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Pivotal role of VASP in Arp2/3 complex–mediated actin nucleation, actin branch-formation, and *Listeria monocytogenes* motility

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The *Listeria monocytogenes* ActA protein mediates actin-based motility by recruiting and stimulating the Arp2/3 complex. In vitro, the actin monomer-binding region of ActA is critical for stimulating Arp2/3-dependent actin nucleation; however, this region is dispensable for actin-based motility in cells. Here, we provide genetic and biochemical evidence that vasodilator-stimulated phosphoprotein (VASP) recruitment by ActA can bypass defects in actin monomer-binding. Furthermore, purified VASP enhances the actin-nucleating activity of wild-type ActA and the Arp2/3 complex while also reducing the frequency of actin branch formation. These data suggest that ActA stimulates the Arp2/3 complex by both VASP-dependent and -independent mechanisms that generate distinct populations of actin filaments in the comet tails of *L. monocytogenes.* The ability of VASP to contribute to actin filament nucleation and to regulate actin filament architecture highlights the central role of VASP in actin-based motility.

Introduction

Listeria monocytogenes is a facultative intracellular Gram-positive bacterium that can invade a broad range of cell types and can cause a variety of syndromes in humans and animals. Intracellular *L. monocytogenes* replicate in the cytoplasm of host cells and induce the polymerization of host actin filaments at the bacterial surface. Polarized polymerization of actin propels the bacteria through the cytoplasm and into pseudopod-like structures that are engulfed by neighboring cells. Actin-based motility allows *L. monocytogenes* to spread from cell to cell without leaving the protective intracellular niche, and is essential for pathogenesis (Cossart and Bierne, 2001).

Actin polymerization induced by *L. monocytogenes* has been intensively studied because it represents a simplified model for understanding the regulation of actin dynamics (Frischknecht and Way, 2001). A single bacterial surface protein, ActA, is necessary and sufficient to induce actin-based motility in cells and cell lysates (Domann et al., 1992; Kocks et al., 1992, 1995; Smith et al., 1995; Cameron et al., 1999). ActA does not directly induce polymerization of purified actin. Instead, ActA recruits and stimulates the actin-nucleating activity of a highly conserved host protein complex that contains actin-related proteins 2 and 3 and five other subunits (Arp2/3 complex) (Machesky et al., 1994; Welch et al., 1997, 1998). The Arp2/3 complex is required for actin-based motility of *L. monocytogenes* (Yarar et al., 1999; May et al., 1999). In the host, the Arp2/3 complex is thought to play a critical role in cellular migration, phagocytosis, and vesicle motility through interactions with proteins of the Wiscott-Aldrich Syndrome protein (WASP)* family (Machesky and Insall, 1998; May et al., 2000; Rozelle et al., 2000).

The NH_2-terminal domain of ActA is required for actin-based motility (Lasa et al., 1995; Pistor et al., 1995), as it directly binds the Arp2/3 complex and stimulates its actin-nucleating activity (Welch et al., 1998; Skoble et al., 2000; Zalevsky et al., 2001). Within the NH_2-terminal domain of ActA are three regions that contribute to actin nucleation: an acidic stretch, an actin monomer-binding region, and a cofilin homology sequence (Skoble et al., 2000). These three regions have functional or sequence similarity to domains in WASP family proteins (Bi and Zigmond, 1999; Pistor et al., 2000). The actin monomer-binding region in ActA, located

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*Abbreviations used in this paper: Ena/VASP, enabled/vasodilator-stimulated phosphoprotein; PtK2 *Potoroo tridactylis* kidney epithelial; SOE, splice-by-overlap extension; WASP, Wiscott-Aldrich syndrome protein.

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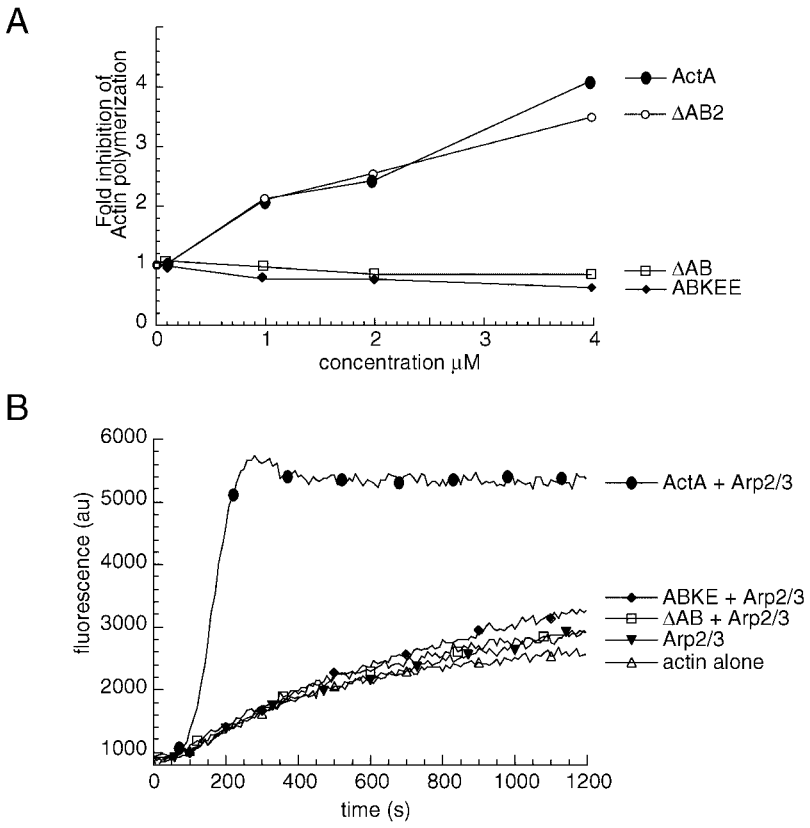

Figure 1. **Effects of mutations in the actin monomer-binding region of ActA.** (A) Point mutations in the actin monomer-binding region prevent sequestration of actin monomer. The effect of the indicated concentrations of ActA and ActA derivatives on the polymerization kinetics of 2 μM actin was monitored using pyrene-actin polymerization assays. The fold inhibition was calculated by dividing the maximal rate of actin polymerization by the maximal rate of actin polymerization in the presence of ActA and ActA derivatives. (B) Mutations in the actin monomer-binding region of ActA decrease the efficiency of Arp2/3 complex–mediated actin nucleation. Graphs of fluorescence intensity (measured in arbitrary units) versus time in pyrene-actin polymerization assays. Fluorescence of pyrene-labeled actin increases when it is incorporated into actin polymer. Thus, an increase in fluorescence represents an increase in actin polymer mass. Initiation of polymerization of 2 μM actin was induced at time 0 in the presence or absence of 20 nM Arp2/3 complex and 20 nM ActA derivatives.

between residues 60 and 101, is sufficient for monomer-binding activity (Lasa et al., 1997, Cicchetti et al., 1999; Skoble et al., 2000) and when this region is deleted, ActA has no monomer-binding activity (Skoble et al., 2000). This actin monomer-binding region is required for inducing actin nucleation in vitro, but is not essential for actin polymerization or motility in cells (Skoble et al., 2000). This apparent paradox suggests that the actin monomer-binding region in ActA may serve a redundant function in the context of the host cytoplasm.

A second putative actin monomer-binding site, located between residues 121 and 138, was recently identified in NH_2-terminally truncated molecules of ActA lacking the first actin-binding site (Zalevsky et al., 2001). However, these truncations caused ActA to bind actin more tightly than full-length protein and caused ActA to prevent both barbed and pointed end elongation, suggesting a nonspecific interaction with actin monomer. The fact that these truncated molecules have different binding properties than wild-type ActA and that deletions in the NH_2-terminal domain of ActA can alter the secondary structure (Cicchetti et al., 1999) suggests that this interaction may be caused by misfolding.

The central domain of ActA does not play a direct role in stimulating the actin-nucleating activity of purified Arp2/3 complex (Welch et al., 1998; Skoble et al., 2000; Zalevsky et al., 2001). Although actin-based motility can occur in the absence of the central domain, motility is greatly reduced in velocity and efficiency (Lasa et al., 1995; Smith et al., 1996; Niebuhr et al., 1997). The central domains of ActA from wild-type isolates vary in that they contain either three or four proline-rich repeats (Wiedmann et al., 1997) that recruit members of the Enabled/vasodilator-stimulated phosphoprotein (Ena/VASP) family (Chakraborty et al., 1995; Pistor et al., 1995; Gertler et al., 1996; Smith et al., 1996; Niebuhr et al., 1997). Ena/VASP family proteins are tetrameric F-actin–binding proteins that can nucleate actin assembly in vitro but have not been demonstrated to play a role in actin nucleation in vivo (Bachmann et al., 1999; Huttelmaier et al., 1999; Bear et al., 2000; Bearer et al., 2000). Ena/VASP proteins also function to recruit the actin monomer-binding protein profilin to the bacterial surface (Reinhard et al., 1995; Smith et al., 1996). Depletion of Ena/VASP family proteins from cell-free extracts prevents *L. monocytogenes* motility but not actin polymerization at the bacterial surface, whereas profilin depletion reduces the velocity of actin-based motility (Marchand et al., 1995; Laurent et al., 1999). Neither VASP nor profilin is required for the reconstitution of actin-based motility with purified proteins, but both contribute independently to increase the efficiency of motility (Loisel et al., 1999).

In this study, we tested the hypothesis that Ena/VASP recruitment by the central domain of ActA functions redundantly with the actin monomer-binding region of ActA to stimulate actin polymerization. We found that VASP recruitment by ActA is required to initiate actin polymerization in the absence of actin monomer-binding activity. Furthermore, we found that VASP enhances the ability of wild-type ActA to stimulate Arp2/3 complex-mediated actin nucleation and decreases filament branching.

Results

ActA contains a single actin monomer-binding site

In combination with the Arp2/3 complex, ActA is a potent stimulator of actin nucleation. However, ActA alone sequesters actin monomer and causes a concentration-dependent

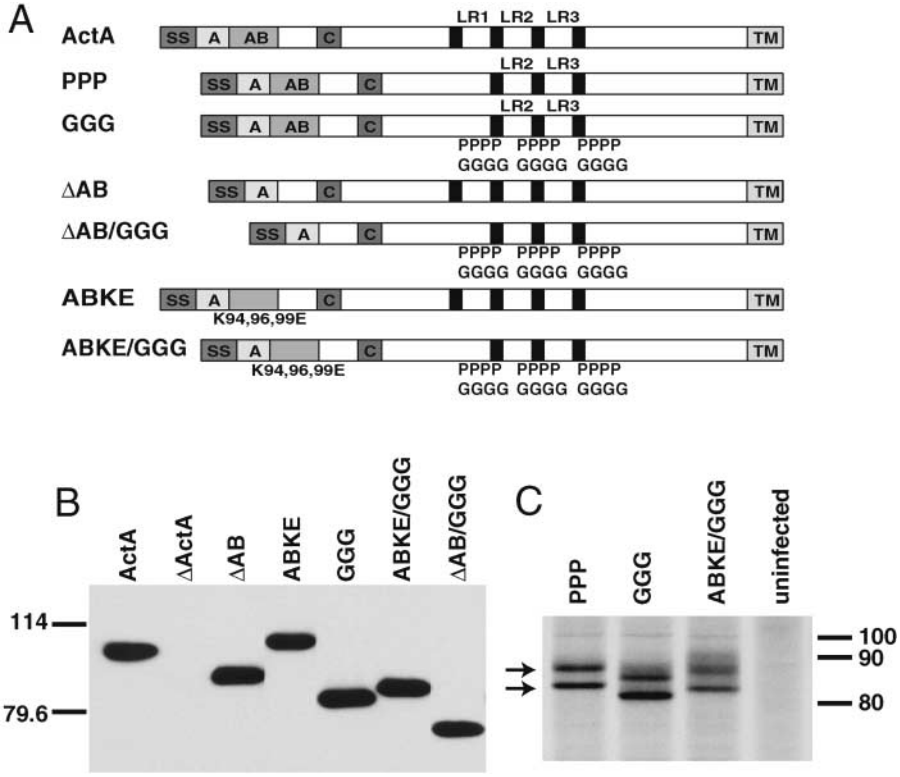

Figure 2. **Mutations in the actin monomer-binding region and proline-rich repeats do not alter levels of ActA.** (A) Schematic diagram of ActA derivatives. Functional domains of ActA are labeled: (SS) signal sequence, (A) acidic stretch, (AB) actin monomer-binding region, (C) cofilin homology sequence, (LR) long repeats, (TM) transmembrane domain, black boxes represent proline-rich repeats. (B) Surface-associated ActA from actA mutants visualized by Western blotting. Surface proteins were extracted from an equivalent number of late log phase bacteria grown in Luria-Bertani broth. Proteins were separated on a 7.5% polyacrylamide gel. ActA was detected by Western blotting using polyclonal anti-ActA antibodies made to the first 18 residues of the mature protein (Mourrain et al., 1997). All lanes shown are from a single exposure of one blot. (C) Metabolic labeling of ActA from mutant bacteria grown within host cells. Strains expressing derivatives of ActA (containing an equal number of methionine residues) were grown in J774 macrophages. Host protein synthesis was inhibited with cyclohexamide and anisomycin and bacterial proteins were labeled with [^{35}S] methionine. SDS-extracted proteins were resolved on a 7.5% gel and visualized by autoradiography. ActA and its phosphorylated forms are labeled with arrows.

decrease in the rate of spontaneous actin polymerization (Lasa et al., 1997; Welch et al., 1998; Skoble et al., 2000; Zalevsky et al., 2001). An actin monomer-binding site between amino acids 60 and 101 in ActA has been identified previously (Lasa et al., 1997; Cicchetti et al., 1999; Skoble et al., 2000). In-frame deletion of this region (ΔAB) renders ActA incapable of inhibiting spontaneous actin polymerization (Skoble et al., 2000). To assess the contribution of the first actin-binding domain within the context of full-length ActA, we generated a substitution mutation in which three lysine residues (at positions 94, 96,and 99) were changed to glutamate (ABKE) (see Fig. 2 A). These residues were chosen because they were shown previously to be essential for the actin-binding activity of a peptide derived from this region (Lasa et al., 1997). We found that the ABKE derivative of ActA was completely defective at inhibiting spontaneous actin polymerization at all concentrations tested (Fig. 1 A), confirming that this site is the only functional actin monomer-binding site in full-length ActA. To determine the contribution of the second putative actin monomer-binding site, we generated an in-frame deletion that removes this entire region (ΔAB2). We found that this deletion had no effect on the ability of ActA to bind actin monomer. Together, these data suggest that there is a single actin monomer-binding site in full-length ActA, or that the second monomer–binding site is masked in the context of an intact NH2-terminal domain.

Actin monomer-binding activity is required to stimulate actin nucleation

To determine whether actin monomer-binding activity is required to stimulate Arp2/3 complex–mediated actin nucleation, we tested the derivatives of ActA incapable of binding actin monomer in the pyrene-actin polymerization assay (Fig. 1 B). As previously described, equimolar concentrations of full-length ActA and the Arp2/3 complex stimulated actin nucleation, whereas the ΔAB derivative of ActA was dramatically reduced in its ability to activate the Arp2/3 complex (Skoble et al., 2000). The ABKE derivative was also defective in stimulating actin nucleation, suggesting that the actin nucleation defects observed with these derivatives are primarily due to the defect in actin monomer-binding activity.

Mutants that cannot bind actin monomer or VASP

Deletion of the actin monomer-binding region in ActA greatly reduces its ability to nucleate with the Arp2/3 complex in vitro; however, when this modified protein is present at the surface of intracellular *L. monocytogenes*, it mediates actin polymerization and actin-based motility (Skoble et al., 2000). This suggested that host actin-binding proteins can function to rescue the defects associated with this mutation. Therefore, we tested whether preventing recruitment of host actin-binding proteins to the surface of *L. monocytogenes* would block the rescue observed in vivo. F-actin binding proteins of the Ena/VASP family bind to the polyproline II helix formed by each of the proline-rich repeats in ActA (Prehoda et al., 1999), and then recruit the actin monomer-binding protein profilin (Reinhard et al., 1995; Smith et al., 1996). To test whether recruitment of Ena/VASP and profilin was required to rescue the actin polymerization defect of actin monomer-binding mutations, we generated derivatives of ActA in which all of the proline-rich repeats were mu-

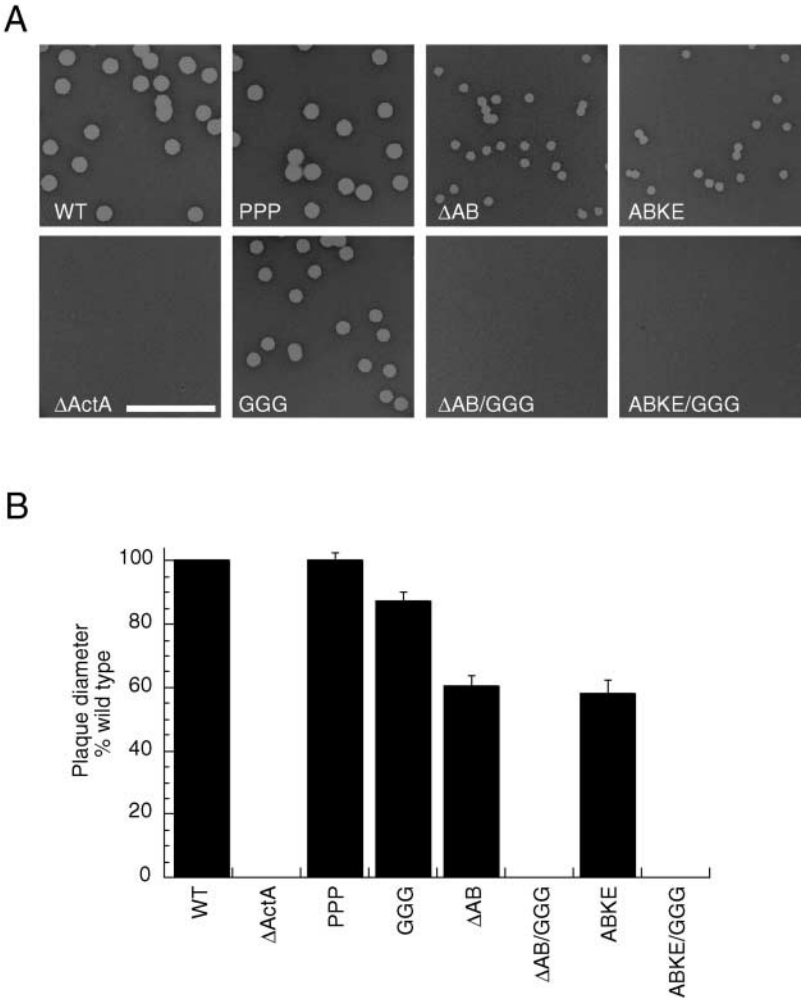

Figure 3. **In the absence of actin monomer-binding activity, intact proline-rich repeats of ActA are required for intercellular spread of *L. monocytogenes*.** (A) Plaque assays. Images of plaques formed in L2 mouse fibroblast monolayers after 4 d of infection with the indicated *L. monocytogenes* strains. Media contains 0.7% agarose and 10 μg/ml gentamicin. Live cells are stained with neutral red. Bar, 10 mm. (B) Quantification of intercellular spread. Plaque diameters were measured for each strain and are expressed as percent of wild type. Error bars represent the SD between five and twelve independent assays.

tated. As repeated sequences are difficult to mutagenize we used a background strain containing three proline-rich repeats (PPP) that is fully virulent (Smith et al., 1996), and in the remaining three repeats, all of the proline residues were changed to glycine (GGG) (Fig. 2 A). Proline-to-glycine substitutions have been shown to prevent VASP binding to a peptide corresponding to this region of ActA (Niebuhr et al., 1997), and to prevent VASP and profilin association with *L. monocytogenes* expressing an *actA* allele with only two glycine-rich repeats (Smith et al., 1996). We then combined this GGG mutation with either the ΔAB or the ABKE mutations (ΔAB/GGG, ABKE/GGG), and replaced the wild-type chromosomal *actA* gene in *L. monocytogenes* with the mutated versions using allelic exchange. To confirm that each of these mutated derivatives was expressed at the surface of *L. monocytogenes* to the same extent as wild-type ActA, we performed Western analysis of proteins extracted from the surface of equivalent numbers of bacteria using antibodies that recognize the first 18 residues in mature ActA (Fig. 2 B) (Mourrain et al., 1997). To ensure that the combination of mutations did not decrease the stability of mutant ActA derivatives in the cytoplasmic environment, [^{35}S] labeling of bacterial proteins from mutants grown inside J774 macrophage-like cells was performed. By comparing the steady state levels of ActA derivatives, we found that neither the proline-to-glycine substitution in the central domain nor the combination of this mutation with the charge reversal within the actin-binding region altered the levels of ActA at the surface of intracellular bacteria (Fig. 2 C).

To quantitatively measure the ability of *L. monocytogenes* expressing each of these mutant derivatives of ActA to spread from cell to cell, we performed a plaquing assay using an L2 cell monolayer. In this assay, the presence of gentamicin in the overlay kills extracellular bacteria, thus the plaque diameter represents the ability of the bacteria to spread intercellularly by actin-based motility over a period of 4 d. Bacteria expressing the ΔAB mutation or the ABKE mutation had similar plaque defects (60% and 58% of wild type) (Fig. 3, A and B). The PPP mutants, with three proline-rich repeats, produced plaques that were equal in diameter to wild type (100%), as reported previously (Smith et al., 1996). The GGG mutants, expressing ActA with no functional proline-rich repeats, made plaques that were 88% of wild type. Although this plaque size is larger than the 60% reported previously for mutants expressing a deletion of this region (Smith et al., 1996; Niebuhr et al., 1997), we believe that this substitution mutation more accurately reflects the contribution of Ena/VASP recruitment than large deletions which would also inhibit binding of other factors including LaXp180 (Pfeuffer et al., 2000). In contrast to the plaques

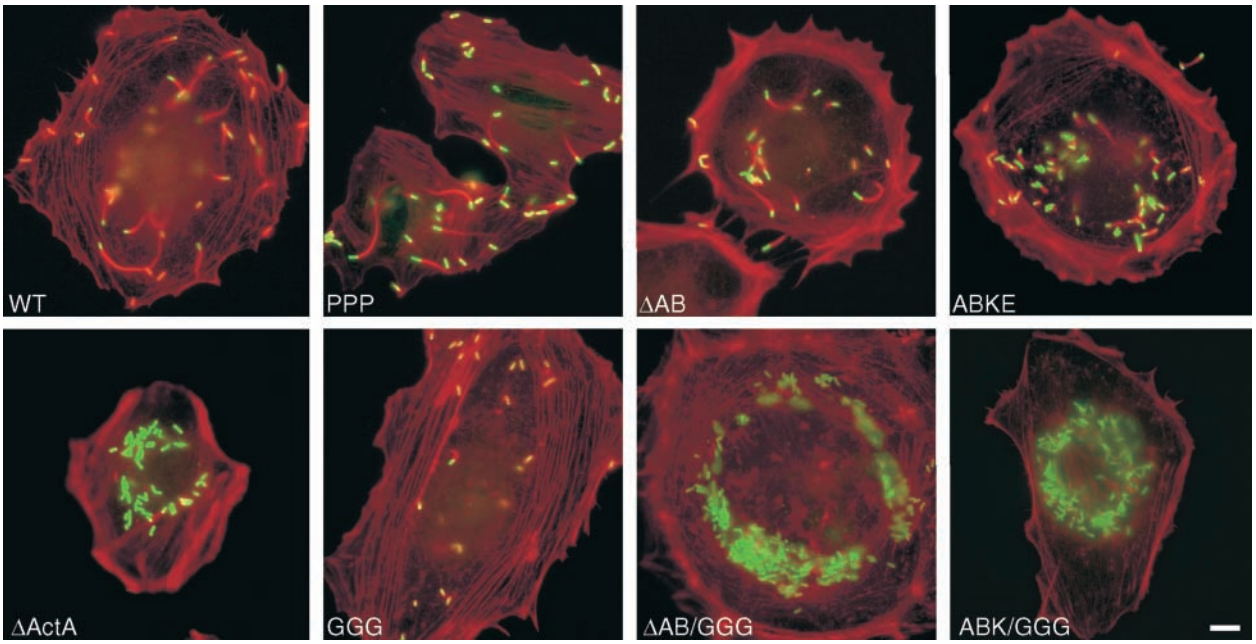

Figure 4. **Ability of ActA to induce actin polymerization in vivo requires actin monomer-binding activity or intact proline-rich repeats.** PtK2 cells infected for 3.5 h with wild-type *L. monocytogenes* or mutants expressing the indicated derivatives of ActA. F-actin was visualized by staining with rhodamine-conjugated phalloidin and bacteria were detected by indirect immunofluorescence using polyclonal anti–*L. monocytogenes* primary antibodies followed by FITC-conjugated secondary antibodies. Bar, 10 μm.

formed by each of the single mutations, *L. monocytogenes* expressing ActA molecules that were incapable of recruiting actin monomer and had mutated proline-rich repeats (ΔAB/GGG and ABKE/GGG) formed pinpoint lesions that were indistinguishable from an ActA-null mutant (ΔActA). These data demonstrate that mutations in the proline-rich repeats act synergistically with mutations in the actin monomer-binding region to prevent intercellular spread.

To quantify the ability of each mutant to initiate actin polymerization in host cells within the first 3.5 h after infection, we infected *Potoroo tridactylis* kidney epithelial (PtK2) cells because their flat morphology facilitates counting bacteria (Fig. 4). We found that >99% of wild type and the PPP mutant bacteria were associated with F-actin, and that 55 and 48%, respectively, generated actin tails. 93% of the ΔAB and the 90% of the ABKE mutants were associated with actin, and 38 and 36%, respectively, formed actin tails. Bacteria expressing ActA with the GGG mutation were 99% associated with actin, but only 16% generated actin comet tails. Although the majority of these GGG mutants were associated with actin clouds, in many cases the actin staining in the clouds was faint compared with wild type, similar to deletions of this region (Smith et al., 1996; Niebuhr et al., 1997). In contrast, only 1% of the mutants expressing the double mutations ΔAB/GGG and ABK/GGG were associated with any cellular actin, and most grew in perinuclear microcolonies that resembled those formed by an ActA-null strain (ΔActA). These data demonstrate that, in the absence of actin monomer-binding activity, the proline-rich repeats of ActA are necessary for actin nucleation in vivo.

To verify that VASP is no longer recruited to the surface of *L. monocytogenes* strains expressing ActA derivatives with the mutated proline-rich repeats, we infected HeLa cells and stained for VASP with anti–human VASP antibodies. Wild-type bacteria or mutants in the actin-binding region had intense polarized VASP staining at the bacterial surface (Fig. 5). Bacteria expressing ActA with the GGG mutation did not colocalize with VASP, nor did the double mutants, ΔAB/GGG and ABKE/GGG. VASP localization to the surface of *L. monocytogenes* is independent of the presence of F-actin, as mutations in the cofilin homology sequence prevent actin polymerization but do not affect VASP localization to the bacterial surface (Skoble et al., 2000). Together, these data provide genetic evidence that VASP recruitment is required to initiate actin polymerization and intercellular spread of *L. monocytogenes* in the absence of the actin monomer-binding activity of ActA.

VASP contributes to actin nucleation

The genetic evidence suggested that VASP recruitment was required to initiate actin polymerization in the absence of monomer-binding activity. Thus, we used the pyrene-actin polymerization assay to test directly whether VASP and/or profilin could rescue the actin-nucleating defect caused by the deletion of the actin monomer-binding region of ActA in vitro. In this assay, an increase in actin nucleation is best represented by an increase in the slope of the curves. Indeed, the addition of equimolar concentrations of purified recombinant human VASP and ΔAB together were able to stimulate Arp2/3 complex–mediated actin nucleation with kinetics similar to full-length ActA (Fig. 6 A). Addition of an equimolar concentration of profilin to this reaction did not enhance the ability of VASP to rescue the nucleation defect, but rather slowed polymerization kinetics. Without VASP, profilin also slowed the kinetics of actin polymerization by the Arp2/3 complex and the ΔAB derivative (unpublished

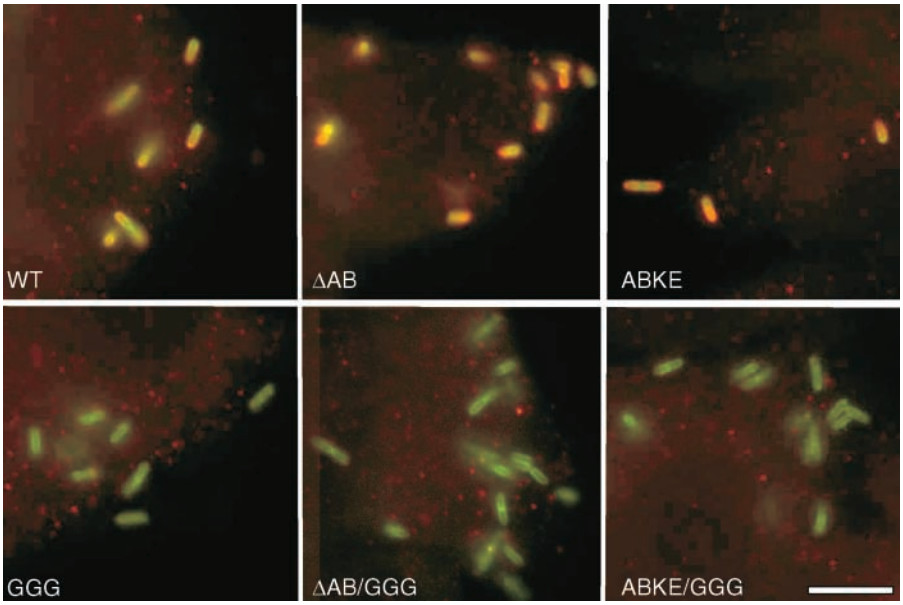

Figure 5. **VASP recruitment is impaired in mutants expressing ActA with mutated proline-rich repeats.** HeLa Cells were infected for 3.5 h with wild-type or indicated mutant strains. After fixation, VASP was detected with affinity-purified polyclonal anti–human VASP antibodies, followed by rhodamine-conjugated secondary antibodies. *L. monocytogenes* were detected by staining with FITC-conjugated polyclonal anti–*L. monocytogenes* antibodies. Bar, 10 μm.

data), a result that is consistent with profilin's known ability to bind and sequester actin monomer. As reported previously, VASP alone was capable of weakly nucleating actin filaments (Huttelmaier et al., 1999; Bearer et al., 2000; Harbeck et al., 2000), but the kinetics of nucleation by VASP were not altered by the addition of the Arp2/3 complex. This suggests that VASP is not capable of stimulating Arp2/3 complex–mediated actin nucleation in the absence of ActA.

Because VASP is a tetramer (Bachmann et al., 1999) and full-length ActA has four proline-rich repeats, it is theoretically possible for 16 VASP molecules to associate with each ActA molecule. To determine the amount of VASP that stimulates ActA maximally, we added increasing concentrations of VASP to equimolar concentrations of the ΔAB mutant and Arp2/3 complex (Fig. 6 B). We found that the ability of VASP to rescue the Arp2/3 complex–mediated actin nucleation defect of ΔAB was saturated at a 2:1 molar excess of VASP. This suggests that ActA may function as a dimer and that each ActA dimer binds one VASP tetramer.

Actin filaments increase the nucleation rate of stimulated Arp2/3 complex (Machesky et al., 1999; Zalevsky et al., 2001). Because VASP is capable of nucleating assembly of actin filaments on its own, we tested whether the observed rescue of the actin nucleation defect by addition of VASP to the ΔAB derivative was due to the increased initial concentration of F-actin. We found that addition of a low concentration of phalloidin-stabilized F-actin seeds resulted in polymerization kinetics similar to VASP alone; however, unlike VASP, the addition of the F-actin seeds did not rescue the defect of the ΔAB mutant derivative (Fig. 6 C).

The results described above clearly show that VASP can rescue the nucleation defect of an ActA derivative that cannot bind actin monomer. To determine whether the stimulatory effect of VASP requires a defective actin monomer-binding region or is a separate stimulatory mechanism, we directly evaluated the effect of VASP on full-length ActA. VASP dramatically enhanced the actin-nucleating activity of wild-type ActA, and this stimulation was more extensive than the levels achieved by the addition of F-actin seeds (Fig. 6 D). This suggests that VASP may not be simply substituting for the actin monomer-binding region, but may instead be functioning to independently stimulate nucleation.

VASP decreases branching of actin filaments

The Arp2/3 complex binds to the sides of actin filaments, caps the pointed ends of actin filaments, and has been localized to the branch points of dendritic actin arrays in vitro and in vivo (Mullins et al., 1998; Svitkina and Borisy, 1999; Cameron et al., 2001). Stimulation of actin nucleation by WASP-family proteins increases the number of actin branches formed by the Arp2/3 complex (Blanchoin et al., 2000). To determine whether ActA stimulates the actin-branching activity of the Arp2/3 complex, we visualized the structures formed in vitro by staining actin filaments with rhodamine-phalloidin. The minimal concentration at which the nucleating activity of the Arp2/3 complex is saturated by ActA is 10:1 (Skoble et al., 2000), thus we used this ratio to determine whether ActA stimulated Arp2/3 complex–mediated actin branch formation, and found that 56.4% of the actin filaments generated were associated with branched structures (Fig. 7, A and B). In contrast, actin filaments polymerized in the presence of the Arp2/3 complex alone or the Arp2/3 complex and the ΔAB derivative were infrequently branched (2.0% and 3.6%, respectively). This demonstrates that ActA stimulates the branching activity of the Arp2/3 complex and that the actin monomer-binding region of ActA is required for this function.

Next, we wanted to evaluate whether VASP had any effect on actin filament architecture. The vast majority of actin filaments that were nucleated in the presence of VASP and the Arp2/3 complex were unbranched (2.6%) (Fig. 7, A and B). In the VASP-containing reactions there were occasional filaments that appeared thicker and many-fold brighter (unpublished data), suggesting that VASP may bundle actin filaments into parallel arrays. Because VASP was able to rescue the defect in nucleation of the ΔAB derivative, we wanted to test whether VASP could also rescue the branching defect of this mutant. However, actin filaments nucleated in the presence of the Arp2/3 com-

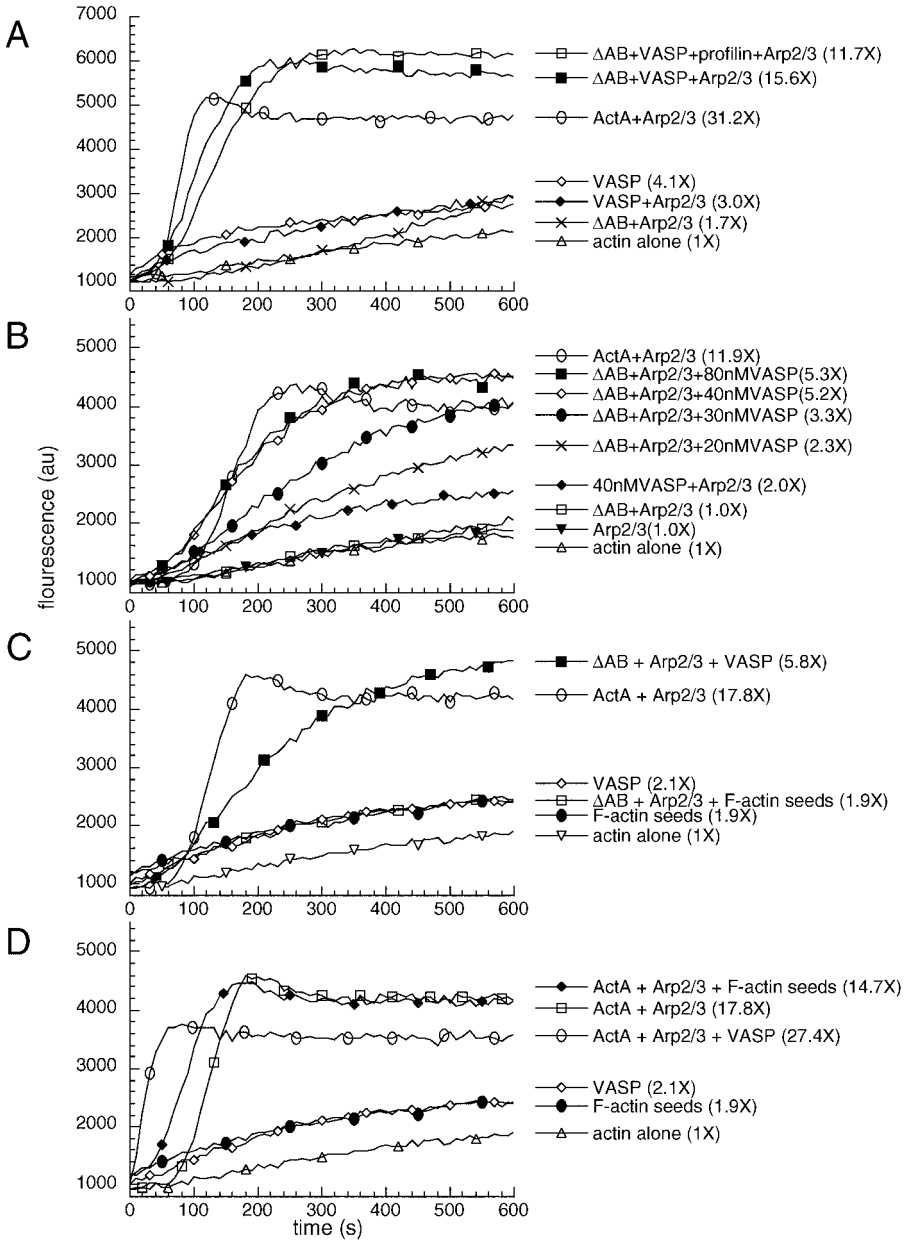

Figure 6. **Purified VASP rescues the actin nucleation defect of an ActA derivative deficient for actin monomer binding.** (A) Pyrene-actin polymerization assays carried out in the presence of VASP and profilin. Kinetics of 2 μM actin polymerization was monitored in the presence or absence of 100 nM ActA, ΔAB derivative, VASP, or profilin and in the presence or absence of 20 nM Arp2/3 complex as indicated. The fold increase in polymerization rate was determined by calculating the maximal rate of polymerization and dividing that value by the rate of spontaneous actin polymerization and is presented in parentheses. (B) VASP rescue of actin nucleation defect saturates at a ratio of 2:1 with ΔAB. Pyrene-actin polymerization assays were carried out with 2 μM actin and 20 nM Arp2/3 complex, with either 20 nM ActA or ΔAB in the presence of the indicated concentration of VASP. The fold increase in polymerization rate is presented in parentheses. (C) Actin filaments do not rescue the actin nucleation defect of the ΔAB derivative. Pyrene-actin polymerization assays were carried out with 2 μM actin in the presence or absence of 20 nM Arp2/3 complex, ActA or ΔAB, 40 nM VASP, or 200 nM phalloidin-stabilized F-actin seeds, as indicated. The fold increase in polymerization rate is presented in parentheses. (D) VASP stimulates actin nucleation by ActA and the Arp2/3 complex Pyrene-actin polymerization assays were carried out with 2 μM actin in the presence or absence of 20 nM Arp2/3 complex, ActA or ΔAB, 40 nM VASP, or 200 nM phalloidin-stabilized F-actin seeds, as indicated. The fold increase in polymerization rate is presented in parentheses.

plex, VASP, and the ΔAB derivative were only 3.2% branched. This demonstrates that although VASP can rescue the actin nucleation defect of the ΔAB derivative, the actin filaments produced have a dramatically different organization than those nucleated by wild-type ActA and the Arp2/3 complex. Furthermore, VASP also reduced branching by full-length ActA and the Arp2/3 complex by greater than fourfold, to 12.8%.

These observations suggest that actin filaments nucleated directly by ActA and the Arp2/3 complex form branched arrays, but that those nucleated in the presence of VASP form primarily unbranched structures that can be bundled into parallel arrays. The NH_2-terminal domain of ActA (A263), which has been shown to be fully capable of stimulating Arp2/3 complex–mediated actin nucleation (Welch et al., 1998; Skoble et al., 2000), also stimulated Arp2/3-dependent actin branching to 47.0% (Fig. 7, A and B). Despite the fact that this derivative lacks the proline-rich repeats of ActA, addition of VASP to this reaction also decreased actin branching, but less than twofold, to 28.7%. These data suggest that in vitro, the ability of VASP to inhibit actin branching is only partially dependent on the ability to be recruited by ActA.

Discussion

L. monocytogenes exploits a highly conserved mechanism of actin-based motility and provides a unique tool for dissection of the host factors involved in this process. The interaction of ActA with the Arp2/3 complex, VASP, and monomeric actin suggests that it recruits each of these proteins to play a critical role in motility. The results of this study provide both genetic and biochemical evidence that VASP recruitment to the surface of *L. monocytogenes* by the proline-rich repeats of ActA functions to stimulate actin nucleation. This finding was unexpected because the central domain of ActA was found to play no role in directly stimulating the

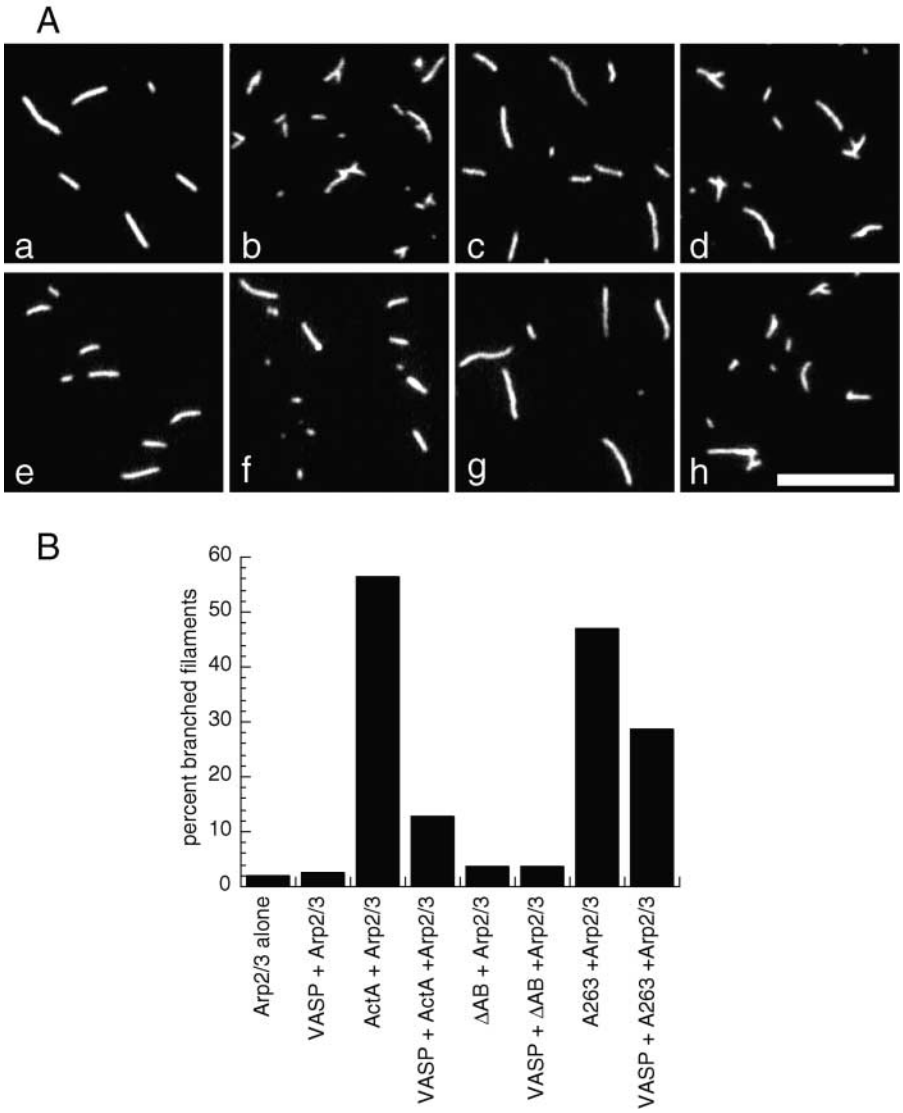

Figure 7. **VASP recruitment decreases actin branch formation by ActA and the Arp2/3 complex.** (A) Images of actin filament structures nucleated in the presence or absence of VASP. 4 μM actin was polymerized in the presence of 20 nM Arp2/3 complex (top) or 20 nM Arp2/3 complex and 200 nM VASP (bottom) with no ActA (a and e), 200 nM ActA (b and f) , 200 nM ΔAB (c and g), and 200 nM A263 (d and h). Actin structures were stabilized and labeled with addition of 4 μM rhodamine-phalloidin and visualized by direct microscopic observation. (B) Quantification of actin branches formed in presence of VASP. The values are presented as a percent of the total population of actin filaments that appear in branched structures. All of the filaments in a given field were counted; when two or more actin filaments were found to intersect, each was scored as branched. The data represent a minimum of 800 filaments for each condition from two to five independent experiments.

Arp2/3 complex (Skoble et al., 2000; Zalevsky et al., 2001). In fact, numerous studies have demonstrated that the central domain of ActA is not sufficient to induce actin polymerization in host cell cytoplasm (Friederich et al., 1995; Lasa et al., 1995, 1997; Pistor et al., 1995, 2000; Smith et al., 1996; Niebuhr et al., 1997; Bear et al., 2000; Skoble et al., 2000), although a recent report has demonstrated that filamentous actin can be recruited to the surface of beads coated with only this domain of ActA (Fradelizi et al., 2001). Previously, it had been proposed that this region functions to enhance the efficiency of motility by delivering profilin/actin complexes to elongating filaments (Theriot et al., 1994), or by recruiting Ena/VASP to anchor elongating filaments at the bacterial surface (Laurent et al., 1999). However, we find that in the absence of actin monomer-binding activity, the proline-rich repeats of ActA are required for the initiation of actin polymerization at the bacterial surface. In vitro, we find that addition of purified recombinant VASP rescues the actin nucleation defect of an ActA mutant that cannot bind actin monomer, and that profilin is not required for this rescue. These data suggest that VASP recruitment directly facilitates actin nucleation, and are consistent with the finding that VASP and profilin can independently increase the efficiency of actin-based motility (Loisel et al., 1999). Our findings bring to light the interplay between the central domain and the NH_2-terminal domain of ActA in actin nucleation; however, our observations do not exclude the possibility that profilin recruitment may further enhance motility.

ActA and WASP family proteins both require actin monomer-binding regions to directly stimulate actin nucleation with Arp2/3 complex (Machesky et al., 1999; Skoble et al., 2000). Thus, the observation that VASP, an F-actin binding protein, can rescue the actin nucleation defect caused by an actin monomer-binding region mutation was initially surprising. However, we also find that VASP stimulates actin nucleation by full-length ActA, suggesting that VASP is not merely substituting as a replacement for the actin-binding region, but rather that VASP stimulates nucleation by a distinct mechanism, perhaps by delivering actin filaments to the Arp2/3 complex. In support of this hypothesis, a number of F-actin binding proteins have recently been described that stimulate the nucleating activity of the Arp2/3 complex,

including cortactin, Myo1p, and Abp1p (Lee et al., 2000; Goode et al., 2001; Uruno et al., 2001; Weaver et al., 2001). The mechanism by which F-actin binding proteins stimulate the nucleating activity of the Arp2/3 complex is not well understood. It is possible that this class of nucleating proteins function by presenting actin filaments to the Arp2/3 complex, which stimulates dendritic nucleation. In the case of cortactin, stimulation of nucleation leads to increased actin branching (Weaver et al., 2001); however, we find that VASP decreases branching. Thus, whereas these F-actin binding proteins may stimulate nucleation via a similar mechanism, they appear to give rise to different filament structures, suggesting that Arp2/3-dependent actin branching may be separable from nucleation.

The different architecture of the actin filaments we observed in the presence or absence of VASP is reminiscent of the divergent structures observed in *L. monocytogenes* tails. These comet tails are composed of distinct populations of actin filaments: an outer cortex of long parallel actin filaments and a core containing shorter more orthogonal actin structures (Zhukarev et al., 1995; Sechi et al., 1997). The similarity of the filament populations observed in vitro with those formed in vivo, suggest that VASP may be responsible for organizing actin filaments into the parallel structures found at the cortex of comet tails, whereas the orthogonal filament architecture at the core may be organized by the branching properties of ActA and the Arp2/3 complex. Although VASP is most concentrated at the older bacterial pole, it is also localized throughout the longitudinal surface of intracellular *L. monocytogenes*. Thus, actin filaments parallel to the longitudinal axis of motile bacteria would encounter more VASP molecules than those extending from the rear of bacteria. Highlighting the structural role of VASP in actin-based motility, depletion of VASP from cell lysates prevents actin-based motility of *L. monocytogenes* but does not prevent actin cloud formation (Laurent et al., 1999), and mutants that cannot recruit VASP have defects in cloud-to-tail transition and in initiating motility (Smith et al., 1996; Niebuhr et al., 1997). Thus, VASP localization to the bacterial surface may play a critical role in enhancing actin nucleation and in generating an actin filament architecture that is optimal for motility. Consistent with this hypothesis, mutant *L. monocytogenes* that cannot form aligned longitudinal actin arrays are not motile (Kuhn et al., 1990; Zhukarev et al., 1995), and a mutant that cannot bind VASP polymerizes actin filaments that are threefold less aligned than wild type (Smith et al., 1996). Thus, it is possible that although actin polymerizes at the surface of these bacteria, without VASP the filaments are less capable of forming a structure that is productive for actin-based motility.

L. monocytogenes is studied as a simplified model system for the investigation of actin-based motility of host cells; however, cellular migration is significantly more complex than bacterial motility in that it requires precise directional coordination of protrusive forces. Although Ena/VASP proteins clearly enhance *L. monocytogenes* motility, these proteins have been recently found to have a negative regulatory effect on cellular migration (Bear et al., 2000). Our data provide at least two possible mechanisms for the enigmatic role of VASP in cellular migration. Overexpression of VASP could inhibit cellular migration by artificially stimulating actin nucleation at unproductive sites. Conversely, when VASP is sequestered this may increase the polarization of protrusion sites by increasing the threshold of actin nucleating factors required for lamellipodia formation. Alternatively, changing the local concentration of Ena/VASP may alter the architecture of actin filaments, making them more or less productive for motility. In support of this latter hypothesis, recent ultrastructural analyses of actin filament architecture in lamellipodia of cells absent of Ena/VASP family proteins demonstrates that these cells appear to have more highly branched actin structures. Furthermore, overexpression of Ena/VASP results in lamellipodia in which actin filaments appear to have fewer branches and are more parallel (F. Gertler, personal communication). Ultrastructural analyses of *L. monocytogenes* comet tails in these cells may provide further insight into the role of Ena/VASP family proteins in actin-based motility.

Materials and methods

Expression and purification of 6× His-tagged derivatives of ActA

L. monocytogenes strains secreting 6× His-tagged full-length ActA, the A263 derivative, and the ΔAB derivative (deleted of amino acids 60–101) were described previously (Table I). The deletion of residues 102–135 (ΔAB2) that remove the second putative actin-binding site was accomplished by splice- by-overlap extension (SOE) PCR (Horton et al., 1990) using primer 5′-GCAAAAGCAGAGAAAGGTCCGGGTCTGTCATCGGATAGCGCA-3′ and its reverse compliment as described in Skoble et al. (2000). The ABKE substitution (in which codons for lysine residues 94, 96, and 99 were changed to codons for glutamate) was generated using the Quickchange mutagenesis kit (Stratagene) according to manufacturer recommendations. pDP-3934 (Skoble et al., 2000) was used as template and primers 5′-GCAGACCTAATAGCAATGTTGGAAGCAGAAGCAGAGGAAGGTCCGAATAACAATAATAAC-3′ and its reverse complement were used to generate p-ABKE (pDP-4230). An HpaI/KpnI restriction fragment surrounding this mutation was cloned into pDP-2717 (Welch et al., 1998) which contains the full-length His-tagged ActA and the DNA sequence of the resulting plasmid pABKEHis (pDP-4231) was confirmed. This plasmid was transformed into the overexpression strain of *L. monocytogenes* DP-L3935 and 6× His-tagged proteins were purified from *L. monocytogenes* culture supernatants as described previously (Skoble et al., 2000).

Generation of isogenic ActA mutants

Isogenic 10403S-derived mutants expressing the ΔActA, ΔAB, and the PPP alleles were described previously (Table I). To replace the wild-type chromosomal copy of *actA* with the ABKE mutation, the temperature-sensitive plasmid pABKE (pDP-4230, above) was used. To generate the GGG mutation (from which residues 299–333 were deleted and proline residues 266, 267, 268, 269, 336, 337, 338, 339, 381, 382, and 384 were changed to glycine), a plasmid encoding the GGG *actA* allele was constructed by performing three consecutive rounds of SOE PCR (Horton et al., 1990). For the first round of PCR, pDP2118, which contains a deletion of proline-rich repeat 2 corresponding to the deletion in PPP, was used as the template (Smith et al., 1996). Primers 5′-CTCTTCTTCTGTTCCGATTCCTCCGAAATCAGAGAAATTTTC-3′ and its reverse compliment were used to mutagenize proline-rich repeat 3. This mutated fragment was gel purified, and then used as the template for the next round of SOE PCR to mutate proline-rich repeat 1 as described previously (Smith et al., 1996). The resultant fragment was purified and used as template for mutagenesis of proline-rich repeat 4 as described previously (Smith et al., 1996). The final PCR product was digested using SalI and KpnI and ligated into similarly digested pDP-3934 to generate pGGG (pDP-4035). Plasmids encoding the mutation ΔAB/GGG (pDP-4234) and ABKE/GGG (pDP-4238) were generated by sublcloning SphI/KpnI fragments from pΔAB (pDP-3993) and pABKE (pDP-2430) into pGGG (pDP-4035). To replace the chromosomal copy of *actA* with the mutated alleles, allelic exchange was performed as described by Skoble et al. (2000). To verify that each strain contained the desired de-

Table I. ***L. monocytogenes*** **strains used in this study**

actA allele	Strain number: surface-associated ActA	Strain number: secreted 6× His-tagged ActA	Reference
WT	10403S	DP-L2723	Bishop and Hinrichs, 1987; Welch et al., 1998
ΔActA	DP-L3078	–	Skoble et al., 2000
ΔAB	DP-L3944	DP-L3987	Skoble et al., 2000
ABKE	DP-L4232	DP-L4233	This study
ΔAB2	–	DP-L4226	This study
PPP	DP-L2157	–	Smith et al., 1996
GGG	DP-L4032	–	This study
ΔAB/GGG	DP-L4236	–	This study
ABKE/GGG	DP-L4248	–	This study
A263	–	DP-L4007	Welch et al., 1998

letion, chromosomal DNA was amplified by PCR and the region flanking each deletion was sequenced.

Analysis of ActA expression

To confirm that the desired allele of *actA* was expressed on the surface of *L. monocytogenes*, bacteria were grown to an OD_{600} of ~0.5, and then washed once with PBS containing 10 mM EDTA. Surface proteins were extracted from samples corresponding to equivalent bacterial numbers (calculated by OD_{600}) as described previously (Mourrain et al., 1997; Skoble et al., 2000). Extracted proteins were separated on a 7.5% SDS-PAGE gel and transferred to a nitrocellulose membrane (Bio-Rad Laboratories). ActA was detected by immunoblotting using affinity-purified rabbit polyclonal antibodies (A18K) raised against a peptide derived from the first 18 residues of mature ActA (Mourrain et al., 1997) , provided by Pascale Cossart (Institut Pasteur, Paris, France), followed by detection with ECL-plus detection system as described by the manufacturer (Amersham Pharmacia Biotech).

[^{35}S] methionine–labeling of bacterial surface proteins from *L. monocytogenes* strains grown within J774 macrophage-like cells was performed as described (Brundage et al., 1993).

Analysis of *L. monocytogenes*infected tissue culture cells

HeLa and PtK2 cells were grown on glass coverslips in RPMI 1640 medium (Life Technologies) supplemented with 10% FBS, 2 mM glutamine and, 1 mM pyruvate. Subconfluent monolayers were infected with *L. monocytogenes* as described (Smith et al., 1996). For VASP staining, HeLa cells were fixed with 3.2% paraformaldehyde at 3.5 h after infection. VASP was detected with affinity-purified rabbit polyclonal anti–human VASP primary antibodies (Smith et al., 1996), followed by rhodamine-conjugated goat anti–rabbit secondary antibodies (Jackson ImmunoResearch Laboratories). *L. monocytogenes* were stained directly with fluorescein-conjugated anti–*L. monocytogenes* antibodies (DIFCO Laboratories). For quantification of bacteria associated with F-actin, infected PtK2 Cells were fixed as above and stained with rhodamine-phalloidin and rabbit polyclonal anti-*Listeria* antibodies (DIFCO Laboratories) followed by FITC-conjugated donkey anti–rabbit secondary antibodies. Infected cells were visualized using a TE300 inverted microscope (Nikon). Images were captured with a CCD camera (Hammamatsu) and pseudocolored and merged using Metamorph software (Universal Imaging).

Plaque assays were performed in monolayers of L2 fibroblast cells as described previously (Jones and Portnoy, 1994). Plaque size was determined by capturing images using a digital camera and measuring the diameter of at least 15 plaques per experiment using Canvas software (Deneba). Mutant plaque size was compared with wild type for each experiment and the mean percent plaque size was averaged from between five and twelve independent experiments.

Purification of human VASP and profilin

Human VASP was expressed in *Spodoptera frugiperda* Sf9 cells using the Bac-to-Bac baculovirus expression system (Invitrogen). To generate an appropriate baculovirus strain, the VASP cDNA (EST clone ID 844271; Incyte Genomics) was amplified by PCR using the following primers: 5′-CGAGGATCCATGAGCGAGACGGTCATCTG-3′, and 5′-CGAGGTACCTCAGGGAGAACCCCGCTTCCTCAG-3′. The PCR product was digested with BamHI and KpnI and subcloned into the corresponding sites in the pFastBacHTb vector (Invitrogen). A baculovirus strain was prepared from this expression vector according to procedures supplied with the Bac-to-Bac system.

For VASP expression, Sf9 cells were infected for 72 h at 27°C with the baculovirus strain expressing VASP. Infected cells were pelleted by centrifugation at 250 g for 10 min, washed once with PBS, and pelleted again. Pellets were quick frozen in liquid N_2, and cells were lysed by thawing in an equal volume 50 mM KPO_4, 300 mM KCl, and LPC (10 μg/ml leupeptin, pepstatin A, and chymostatin from Chemicon International). The cellular debris was pelleted at 250,000 *g* and the extract was supplemented with 20 mM imidazole. 1 ml of NiNTA agarose, equilibrated in wash buffer (50 mM KPO_4, 300 mM KCl, and 20 nM imidazole), was incubated with the extract for 1 h at 4°C. The beads were pelleted by centrifugation at 200 *g*, washed three times with wash buffer, and then transferred to a 2-ml capacity quick spin column. The beads were washed with three-column volumes of wash buffer supplemented with a final concentration of 1 M KCl, and three times in wash buffer by gravity flow. Excess buffer was removed by centrifugation at 200 *g* for 1 min. Bound proteins were eluted by the addition of 1 M imidazole. VASP was further purified by gel filtration chromatography over a Superdex 200 column (Pharmacia) equilibrated in control buffer (20 mM MOPS pH 7.0, 100 mM KCl, 2 mM $MgCl_2$, 5 mM EGTA, 1 mM EDTA, 0.2 mM ATP, 0.5 mM DTT, 10% vol/vol glycerol). VASP was then concentrated using a 30-kD centrifugal filtration device (Millipore). Profilin was purified from human platelets as described previously (Janmey, 1991).

Pyrene-actin polymerization assays

Pyrene-actin polymerization assays were performed as described previously (Skoble et al., 2000) with the following modifications. Pyrene-actin and unlabeled actin were mixed in G-buffer (2 mM Tris pH 7.6, 0.2 mM $CaCl_2$, 0.2 mM ATP, 0.2 mM DTT) to generate a 6-μM monomeric actin solution with <10% pyrene-actin. 6 μl of control buffer (20 mM MOPS, pH7.0, 100 mM KCl, 2 mM $MgCl_2$, 5 mM EGTA, 1 mM EDTA, 0.2 mM ATP, 0.5 mM DTT 10% v/v glycerol) or Arp2/3 complex, VASP, or Profilin in control buffer, was mixed with 6 μl 10 × initiation buffer (20 mM $MgCl_2$, 10 mM EGTA, 5 mM ATP) and 28 μl G-buffer or up to 28 μl ActA in G-buffer. This 40 μl solution was mixed with 20 μl of monomeric actin solution to initiate polymerization. Assembly kinetics were monitored using a Fluorolog 3 fluorometer (excitation wavelength 365 nm, emission wavelength 407 nm; Instruments S.A.) maintained at a temperature of 25°C. The maximal rate of polymerization was determined by calculating the linear regression of the maximal slope using Kaleidagraph software (Synergy Software). Fold inhibition of polymerization by ActA was calculated by dividing the maximal rate of polymerization in the absence of ActA by the maximal rate in the presence of ActA.

Branching assay

The branching assay was carried out essentially as described in by Blanchoin et al. (2000) and Amann and Pollard (2001). All proteins except actin monomer were mixed at room temperature in 1× KMEI (50 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 10 mM imidazole pH 7.0) with 0.2 mM ATP and 1 mM DTT. Actin monomer in G-buffer (5 mM Tris pH 7.4, 0.2 mM $CaCl_2$, 0.2 mM DTT, 0.2 mM ATP) was added to a final concentration of 4 μM. After polymerization proceeded for 1 min, rhodamine-phalloidin was added to a final concentration of 4 μM. Polymerization was allowed to proceed at room temperature for 5 min after the addition of rhodamine-phalloidin. Samples were diluted 625-fold into fresh fluorescence buffer (50 mM KCl, 1 mM $MgCl_2$, 100 mM DTT, 10 mM imidazole, pH 7.0, 0.5% methylcellulose, 20 μg/ml catalase, 100 μg/ml glucose oxidase, 3 mg/ml glucose) and 1.3 μl diluted samples were applied to coverslips precoated with 0.1% nitrocellulose in amyl acetate. Branches were visualized by fluorescence microscopy and images were analyzed using Metamorph software (Universal Imaging).

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