expressing CFP-VASP, profilin colocalized with VASP at the surface of motile bacteria (arrowheads in C and C′) but not stationary ones (arrows in C and C′). In contrast, in cells expressing CFP-VASP ΔPRR, profilin was absent from the surface of both stationary (arrow in D′) and motile *Listeria* (arrowhead in D′). Bar, 1 μm.

has been shown to bind to profilin (Kang *et al.*, 1997), whereas the GFP-VASP ΔPRR construct includes two additional small deletions (also characterized by a GP_5 motif) flaking the triple GP_5 sequence (Figure 3A). As appraised by fluorescence microscopy, wild-type *Listeria* were associated with short actin comet tails that were morphologically similar to those induced by these bacteria in the parental MV^{D7} cells (Figure 4, A–A″). Moreover, video microscopy analysis showed that the speed of *Listeria* was greatly reduced in cells expressing GFP-Mena ΔPRR and GFP-VASP ΔPRR compared with the full-length counterparts (Figure 4B). The deletion of the proline-rich stretches in the GP_5 motif in VASP also resulted in an intermediate but still significant reduction of bacterial motility (Mann-Whitney *U* test, $p < 0.05$; GFP-VASP, $n = 128$; GFP-VASP ΔPRR, $n = 111$) that was characterized by the formation of actin tails slightly longer than those induced by wild-type *Listeria* in cells expressing Ena/VASP ΔPRR mutants (Figure 4, A″ and B).

Because the effect of GFP-Mena ΔPRR and GFP-VASP ΔPRR on *Listeria* motility may be due to their inability to bind profilin, we cotransfected MV^{D7} cells with CFP-tagged wild-type VASP or VASP ΔPRR and profilin II-YFP, infected them with *Listeria* and analyzed their localization at the bacterial surface by fluorescence microscopy. As expected, both CFP-tagged VASP constructs properly localized at the *Listeria* surface (Figure 4, C and D). In agreement with previous findings (Geese *et al.*, 2000), profilin-YFP localized at the bacterial surface of motile, but not stationary *Listeria* in cells expressing full-length CFP-VASP (Figure 4, C and C′), whereas in cells expressing CFP-VASP ΔPRR, profilin-YFP

could be detected neither around motile nor stationary bacteria (Figure 4, D and D′).

Overall, these results clearly indicate that the proline-rich region of Ena/VASP proteins is essential for efficient *Listeria* motility and that the deletion of the PRR correlated with a lack of profilin recruitment at the bacterial surface.

Deletion of Thymosin β4-like-Motif of Mena Reduces Listeria Motility

The deletion of the proline-rich region in Ena/VASP proteins did not cause a reduction in speed to the values measured in the parental MV^{D7} cells (Figure 4B), suggesting that Ena/VASP proteins play additional roles in *Listeria* motility other than recruiting profilin–G-actin complexes at the bacterial surface and that other regions of Ena/VASP proteins contribute to *Listeria* motility. Therefore, we analyzed the contribution of three regions contained in the EVH2 domain to this process.

The amino-terminal part of the EVH2 domain of VASP and Mena harbors a motif that is similar to the G-actin-binding site KLKR found in thymosin β4 (Gertler *et al.*, 1996; van Troys *et al.*, 1996). Similar sequences are also found in the headpiece of the actin-binding proteins villin and dematin (van Troys *et al.*, 1996). In MV^{D7} cells expressing GFP-Mena ΔTLM (for thymosinβ4-like-motif) *Listeria* moved significantly slower (Mann-Whitney *U* test, $p < 0.05$; GFP-Mena, n = 93; GFP-Mena ΔTLM, n = 73) than in cells expressing full-length GFP-Mena and induced the formation of short actin comet tails (Figure 5, A and B). Based on these results and on the observation that VASP rescues the ability of an ActA mutant, which lacks the G-actin–binding site, to support both the actin-nucleating activity of the Arp2/3 complex and the accumulation of actin at the *Listeria* surface (Skoble *et al.*, 2001), we speculated that the TLM motif substitutes for the activity of the G-actin–binding site of ActA in these events.

To test this hypothesis, we infected MV^{D7} cells and MV^{D7} cells expressing GFP-Mena or GFP-Mena ΔTLM with wild-type bacteria or with a *Listeria* mutant that expresses a mutated version of ActA that lacks the G-actin–binding site (Δ12; deletion spanning amino acids 68–109; Pistor *et al.*, 2000). In all these cell lines, wild-type bacteria induced a normal accumulation of actin at their surface as judged after labeling with fluorescent phalloidin. In contrast, the ability of the Δ12 mutant to induce a normal actin accumulation at the bacterial surface was impaired only in MV^{D7} cells and in cells expressing GFP-Mena ΔTLM (our unpublished data). The number of Δ12 bacteria associated with deficient actin accumulation was higher than that of wild-type *Listeria* in MV^{D7} cells and in MV^{D7} cells expressing GFP-Mena ΔTLM (Table 1, compare with MV^{D7} cells expressing GFP-Mena). Because Ena/VASP proteins may rescue the ability of the Δ12 mutant to induce actin accumulation at its surface also via a PRR-dependent recruitment of G-actin, we infected with this mutant MV^{D7} cells expressing GFP-Mena or GFP-Mena ΔPRR. As shown in Table 1, the deficient actin accumulation at the surface of the Δ12 mutant was much less pronounced in MV^{D7} cells expressing GFP-Mena ΔPRR, indicating that the effect observed was mainly due to the deletion of the TLM motif. Thus, these results suggest that the TLM motif is implicated in the regulation of actin filament nucleation at the bacterial surface.

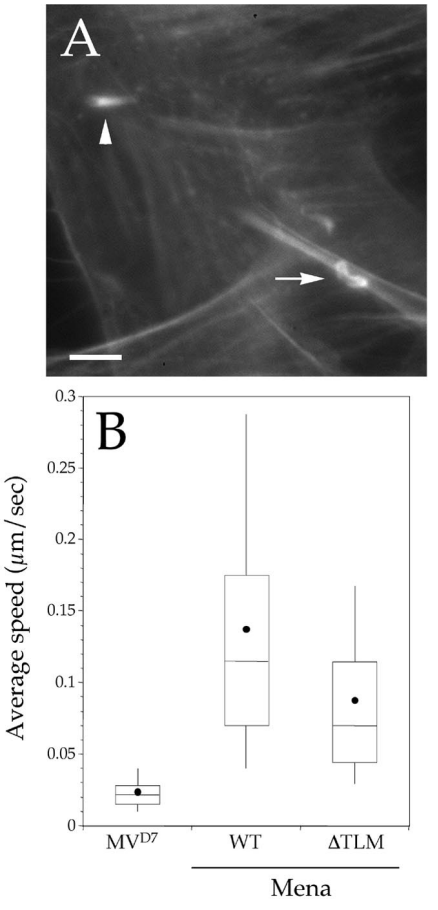

Figure 5. (A) Deletion of the thymosin β4-like motif of Mena causes a significant decrease of *Listeria* motility. MV^{D7} cells expressing high levels of GFP-Mena ΔTLM were infected with wild-type *Listeria*, fixed, and stained with fluorescent phalloidin. In these cells, *Listeria* induced the formation of short actin tails (arrowheads). Bar, 2 μm. (B) Box and whiskers plots of bacterial speed.

Deletion of F-Actin–binding Site of Ena/VASP Proteins Enhances Listeria Motility

The EVH2 domain of Ena/VASP proteins has also been implicated in the interaction of this protein family with F-actin in vitro (Bachmann *et al.*, 1999; Hüttelmaier *et al.*, 1999; Harbeck *et al.*, 2000). Moreover, Ena/VASP proteins stimulate actin polymerization by shortening the lag phase of actin filament formation, an effect that can be reversed by adding a peptide (corresponding to amino acids 261–283 of EVL) that has been shown to interact with F-actin in sedimentation assays (Laurent *et al.*, 1999; Harbeck *et al.*, 2000; Lambrechts *et al.*, 2000). To test whether the binding of Ena/VASP proteins to F-actin is required for *Listeria* motility, we expressed GFP-Mena ΔFAB (for F-actin binding) or GFP-VASP ΔFAB in MV^{D7} cells. These fusion proteins did not cause morphological changes in actin comet tails, as appraised by fluorescence microscopy (Figure 6, A and B). Unexpectedly, the speed of *Listeria* in MV^{D7} cells expressing these fusion proteins was significantly higher than that of bacteria in MV^{D7} cells expressing full-length Ena/VASP

Table 1. TLM motif contributes to the regulation of actin filament nucleation at the bacterial surface

	Wild type	Δ12
	% bacteria with deficient actin accumulation/(total bacteria)	% bacteria with deficient actin accumulation/(total bacteria)
MV^{D7}	38 (125)	96 (91)
MV^{D7} + Mena WT	9 (47)	13 (52)
MV^{D7} + Mena ΔTLM	11 (55)	81 (48)
MV^{D7} + Mena ΔPRR	9 (68)	16 (67)

proteins (Figure 6C; Mann-Whitney *U* test, $p < 0.05$; GFP-Mena, n = 93; GFP-Mena ΔFAB, n = 78; GFP-VASP, n = 128; GFP-VASP ΔFAB, n = 85), indicating that the interaction between Ena/VASP proteins and actin filaments is not required for intracellular *Listeria* motility.

A Coiled-Coil Motif in EVH2 Domain of Ena/VASP Proteins Is Not Required for Listeria Motility

Various experimental approaches indicate that the most C-terminal 35 amino acids in the EVH2 domain, which is predicted to form coiled-coil structures, can mediate the formation of Ena/VASP multimers (Haffner *et al.*, 1995; Ahern-Djamali *et al.*, 1998; Bachmann *et al.*, 1999; Carl *et al.*, 1999). In the *Listeria* context, it has been hypothesized that the formation of Ena/VASP multimers at the bacterial surface increases the recruitment of profilin–G-actin complexes and, as a consequence, *Listeria* motility (Kang *et al.*, 1997).

In MV^{D7} cells expressing GFP-Mena ΔCo-Co (for coiled-coil) and GFP-VASP ΔCo-Co *Listeria* induced the formation of normal actin comet tails (Figure 7, A and B). The visual impression that *Listeria* motility is not grossly altered in the presence of Ena/VASP ΔCo-Co proteins was confirmed by video microscopy analysis. The average speed of the bacteria in cells expressing GFP-Mena ΔCo-Co was not different from that measured in cells expressing full-length Mena (Figure 7C; Mann-Whitney *U* test, p = 0.24; GFP-Mena, n = 93; GFP-Mena ΔCo-Co, n = 90), whereas *Listeria* moved at a higher average speed in MV^{D7} cells expressing GFP-VASP ΔCo-Co (Figure 7B; Mann-Whitney *U* test, $p < 0.05$; GFP-VASP, n = 128; GFP-VASP ΔCo-Co, n = 85). Thus, the formation of Ena/VASP multimers is not required for *Listeria* motility.

Phosphorylation of Serine and Threonine Residues of Ena/VASP Proteins Increases Listeria Motility

VASP and Mena harbor three (S157, S239, and T278) and two (S236 and S376) phosphorylation sites, respectively, that can be phosphorylated in a cAMP- and cGMP-dependent manner (Halbrugge *et al.*, 1990). In vitro, the phosphorylation state of Ena/VASP proteins influences their ability to interact with F-actin and some SH3-containing proteins (Laurent *et al.*, 1999; Harbeck *et al.*, 2000; Lambrechts *et al.*, 2000).

To assess whether the phosphorylation of Ena/VASP proteins plays a role in *Listeria* motility, we mutated all phosphorylation sites of VASP and Mena to alanine to block phosphorylation or to aspartic acid to mimic the constitutively phosphorylated forms of these proteins, respectively (Figure 3A). None of these phosphorylation mutants grossly affected the formation of actin comet tails as judged by fluorescence microscopy (our unpublished data). Compared with MV^{D7} cells expressing wild-type GFP-Mena, GFP-Mena AA caused a slight but significant reduction in *Listeria* motility, whereas the speed of the bacteria in MV^{D7} cells expressing GFP-Mena DD was significantly increased (Figure 8A; Mann-Whitney *U* test, p = 0.04 for WT vs. AA, $p < 0.05$ for WT vs. DD; GFP-Mena, n = 93; GFP-Mena, AA, n = 67; GFP-Mena DD, n = 72). The slight reduction in *Listeria* speed is mainly due to the first Ser → Ala mutation as indicated by the observation that the speed of *Listeria* in MV^{D7} cells expressing GFP-Mena AS is not different from that measured in cells transfected with GFP-Mena AA (Figure 8A; Mann-Whitney *U* test, p = 0.058 for AS vs. AA; GFP-Mena AS, n = 126; GFP-Mena, AA, n = 67). Moreover, the increase in bacterial speed caused by GFP-Mena DD seems to be mostly dependent on the second Ser → Asp mutation as suggested by observation that there is no significant difference between *Listeria* motility measured in GFP-Mena WT and GFP-Mena DS (Figure 8A; Mann-Whitney *U* test, $p > 0.05$ for WT vs. DS; GFP-Mena, n = 93; GFP-Mena DS, n = 69).

The effect of similar mutations in VASP was slightly different. In particular, in MV^{D7} cells expressing GFP-VASP AAA the average bacterial speed was comparable with that measured in cells expressing wild-type GFP-VASP. Conversely, the expression of GFP-VASP DDD caused a significant enhancement of *Listeria* motility (Figure 8B; Mann-Whitney *U* test, p = 0.48 for WT vs. AAA, $p < 0.05$ for WT vs. DDD; GFP-VASP, n = 128; GFP-VASP AAA, n = 88; GFP-VASP DDD, n = 92). Because the first two Ser → Asp mutations mainly cause the enhancement of *Listeria* motility, these residues seems to be critical for this process (Figure 8B).

DISCUSSION

In this study we have characterized the contributions of four Ena/VASP protein domains to the intracellular motility of *L. monocytogenes*. In particular, we showed that the interaction of Ena/VASP proteins with F-actin and their potential ability to form multimers are both dispensable for their function in actin-based *Listeria* movement, whereas the proline-rich region, the putative G-actin binding site and the Ser/Thr phosphorylation of Ena/VASP proteins contribute to efficient *Listeria* motility.

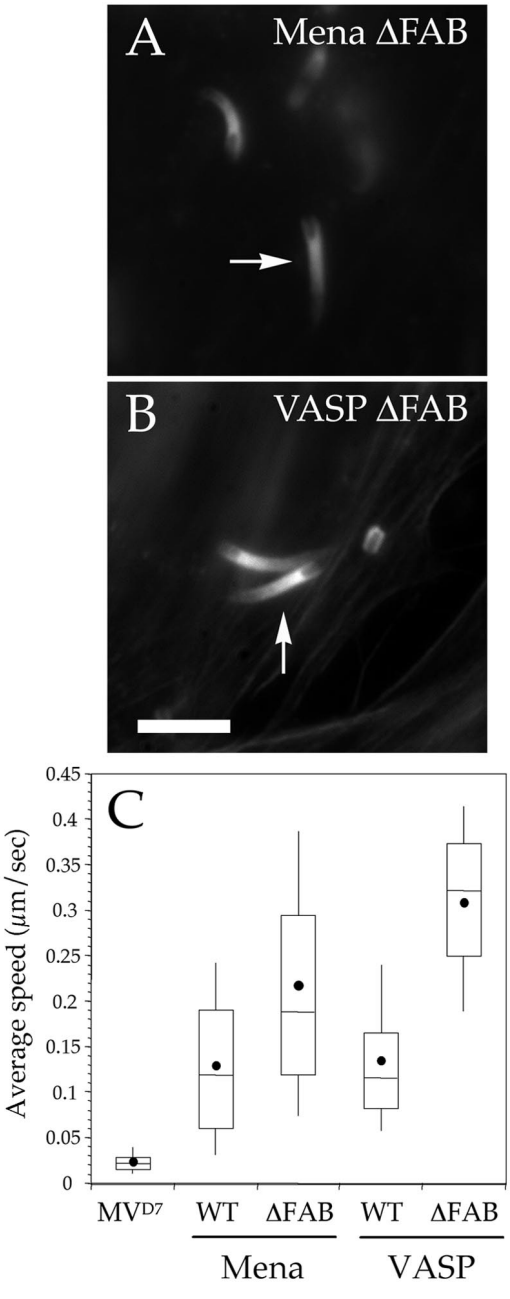

Figure 6. (A and B) Deletion of the F-actin–binding site of Mena and VASP increases *Listeria* motility. MV^{D7} cells expressing high levels of GFP-Mena ΔFAB (A) or GFP-VASP ΔFAB (B) were infected with wild-type *Listeria*, fixed, and stained with fluorescent phalloidin. In both cases, *Listeria* induced the formation of actin tails (arrows in A and B) that were not distinguishable from those induced by the same bacterium in MV^{D7} cells expressing wild-type Ena/VASP proteins (compare to Figure 2). Bar, 2 μm. (C). Box and whiskers plots of bacterial speed.

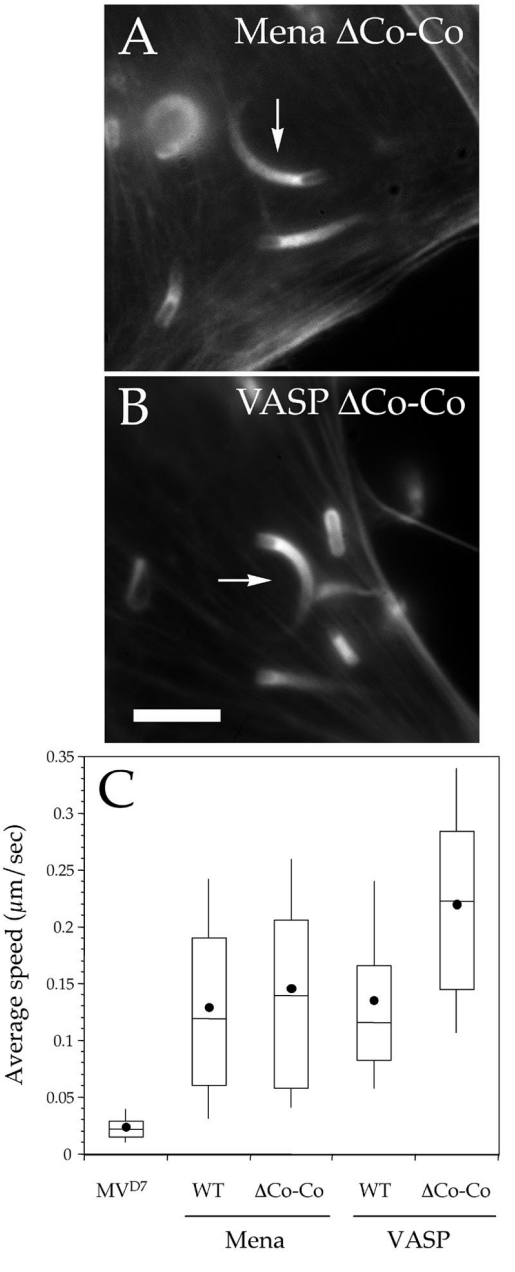

Figure 7. (A and B) Potential multimerization of Ena/VASP proteins is not required for *Listeria* motility. MV^{D7} cells expressing high levels of GFP-Mena ΔCo-Co (A) or GFP-VASP ΔCo-Co (B) were infected with wild-type *Listeria*, fixed, and stained with fluorescent phalloidin. In both cell lines, the bacteria induced the formation of actin tails (arrows in A and B) that were not distinguishable from those induced by the same bacterium in MV^{D7} cells expressing wild-type Ena/VASP proteins (compare to Figure 2). Bar, 2 μm. (B) Box and whiskers plots of bacterial speed.

Ena/VASP proteins were originally thought to regulate actin filament remodeling through their ability to interact with the G-actin–binding protein profilin (Reinhard *et al*., 1995; Gertler *et al*., 1996; Lambrechts *et al*., 2000). This notion is clearly supported by genetic studies, which suggest a physiological role for the interaction between Mena and profilin during the actin-based process of neurulation

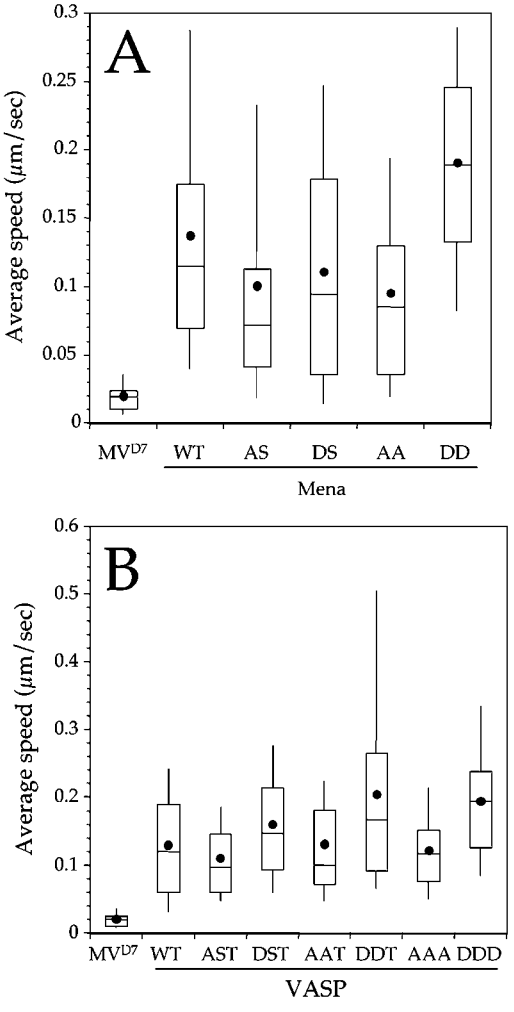

Figure 8. (A and B) Phosphorylation of the serine and threonine residues of Mena (A) and VASP (B) increases *Listeria* motility. Box and whiskers plots of *Listeria* speed showing the influence of Mena and VASP phosphorylation state on bacterial motility.

(Lanier *et al.*, 1999). Moreover, the injection of the proline-rich region of VASP into *Listeria*- and *Shigella*-infected cells causes the arrest of bacterial movement (Zeile *et al.*, 1996; Kang *et al.*, 1997), whereas a similar VASP peptide favors the disassociation of profilin–G-actin complexes leading to the enhancement of nucleation and elongation of actin filaments, in vitro (Jonckheere *et al.*, 1999). On the other hand, Ena/VASP proteins enhance *Listeria* motility in cell-free systems in absence of profilin (Loisel *et al.*, 1999), and Ena/VASP-profilin interaction is not required for the function of this protein family in whole cell motility (Loureiro *et al.*, 2002). Thus, although this study demonstrates that the interaction between Ena/VASP proteins and profilin at the *Listeria* surface is important for supporting the efficient bacterial motility, it is possible that the binding of Ena/VASP proteins to profilin is not required for, or plays a minor role in, the contribution of these proteins to other actin-based processes. Although none of the known proteins that contain SH3 and WW domains has been involved in *Listeria* motility, at present we cannot rule out that the reduced bacterial motility we observed in cells expressing Ena/VASP ΔPRR is in part due to the lack of recruitment of these proteins.

Ena/VASP proteins harbor a short sequence that is similar to the G-actin–binding motif of the actin-sequestering molecule thymosin β4 (van Troys *et al.*, 1996). Although a direct binding between G-actin and Ena/VASP proteins has not yet been demonstrated, we show that the deletion of this site in Mena causes a small but still significant reduction in *Listeria* motility. It has recently been shown that VASP exerts a weak actin nucleating activity in vitro (Harbeck *et al.*, 2000), suggesting that the deletion of the putative G-actin–binding site in Mena could result in a decrease in actin filament formation. This possibility seems unlikely in light of many observations demonstrating that *Listeria* mutants that are not able to bind to the Arp2/3 complex, but are still fully competent for interacting with Ena/VASP proteins, cannot induce the formation of actin clouds at their surface (Lasa *et al.*, 1995, 1997; Pistor *et al.*, 1995, 2000; Smith *et al.*, 1996; Skoble *et al.*, 2000). Although Ena/VASP proteins are not able to nucleate actin, recent data suggest that they may support the nucleation activity of the Arp2/3 complex. In particular, Skoble *et al.* (2001) showed that VASP can rescue the ability to activate the Arp2/3 complex of an ActA mutant that lacks the G-actin–binding site, and suggested that the F-actin-binding site of VASP is required for this process. In contrast with their conclusion and based on our data showing that the deletion of the F-actin–binding site of Ena/VASP proteins does not affect *Listeria* motility and that the deletion of the TLM site clearly impairs actin accumulation at the bacterial surface, we suggest that Ena/VASP proteins may stimulate actin filament nucleation at the bacterial surface by supplying actin monomers to the Arp2/3 complex. Our hypothesis is consistent with the observation that stimulators of the Arp2/3 complex such as WASp/Scar proteins require binding to G-actin to activate this complex, and that Ena/VASP proteins enhance *Listeria* motility in cell-free systems in the absence of profilin (Loisel *et al.*, 1999; Machesky *et al.*, 1999). It will be important to characterize the function of the TLM motif in detail and test whether it actually binds G-actin.

The EVH2 domain of Ena/VASP proteins has been implicated in the interaction of this protein family with F-actin in vitro (Bachmann *et al.*, 1999; Hüttelmaier *et al.*, 1999). Kuo and McGrath (2000) have recently demonstrated that *Listeria* are tightly linked to their own actin tails raising the possibility that this tight interaction limits bacterial motility. Accordingly, we show herein that the deletion of the FAB site in Ena/VASP proteins seems to remove this physical constraint and thereby increase bacterial speed. In vitro, VASP seems to protect actin filaments from the actin-severing activity of gelsolin (Bearer *et al.*, 2000). In addition, gelsolin, which localizes at the interfaces between bacteria and actin tails, enhances *Listeria* motility when overexpressed or injected in fibroblasts (Laine *et al.*, 1998). The conclusion that could be made from these results is that the deletion of the FAB region of Ena/VASP proteins makes actin filaments more susceptible to gelsolin's action, resulting in higher bacterial speed. Laurent *et al.* (1999) reported that a GST-tagged EVH2 domain inhibits *Listeria* motility in platelets

extracts concluding that the interaction between F-actin and Ena/VASP proteins is essential for this process. We believe, however, that their interpretation was more likely to arise by the interference with both binding activities of thymosin β4-like motif and F-actin-binding site of endogenous VASP. Alternatively, other regions within the EVH2 domain of hitherto unknown function could also be responsible for such effect.

The binding of Ena/VASP proteins to F-actin in vitro seems to be dependent on their phosphorylation state (Laurent *et al*., 1999; Harbeck *et al*., 2000; Lambrechts *et al*., 2000). We have shown that deletion of the F-actin–binding site of Mena and VASP results in an enhancement of *Listeria* motility. Similarly, the expression of Ena/VASP mutants that mimics the full phosphorylation state of these proteins increases bacterial speed. Thus, it is possible that the phosphorylation of Ena/VASP proteins weakens their binding to F-actin, resulting in faster *Listeria* motility. This possibility would be in agreement with the findings of Lambrechts *et al.* (2000) and Harbeck *et al*., (2000), who showed that fully phosphorylated EVL and VASP bind less efficiently to F-actin. The possibility that Ena/VASP phosphorylation plays a role in actin-based processes is supported by the observation that VASP phosphorylation directly correlates with spreading of neutrophils (Lawrence and Pryzwansky, 2001). Moreover, Mena phosphorylation is required for its function as negative regulator of cell motility in fibroblasts (Loureiro *et al*., 2002).

A number of studies indicate that the EVH2 domain can mediate the formation of Ena/VASP multimers and that they may be required for the function of this protein family in vivo (Ahern-Djamali *et al*., 1998; Bachmann *et al*., 1999; Carl *et al*., 1999). In particular, Ena/VASP multimers seem to be required for the function of Ena/VASP proteins as suggested by the observation that a truncated form of Ena lacking the EVH2 domain caused lethality of *Drosophila* embryos (Ahern-Djamali *et al*., 1998). Moreover, the Mena mutant ΔCo-Co is only partially able to rescue normal motile properties of MV^{D7} fibroblasts (Loureiro *et al*., 2002), suggesting that Ena/VASP multimerization could play a role in this process. In the context of *Listeria* motility, the formation of Ena/VASP multimers at the bacterial surface has been proposed to increase the availability of polymerization-competent actin monomers and, as a consequence, bacterial motility. Therefore, we expected that the inhibition of Ena/VASP multimerization would result in the decrease of *Listeria* motility due to the limited availability of actin monomers. In contrast, we found that the expression of Ena/VASP ΔCo-Co proteins did not reduce bacterial movement but, in VASP, increased it. Based on our results that deletion of FAB also causes an increase of bacterial motility, it is conceivable that the deletion of the multimerization site in VASP, by causing a reduction in the number of F-actin–binding sites, weakens the interaction of the bacteria with the actin tails and, as a consequence, augments bacterial speed. This hypothesis is consistent with the observation that the deletion of the multimerization motif from the EVH2 domain of VASP decreases its ability to interact with F-actin, in vitro (Bachmann *et al*., 1999).

CONCLUSION

Our data clearly indicate that the proline-rich core, the putative G-actin–binding site, and the phosphorylation state of Ena/VASP proteins are important for *Listeria* motility, whereas, in contrast with previous models for *Listeria* motility, the F-actin–binding and multimerization regions of this protein family are dispensable for this actin-based process. Finally, in light of this study and that of Loureiro *et al.* (2002), it is clear that Ena/VASP protein domains can contribute to different extents in distinct actin-based processes.

How then, can Ena/VASP proteins influence the dynamics of the actin cytoskeleton? Other than affecting the nucleation/elongation of actin filaments, Ena/VASP proteins may influence the architecture of the actin cytoskeleton. This view is consistent with the observation that VASP seems to influence the branching of actin filaments induced by the Arp2/3 complex (Skoble *et al*., 2001) and that the expression of Mena in the MV^{D7} background affects the organization of the actin filaments in lamellipodia (Bear *et al*., 2002). Based on these and our study, we propose that Ena/VASP proteins act as multifunctional organizers of the actin cytoskeleton that regulate both the nucleation/elongation and the architecture of actin networks.

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