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Three Regions within ActA Promote Arp2/3 Complex-mediated Actin Nucleation and *Listeria monocytogenes* Motility

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**Abstract.** *Listeria monocytogenes* ActA protein induces actin-based motility by enhancing the actin nucleating activity of the host Arp2/3 complex. Using systematic truncation analysis, we identified a 136-residue NH$_2$-terminal fragment that was fully active in stimulating nucleation in vitro. Further deletion analysis demonstrated that this fragment contains three regions, which are important for nucleation and share functional and/or limited sequence similarity with host WASP family proteins: an acidic stretch, an actin monomer-binding region, and a cofilin homology sequence. To determine the contribution of each region to actin-based motility, we compared the biochemical activities of ActA derivatives with the phenotypes of corresponding mutant bacteria in cells. The acidic stretch functions to increase the efficiency of actin nucleation, the rate and frequency of motility, and the effectiveness of cell-cell spread. The monomer-binding region is required for actin nucleation in vitro, but not for actin polymerization or motility in infected cells, suggesting that redundant mechanisms may exist to recruit monomer in host cytosol. The cofilin homology sequence is critical for stimulating actin nucleation with the Arp2/3 complex in vitro, and is essential for actin polymerization and motility in cells. These data demonstrate that each region contributes to actin-based motility, and that the cofilin homology sequence plays a principal role in activation of the ActA complex, and is an essential determinant of *L. monocytogenes* pathogenesis.

Key words: bacteria • pathogenesis • cell movement • cytoskeleton • microfilament proteins

**Introduction**

The bacterial pathogen *Listeria monocytogenes* enters mammalian cells and escapes from the phagosome into the host cytosol, where it proliferates rapidly. In the host cytosol, *L. monocytogenes* induces the polymerization of actin filaments at its surface and initiates motility, generating comet tails of actin filaments and actin binding proteins that trail the moving bacteria (for review see Ireton and Cossart, 1997). The propulsive force for intracellular motility is derived from actin filament elongation at the interface between the bacterium and the actin tail (Mogilner and Oster, 1996), which remains fixed in place (Sanger et al., 1992; Theriot et al., 1992). Moving bacteria encounter the host plasma membrane and form filopod-like protrusions that are engulfed by neighboring cells (Tilney and Portnoy, 1989). A ctn-based motility is essential for *L. monocytogenes* pathogenesis (Domann et al., 1992; Kocks et al., 1992; Brundage et al., 1993), and has been studied as a model for understanding the regulation of actin dynamics in eukaryotic cells.

The bacterial cell surface protein ActA is necessary and sufficient for actin-based motility in host cytosol (Domann et al., 1992; Kocks et al., 1992; Pistor et al., 1994; Smith et al., 1995; Cameron et al., 1999). ActA can be divided into three domains that have distinct functions. The COOH-terminal domain (amino acids 391–639) contains a transmembrane sequence that is essential for anchoring ActA to the bacterial surface. The central domain (amino acids 264–390) contains four proline-rich repeats that bind to E-nablvd/vasodilator-stimulated phosphoprotein (Ena/VASP) family proteins (Chakraborty et al., 1995; Gertler et al., 1996; Smith et al., 1996), which in turn bind to actin filaments (R einhard et al., 1992; Bachmann et al., 1999) and the actin-binding protein profilin (Reinhard et al., 1995). The central domain is not required for actin-based motility, but contributes to the rate of movement and the percentage of moving bacteria (Lasa et al., 1995; Pistor et al., 1995; Smith et al., 1996). In contrast, the mature NH$_2$-terminal domain (amino acids 30–263) is essential for actin polymerization...
in host cytosol (Las et al., 1995; Pistor et al., 1995), and can be sufficient for motility if artificially attached to the bacterial membrane (Las et al., 1997). However, the NH₂-terminal domain does not directly stimulate actin polymerization (Welch et al., 1998).

A ctr1 nucleation at the surface of L. monocytogenes is mediated by the NH₂-terminal domain of A ctrA together with the A rp2/3 complex (Welch et al., 1997, 1998), an evolutionarily conserved host protein complex consisting of the actin-related proteins, A rp3, A rp2 and A rp3, and five other subunits (for review see Machesky and Gould, 1999). The A rp2/3 complex is required for bacterial actin-based motility in cell-free extracts (Egile et al., 1999; May et al., 1999; Ylar et al., 1999) and for reconstitution of actin-based motility from purified cytoskeletal proteins (Losel et al., 1999). Three biochemical activities have been assigned to the A rp2/3 complex: pointed-end capping of actin filaments; cross-linking filaments into branched arrays; and weak nucleating activity thought to occur by stabilization of actin dimers (Mullins et al., 1997, 1998). In vitro, the nucleating activity of the A rp2/3 complex is dramatically stimulated by full-length A ctrA and by a truncated derivative consisting only of the mature NH₂-terminal domain (Welch et al., 1998). However, the regions within A ctrA that stimulate the A rp2/3 complex and the contribution of this interaction to motility and pathogenesis, have not been addressed.

In this study, we used deletion and truncation mutations in A ctrA to define the minimal fragment that nucleates actin polymerization with the A rp2/3 complex and to identify the functional elements within this fragment that contribute to nucleation in vitro and actin-based motility in infected cells. We identified three important regions, each of which shares limited sequence similarity or functional similarity with the Wiscott-Aldrich Syndrome protein (WA SP) family of proteins, which has emerged as candidates for mediating actin polymerization at cell membranes by stimulating A rp2/3 complex nucleation activity (for reviews see Machesky and Insall, 1999; Welch, 1999).

By comparing the activities of mutant A ctrA proteins in vitro with their corresponding phenotypes in cells, we have correlated the biochemical function of each region with its role in actin-based motility.

Materials and Methods

Generation of Mutations in the A ctrA Gene

Plasmids encoding full-length 6xHis-tagged A ctrA and the derivative truncated at amino acid 263 were described previously (Welch et al., 1998). A ditional truncations in actrA were generated by PCR using VENT DNA polymerase (New England Biolabs) and the wild-type L. monocytogenes strain 10403S chromosomal DNA as a template. Primer CC CCGGATCTCGT CCCTCGGGAG a GACGA G (DP-1717; BamHI site underlined) anneals upstream of the A ctrA promoter, and was used in combination with the downstream primer GCTCAAAGT AATG TGATG TGGTGG TAGTGG TACGGT CCGGTCGCCGTATTCCTGCT (Xbal and Xmal sites underlined) to amplify a DNA fragment that encodes the first 59 codons of actrA, followed by a 6xHis tag. The BamHI-Xbal fragment was ligated into the gram-positive shuttle vector pMA401 (Wirth et al., 1996) to yield plasmid pDP-3936. Each subsequent truncated derivative (truncated at amino acids 201, 165, 135, and 101) was generated by PCR using primer DP-1717 with primers listed in Table I (Xmal sites underlined). PCR products were digested with Xbal and Xmal, and subcloned into pDP-3936. For each construct, the DNA sequence of the insert was verified.

In-frame deletions in actrA were generated using “splicing by overlap extension PCR” (Horton et al., 1999) as described previously (Smith et al., 1996). For each deletion, two primers were generated that were reverse complements of one another and encoded bases flanking the region to be deleted. The forward primers are listed in Table I. Fragments upstream and downstream of the region to be deleted were amplified, and each pair of amplified fragments was spliced in a third reaction. To generate 6xHis-tagged deletion derivatives, spliced fragments were cloned into pDP-2717, which contains the full-length His-tagged A ctrA (Welch et al., 1998).

Allelic Exchange of In-frame A ctrA Deletions

To replace the 6xHis copy of actrA on the 10403S chromosome with deletion alleles, DNA fragments were subcloned into temperature-sensitive vectors pksV7 (Smith and Yungman, 1992), pCON1 (Moria et al., 1999), or pDP-3934 (a derivative of pksV7 that contains a fragment of L. monocytogenes chromosomal DNA). The resulting plasmids were transformed into 10403S and allelic exchange was performed as described previously (Camilli et al., 1993), yielding strains listed in Table I. This strategy allowed the generation of isogenic strains of L. monocytogenes in which each allele was present in a single copy on the chromosome, maintaining all upstream regulatory elements as well as the endogenous transmembrane domain.

To verify that each strain contained the desired deletion, chromosomal DNA was amplified by PCR and the region flanking each deletion was sequenced. To confirm that the desired allele of actrA was expressed on the surface of L. monocytogenes, bacteria were grown to mid-log phase in LB medium, washed with PBS, and the surface proteins were extracted by boiling in SDS-PAGE GE sample buffer as described previously (Brandman et al., 1993; Mourrain et al., 1997). This treatment does not perturb the cell wall of L. monocytogenes and, thus, proteins in the bacterial cytosol are not released. Extracted proteins from cultures with equivalent cell densities (measured by taking the OD at 600 nm) were separated on a 7% SDS-PAGE gel and transferred to Immobilon-P membranes (Millipore). A ctrA was detected by immunoblotting using rabbit polyclonal antisera (D-P-3997) raised against full-length His-tagged A ctrA.

Expression and Purification of 6xHis-tagged A ctrA Derivatives

To eliminate the possibility that purified A ctrA derivatives were contaminated with endogenous A ctrA, an expression strain of L. monocytogenes was generated in which the entire chromosomal actrA gene was deleted. This strain was produced by transforming L. monocytogenes strain DP-L1545 with a vector encoding a derivative of A ctrA with amino acids 7–633 deleted (D-P-3076) and performing allelic exchange as described above, yielding strain D-P-L3935. The parent strain D-P-L1545 is an mpl derivative of SLCC-5764 (a strain that constitutively expresses high levels of A ctrA), which does not secrete the prfA-regulated metalloprotease that can degrade expressed A ctrA (Robbins et al., 1999). D-P-L3935 was used to express high levels of A ctrA derivatives.

To isolate 6xHis-tagged A ctrA derivatives, plasmids encoding these proteins were transformed into D-P-L3935, and secreted A ctrA derivatives were purified using procedures adapted from Welch et al. (1998). A ctrA derivative derivatives were precipitated from culture supernatants by adding ammonium sulfate to 400 g/liter (~60% saturation). The precipitate was resuspended in wash buffer (20 mM Tris, pH 8.0, 250 mM NaCl, 20 mM imidazole, pH 7.0) and bound to 0.5 ml of nickel-ni-trate agarose (Qiagen) per liter equivalent. The resin was washed 10 times with wash buffer and eluted with three bead volumes of wash buffer supplemented with 1 M imidazole, pH 7.0. Eluted proteins were desalted by passing two times over a G-25 (A sherman Pharmacia Biotech) spin column equilibrated in 2 mM Tris, pH 7.6, 0.2 M CaCl₂ and concentrated using microcon concentrators (Millipore). Protein concentrations were determined in triplicate by the BCA assay (Pierce) using BSA as a standard.

The truncated derivatives of A ctrA were not precipitated by ammonium sulfate, but were purified from culture supernatants by an alternative
method adapted from Cameron et al. (1999). Overnight cultures were diluted 1:100 into modified D10 media supplemented with 10 μg/ml chloramphenicol and incubated for ∼12 h at 37°C with shaking. Secreted proteins were bound to Q-Sepharose fast flow resin (Amersham Pharmacia Biotech) and eluted with 20 mM Tris, pH 8.0, 1 M NaCl. Proteins in the eluate were bound to NiNTA agarose beads and purified as described above.

**Pyrene-Actin Polymerization Assays**

Human platelet Arp2/3 complex (Welch and Mitchison, 1998), rabbit skeletal muscle actin (Spudich and Watt, 1971), and pyrene-labeled actin (Kouyama and Mihashi, 1981) were prepared as described previously. Pyrene-actin polymerization assays were performed as described previously (Cooper et al., 1983) with the following modifications. Pyrene-actin and unlabeled actin were mixed in G-buffer (2 mM Tris, pH 7.6, 0.2 mM CaCl₂, 0.2 mM ATP, 0.2 mM DTT) to generate a 40 μM monomer (g-actin) solution with ∼20% pyrene-actin. 6 μl of 200 nM Arp2/3 complex or 6 μl of control buffer (20 mM MOPS, pH 7.0, 100 mM KCl, 2 mM MgCl₂, 5 mM EGTA, 1 mM EDTA, 0.5 mM DTT, 0.2 mM ATP, 10% vol/vol glycerol, 2 mg/ml BSA and LPC) were incubated with 10% FBS, 2 mM glutamine and 1 mM pyruvate. Subconfluent monolayers were infected with 100 μl CoIP buffer at room temperature for 30 min. The precipitates were washed three times with high salt sonication buffer and three times with CoIP buffer (20 mM HEPES, pH 7, 10 mM MgCl₂, 5 mM EGTA, 1 mM EDTA, 0.5 mM DTT, 0.2 mM ATP, 10% vol/vol glycerol, 2 mg/ml BSA and LPC). The high salt-washed Arp2/3 complex–coated beads were not contaminated by Arp2/3 binding proteins, as determined by SDS-PAGE and silver staining, nor were they contaminated with the Aβ isoform binding proteins or VASP, as determined by immunoblotting (data not shown). For Aβ binding experiments, 50 μl IgG and 100 μl CoIP buffer lacking BSA. Bound proteins were eluted from the beads by the addition of SDS sample buffer, resolved on a 7% SDS-PAGE gel, and transferred to nitrocellulose membranes; Aβ was detected by immunoblotting using a rabbit polyclonal antisera (D P-3997).

**Analysis of L. monocytogenes–infected Tissue Culture Cells**

HELa and Potroo tridactylis kidney epithelia (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 previously (Smith et al., 1996). For fluorescence staining, HELa cells were fixed with 3.2% paraformaldehyde at 3.5 h after infection. Aβ was stained with rhodamine-labeled phalloidin (Molecular Probes). VASP was detected with an affinity-purified rabbit polyclonal anti-VA SP primary antibody (Smith et al., 1996), followed by an FITC–conjugated donkey anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, Inc.). Host and bacterial DNA were stained with DAPI (4,6-diamidino-2-phenylindole). For quantification of bacteria associated with F-actin, infected PtK2 cells were fixed with 3.2% paraformaldehyde at 3.5 h after infection. Aβ was stained with rhodamine-labeled phalloidin (Molecular Probes). VASP was detected with an affinity-purified rabbit polyclonal anti-VA SP primary antibody (Smith et al., 1996), followed by an FITC–conjugated donkey anti-rabbit secondary antibody. Infected cells were visualized using a TE 300 inverted microscope (Nikon). Images were captured with a CCD camera (Hamamatsu), pseudocolored, and were merged using Phase 3 imaging systems software.

**Immunoprecipitation of the Arp2/3 Complex and ActA**

Polyclonal anti-p41 antibody (Y arar et al., 1999) and anti-goat IgG ( Jackson Immunoresearch Laboratories, Inc.) were covalently coupled to A f-fiprep protein-A support (Bi-o-Rad Laboratories) by incubation with 20 mM dimethyl pimelimidate (Pierce Chemical Co.). To generate an Aβ isoform–coated beads, anti-p41 beads were incubated for 30 min at 4°C in human platelet extract that was preincubated with IgG–coated beads for 10 min at 4°C. Platelet extract was prepared by sonicating platelets in high salt sonication buffer (20 mM Tris, pH 8.0, 5 mM EGTA, 1 mM EDTA, 600 mM KCl, 0.1 mM glycerol, 2 mg/ml BSA and LPC). The high salt-washed Arp2/3 complex–coated beads were not contaminated by Arp2/3 binding proteins, as determined by SDS-PAGE and silver staining, nor were they contaminated with the Aβ isoform binding proteins or VASP, as determined by immunoblotting (data not shown). For Aβ binding experiments, 50 μl IgG and 100 μl CoIP buffer lacking BSA. Bound proteins were eluted from the beads by the addition of SDS sample buffer, resolved on a 7% SDS-PAGE gel, and transferred to nitrocellulose membranes; Aβ was detected by immunoblotting using a rabbit polyclonal antisera (D P-3997).
Measurements were terminated if the bacteria stopped moving or encountered the plasma membrane. Plaque assays were performed in monolayers of L2 fibroblast cells as described previously (Sun et al., 1990; 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 (Deneba Software). Mutant plaque size was compared with wild type for each experiment.

Results

A 136–Amino Acid NH$_2$-terminal Fragment of ActA Is Sufficient to Stimulate Arp2/3 Complex Nucleating Activity

To define the regions within the NH$_2$-terminal domain of A ctA that contribute to nucleation, we generated a series of 6xHis-tagged A ctA derivatives that were truncated at amino acids 201, 165, 135, and 101 (A 201, A 165, A 135, and A 101, respectively; Fig. 1 b). Each was truncated at a proline residue, where the secondary structure was predicted to be a turn (Chou and Fasman, 1974), increasing the likelihood that the native secondary structure was preserved in the truncated molecules. Truncated A ctA derivatives were expressed and purified (Fig. 1 c), and the capacity of each derivative to stimulate the nucleating activity of the Arp2/3 complex was measured using the pyrene-actin polymerization assay (Kouyama and Mihashi, 1981; Cooper et al., 1983). As previously reported (Welch et al., 1998), equimolar concentrations of full-length A ctA and Arp2/3 complex acted synergistically to accelerate actin nucleation, whereas A ctA or Arp2/3 complex alone at this concentration had a negligible effect on polymerization kinetics (Fig. 2 a). The A ctA derivatives A 263, A 201, and A 165 were as potent as the full-length protein in their ability to stimulate polymerization with the Arp2/3 complex. In contrast, A 135 and A 101 had virtually no stimulatory effect. This suggests that the region between amino acids 135 and 165, which contains a cofilin homology sequence similar to that found in W A SP family proteins (Fig. 1 a; Bi and Zigmond, 1999), is critical for actin nucleating activity. These derivatives were also tested for their ability to stimulate nucleation when present at a 10-fold molar excess relative to the Arp2/3 complex. As was observed at lower concentrations, A 165 and full-length A ctA were equivalent in stimulating nucleation (Fig. 2 b). A 135 and A 101 stimulated nucleation to a greater extent than they had at lower concentrations, but were less potent than A 165 (Fig. 2 b). By plotting the fold stimulation of the maximum rate of polymerization versus the concentration of full-length A ctA or A 101 (see Fig. 5), we were able to quantitatively compare the relative activities of these polypeptides. Whereas full-length A ctA stimulated polymerization up to 25-fold at 10 times the concentration of Arp2/3 complex (see Fig. 5), A 101 caused only a 3-fold stimulation at concentrations up to 100 times that of A 135.
ActA is predicted to have a turn at proline 59 (Chou and Fasman, 1974), which may separate A101 into two distinct regions. One region (amino acids 30–59) contains a high proportion of acidic residues (11/28 are glutamate or aspartate) that share sequence similarity with the acidic stretch in WASP family proteins (Fig. 1 a). The other (amino acids 59–101) has been previously reported to have actin monomer-binding activity (Lasa et al., 1997; Cicchetti et al., 1999). The above findings indicate that these two regions alone retain some stimulatory capacity, suggesting that they contribute to nucleation in the context of the full-length protein.

### Three Regions within the NH2-terminal Domain of ActA Have Distinct Functions in Actin Nucleation with the Arp2/3 Complex

To better assess the contribution of each region within the NH2-terminal domain of ActA to actin nucleation, we generated and purified a series of 6xHis-tagged in-frame deletion derivatives of ActA (Fig. 3, a and b). The capacity of these derivatives to nucleate polymerization with the Arp2/3 complex was determined using the pyrene-actin polymerization assay. Consistent with the results of the truncation analysis, a derivative lacking the entire NH2-terminal domain (Δ31-262) was unable to enhance nucleation when present at an equal concentration (Fig. 4 a) or at a 10-fold molar excess (Fig. 4 d) with respect to the Arp2/3 complex. A another derivative (Δ202-263), which based on the truncation analysis was missing amino acids that are not critical for nucleation, was as active as full-length ActA (Fig. 4 a), indicating that large deletions within this domain can be tolerated without reducing activity.

A derivative missing the region that harbors the cofilin homology sequence (Δ136-165) had virtually no stimulatory effect on polymerization kinetics when added at equimolar concentrations with the Arp2/3 complex (Fig. 4 b). Other ActA derivatives containing larger deletions encompassing this region (Δ136-200, Δ60-165, and Δ31-165) exhibited comparable activity at this same concentration (Fig. 4 b). To more specifically assess the function of the cofilin homology sequence, a derivative was constructed in which the five basic residues at the core of this sequence were removed (Δ146-150; Fig. 3 a). The activity of this pro-

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**Figure 3.** In-frame ActA deletions. (a) Diagram of secreted 6xHis-tagged derivatives of ActA that contain in-frame deletions within the NH2-terminal domain. Deleted residues are indicated on the left. (b) Purified derivatives of ActA containing in-frame deletions visualized on a 7.5% polyacrylamide gel stained with Coomassie blue.

**Figure 4.** Effects of ActA deletion derivatives and the Arp2/3 complex on the kinetics of actin polymerization. (a–d) Graphs of fluorescence intensity versus time after initiating polymerization in the pyrene-actin polymerization assay. (a–c) 2 μM actin in the presence or absence of 20 nM Arp2/3 complex and 20 nM ActA derivatives. (d) 2 μM actin in the presence or absence of 20 nM Arp2/3 complex and 200 nM ActA derivatives.
tein was identical to those with larger deletions encompassing this region (Fig. 4 b). Because of the relatively conservative nature of its lesion, Δ146-150 was chosen for further analysis. Increasing the concentration of Δ146-150 to 10-fold molar excess over the Arp2/3 complex resulted in an increase in its nucleating activity relative to lower concentrations (Fig. 4 d), although the degree of stimulation was far less than that of the full-length protein. This indicates that Δ146-150 retains some concentration-dependent nucleating activity.

A n A ctA derivative harboring a deletion of the acidic stretch (Δ31-58) was able to accelerate nucleation when added at equal concentrations relative to the A rp2/3 complex, but was less active than full-length A ctA (Fig. 4 c). Increasing the concentration of Δ31-58 to a 10-fold excess over the Arp2/3 complex resulted in a higher degree of stimulation (Fig. 4 d). At both the lower and higher concentrations, Δ31-58 was less potent than the full-length protein, but more potent than mutants lacking the cofilin homology sequence. Finally, a derivative missing the putative actin-binding region (Δ60-101; Lasa et al., 1997; Cicchetti et al., 1999) caused virtually no enhancement of polymerization kinetics when combined at equal concentrations (Fig. 4 c) or a 10-fold excess relative to the Arp2/3 complex (Fig. 4 d). At both concentrations, the activity of Δ60-101 was comparable to that of Δ31-262 (Fig. 4, c and d), which is missing the entire NH2-terminal domain and is essentially inactive.

The relative activities of the derivatives missing the cofilin homology sequence (Δ146-150), the putative actin-binding region (Δ60-101), and the acidic stretch (Δ31-58) were quantified and compared. For increasing concentrations of each derivative, the fold stimulation of the maximum rate of polymerization with the Arp2/3 complex was plotted versus the concentration of the derivative (Fig. 5). Full-length A ctA stimulated the maximal rate of polymerization up to 25-fold, reaching saturation at a concentration 10 times that of the A rp2/3 complex. In contrast, at concentrations 100 times that of A rp2/3 complex, Δ146-150 stimulated polymerization 5-fold (1/5 of the maximum achieved by full-length), Δ60-101 stimulated polymerization 2-fold (1/12 that of full-length), and Δ31-58 stimulated polymerization 13-fold (1/2 that of full-length). Thus, the cofilin homology sequence and the acidic stretch are important for stimulating nucleation, and the putative actin-binding region plays an essential role in this process in vitro.

**ActA Binds Actin Monomer through Its Actin-binding Region**

To confirm that A ctA binds to actin monomer (Lasa et al., 1997; Cicchetti et al., 1999), and to assess the location of the actin-binding region, we evaluated the ability of the full-length A ctA and selected truncation and deletion derivatives to inhibit the rate of actin polymerization (Fig. 6 a). Increasing concentrations of the full-length A ctA in-
cluded in the pyrene-actin assay (in the absence of the Arp2/3 complex) caused a dose-dependent reduction in the maximum rate of polymerization (Fig. 6a), but did not affect the steady state amount of actin polymer (not shown). Deletion of the NH2-terminal domain of ActA (Δ31-262) or the putative actin-binding region (Δ60-101) rendered ActA unable to inhibit actin polymerization even at a two-fold molar excess relative to actin. In contrast, a truncation derivative consisting of the acidic and actin-binding regions (A101) and the deletion derivatives, missing the acidic stretch (Δ31-58) and the cofilin homology region (Δ136-165), was still able to inhibit actin polymerization. Deletion of the acidic stretch slightly enhanced the ability of ActA to inhibit actin polymerization, whereas deletion of the cofilin homology sequence slightly retarded this activity. The inhibition of polymerization was due to actin monomer binding and not filament capping because derivatives did not inhibit filament depolymerization in a pyrene-actin depolymerization assay (data not shown). These results were confirmed by measuring the ability of ActA derivatives to inhibit polymerization when included at a five-fold molar excess relative to actin in a pelleting assay (Fig. 6b). Our results demonstrate that ActA binds to the actin monomer through an actin-binding region located between amino acids 60 and 101. Taken together with the activity of ActA derivatives to inhibit polyactin nucleation, no direct binding interaction between these two factors has yet been demonstrated. To test for a direct interaction, A101 was bound to a five-fold excess of actin, and the deletion derivatives were tested for their ability to pellet with the A101 subunit of the complex. ActA or its deletion derivatives were tested for their ability to pellet with the A101 affinity matrix (Fig. 7). Full-length ActA bound to A101-coated beads, but not to beads coated with nonspecific IgG. In contrast, the Δ31-262 derivative lacking the entire NH2-terminal domain did not bind to the complex, indicating that the NH2-terminal domain is required for this interaction. Derivatives missing the cofilin homology sequence (Δ136-165), the actin-binding region (Δ60-101), and the acidic stretch (Δ31-58) all bound to the A101 complex. These data suggest that no single region within the NH2-terminal domain is solely responsible for binding to the complex.

**ActA Binds Directly to the Arp2/3 Complex**

Although ActA functions with the Arp2/3 complex in actin nucleation, no direct binding interaction between these two factors has yet been demonstrated. To test for a direct interaction, A101 was bound to a five-fold excess of actin, and the deletion derivatives were tested for their ability to pellet with the A101 affinity matrix (Fig. 7). Full-length ActA bound to A101-coated beads, but not to beads coated with nonspecific IgG. In contrast, the Δ31-262 derivative lacking the entire NH2-terminal domain did not bind to the complex, indicating that the NH2-terminal domain is required for this interaction. Derivatives missing the cofilin homology sequence (Δ136-165), the actin-binding region (Δ60-101), and the acidic stretch (Δ31-58) all bound to the A101 complex. These data suggest that no single region within the NH2-terminal domain is solely responsible for binding to the complex.

**Mutations in ActA that Affect Arp2/3**

**Complex-mediated Actin Nucleation In Vitro Cause Defects in Actin Polymerization and Actin-based Motility In Vivo**

To correlate the effect of deletion mutations on actin nucleation in vitro with their effect on actin-based motility in cells, we replaced the wild-type chromosomal actA gene in L. monocytogenes with the mutated versions using allelic exchange (Camilli et al., 1993). We confirmed that each mutant protein was expressed on the bacterial surface at levels comparable to wild type by visualizing surface-extracted ActA using SDS-PAGE and Western blotting (Fig. 8). Each mutant strain was used to infect both HeLa and PtK2 cells, and its capacity to associate with filamentous actin and to undergo actin-based motility was observed and quantified (Fig. 9a and Table II). To confirm that ActA derivatives were properly expressed in host cytoplasm, infected HeLa cells were subjected to immunofluorescence using antibody raised against human VASP, a cellular protein that binds to ActA’s proline-rich repeats (Pistor et al., 1995). VASP colocalized with all strains expressing ActA derivatives containing the proline-rich region (Fig. 9b, not shown), indicating that ActA was expressed on the bacterial surface. All other quantification was carried out in PtK2 cells, whose flat morphology facilitates counting bacteria that are not well separated (Fig. 9a and Table II). Similar results were obtained in HeLa cells (Fig. 9b).

At 3.5 h after infection, nearly all (99%) of wild-type L. monocytogenes were able to polymerize F-actin and the majority (51%) were motile, based on their association with actin comet tails (Theriot et al., 1992). In contrast, none of the mutants carrying deletions of the cofilin homology sequence (Δ7-632, Δ31-165, Δ31-200, Δ60-165, and Δ146-150) were motile or capable of polymerizing actin in these cell types, leading to the accumulation of microcolonies near the center of the cell. Intermediate phenotypes were exhibited by mutants missing the acidic domain (Δ31-58; 73% F-actin positive; 12% with tails), the actin-binding re-
gion (Δ60-101; 75% F-actin positive; 30% with tails), or the region COOH-terminal to the cofilin homology sequence (Δ202-263; 99% F-actin positive; 28% with tails). These data suggest that the cofilin homology sequence is essential for actin polymerization in cells, whereas the other regions contribute to both actin polymerization and motility.

For those mutant strains that formed actin comet tails in infected PtK2 cells, rates of intracellular motility were determined using time-lapse phase microscopy (Table II). Compared with wild-type L. monocytogenes, Δ202-263 (4% reduced), Δ60-101 (18% reduced), and Δ31-58 (34% reduced) mutants exhibited similar or moderately lower mean motility rates. These results suggest that once bacteria are able to initiate actin polymerization, deletions within the NH2-terminal region of ActA cause only small changes in the velocity of movement.

actA Alleles Affect the Ability of L. monocytogenes to Spread from Cell to Cell

To correlate the effects of actA mutations on actin-based motility with their effects on L. monocytogenes cell-to-cell spread, mutant strains were tested for their ability to form a plaque in a monolayer of mouse L2 fibroblast cells (Table II). Wild-type L. monocytogenes (defined as 100% plaque size) and the Δ202-263 mutant formed nearly equivalent-sized plaques. Mutants that expressed derivatives lacking the cofilin homology sequence (Δ7-633, Δ31-165, Δ136-200, Δ60-165, Δ136-165, and Δ146-150), which were unable to polymerize actin in cells, were unable to form plaques. Mutants lacking the actin-binding region (Δ60-101) or the acidic stretch (Δ31-58) exhibited a reduced mean plaque size. Thus, there is a good correlation between the capacity of the mutants to undergo actin-based motility and their ability to spread from cell to cell.

Discussion

A ctn nucleation at the L. monocytogenes surface is mediated by the host Arp2/3 complex together with the NH2-terminal domain of the bacterial ActA protein. Although extensive analysis of the function of the NH2-terminal domain has been conducted (Pistor et al., 1995; Lasa et al., 1997; Mourrain et al., 1997), the regions within this domain that stimulate nucleation with the Arp2/3 complex have not been identified, and their corresponding contribution to actin-based motility and pathogenesis in cells has not been determined. Through systematic truncation and deletion mutagenesis, we provide evidence that three regions in ActA play important roles in stimulating actin nucleation with the Arp2/3 complex and that these regions share similarities with eukaryotic WASP family proteins. Examination of the phenotypes exhibited by mutant L. monocytogenes in infected cells indicates that each region performs a distinct function in actin-based motility, and that a single region centered around the cofilin homology sequence is essential for motility and pathogenesis.

ActA Mimics WASP Family Proteins

L. monocytogenes capitalizes on a host mechanism of actin-based motility to spread from cell to cell, perhaps by

Figure 9. A ctn and L. monocytogenes visualized in infected PtK2 and HeLa cells. (a) PtK2 cells infected for 3.5 h with wild-type L. monocytogenes or the indicated mutants expressing surface-associated deletion derivatives of ActA. F-actin was visualized by staining with rhodamine-phalloidin, and bacteria were detected by indirect immunofluorescence using polyclonal anti-L. monocytogenes primary antibody followed by FITC-conjugated secondary antibody. (b) HeLa cells infected with Δ136-200 mutant L. monocytogenes for 3.5 h. F-actin was stained with rhodamine-phalloidin, DNA with DAPI, and VASP with polyclonal anti-VASP primary antibody followed by FITC-conjugated secondary antibody. Bars, 10 μm.
mimicking the function of endogenous proteins that promote actin nucleation. One such class of host factors is the WA SP family of proteins (including WA SP, N-WA SP, Las17p/Bee1p, and Scar/WAVE), which stimulate actin nucleation with the Arp2/3 complex in vitro (Egile et al., 1999; Machesky et al., 1999; Rohatgi et al., 1999; Winter et al., 1999). These include an acidic stretch and a cofilin homology sequence that share limited sequence similarity with corresponding regions in WASP family proteins (including WASP, N-WASP, Las17p/Bee1p, and Scar/WAVE), which stimulate actin nucleation by similar mechanisms. Interestingly, the acidic stretch in N-WASP is essential for Arp2/3 complex binding, and for stimulating nucleation (Rohatgi et al., 1999), suggesting that ActA may exhibit more redundancy in its binding and activation mechanism.

Compared with the wild type, mutants expressing Δ31-58 exhibit a diminished percentage of bacteria that polymerize actin, a reduced percentage of moving bacteria, a reduced mean rate of motility, and an impaired capacity to spread from cell to cell. These phenotypes point to a direct correlation between the nucleation activity of the Arp2/3 complex and the actin cytoskeleton. L. monocytogenes that express Δ146-150 are capable of associating with actin in M DCK and J774 cells (Lauer, P. J.; Theriot, and D. Portnoy, unpublished results). In addition, mutants that overexpress this derivative form actin clouds, but not actin tails, in Xenopus laevis egg extract (Lasa et al., 1997). The discrepancy between the behaviors of this mutant in different cytoplasmic environments may reflect differences in the concentration of host cytoskeletal proteins. Nevertheless, the mutant phenotypes indicate that the cofilin homology sequence is critical for pathogenesis and, while not necessary for actin nucleation, is required to achieve the threshold of activity needed to initiate actin-based motility.

### The Acidic Stretch Plays a Nonessential Role in Nucleation and Intracellular Motility

A cta fragments consisting only of the acidic stretch and actin-binding region possess stimulatory activity, indicating that these elements play a role in nucleation. Moreover, a derivative lacking the acidic stretch (Δ31-58) retains the capacity to bind the Arp2/3 complex and exhibits a modest reduction in maximum activity compared with full-length ActA in vitro. This suggests that the acidic stretch may function with the cofilin homology sequence to promote nucleation by facilitating a productive interaction with the Arp2/3 complex. Interestingly, the acidic stretch in N-WA SP is essential for Arp2/3 complex binding and for stimulating nucleation (Rohatgi et al., 1999), suggesting that ActA may exhibit more redundancy in its binding and activation mechanism.

The results of our study confirm the actin monomer–binding activity of ActA (Las17p/Bee1p, and Scar/WAVE) and extend previous studies by addressing the role of monomer binding in nucleation and motility. A cta derivative missing the monomer-binding region was virtually unable to stimulate A rp2/3-mediated nucleation in vitro, suggesting that actin binding is critical for nucleation in the context of purified proteins. Similarly, the actin binding WH2 domain of N-WA SP is critical for stimulating nucleation (Rohatgi et al., 1999). The essential function of this region may be to position an actin monomer in close proximity to the A rp2/3 complex, forming a trimeric nucleus (Fig. 10) consisting of the two actin-related proteins in the complex and the actin monomer bound to A cta.

### The Cofilin Homology Sequence Is Critical for Actin Nucleation In Vitro and In Cells

A cta derivative lacking only five amino acids at the core of the cofilin homology sequence (Δ146-150) is severely compromised in its capacity to stimulate nucleation with the A rp2/3 complex, but a mutant that lacks this entire region (Δ136-165) can still bind the complex. Similarly, a derivative of N-WA SP, lacking the cofilin homology sequence, has a dramatically reduced ability to stimulate actin nucleation (Rohatgi et al., 1999), and exhibits a slightly reduced capacity to bind to the A rp2/3 complex (Banazai et al., 2000). Therefore, this sequence element plays a critical role in stimulating nucleation in both proteins, perhaps by participating in binding to the A rp2/3 complex and/or by inducing a conformational change in the complex that facilitates nucleation.

### Table II. Intracellular Phenotypes of L. monocytogenes Expressing Different actA Alleles

| actA allele | Plaque size | F-actin association |
|-------------|-------------|---------------------|
|             | Diameter percent | Intracellular motility |  |
|             | WT SD n* | Mean rate SEM n* | Percent F-actin positive | Percent actin tails n* |
| WT         | 100 na 9 9.8 0.6 41 | 99 51 952 |  |
| D202-263   | 95 2.3 9 9.4 0.7 50 | 99 28 867 |  |
| Δ31-58     | 48 4.6 9 6.5 0.4 54 | 73 12 987 |  |
| Δ60-101    | 59 2.2 9 8 0.5 48 | 75 30 1,195 |  |
| Δ146-150   | 0 0 9 | 0 0 660 |  |
| Δ136-165   | 0 0 8 | 0 0 473 |  |
| Δ136-200   | 0 0 8 | 0 0 654 |  |
| Δ60-165    | 0 0 8 | 0 0 501 |  |
| Δ31-165    | 0 0 8 | 0 0 342 |  |
| Δ7-633     | 0 0 8 | 0 0 751 |  |

*For plaque size, n equals number of independent experiments, whereas for intracellular motility rates and F-actin association, n equals total number of bacteria counted.

The Actin-binding Region Is Necessary for Nucleation In Vitro but Not In Cells

The results of our study confirm the actin monomer–binding activity of ActA (Las17p/Bee1p, and Scar/WAVE) and extend previous studies by addressing the role of monomer binding in nucleation and motility. A cta derivative missing the monomer-binding region was virtually unable to stimulate A rp2/3-mediated nucleation in vitro, suggesting that actin binding is critical for nucleation in the context of purified proteins. Similarly, the actin binding WH2 domain of N-WA SP is critical for stimulating nucleation (Rohatgi et al., 1999). The essential function of this region may be to position an actin monomer in close proximity to the A rp2/3 complex, forming a trimeric nucleus (Fig. 10) consisting of the two actin-related proteins in the complex and the actin monomer bound to A cta.
monocytogenes surface results from an interaction between monomeric actin and the Arp2/3 complex with at least three regions within the NH2-terminal domain of ActA (Fig. 10). Interaction of the Arp2/3 complex with the cofilin homology sequence and the acidic stretch may induce a conformational change in the complex that promotes nucleation. Nucleation may also require correct positioning of an actin monomer in close proximity to the Arp2/3 complex, a function provided by the actin-binding region in ActA or, alternatively, by host proteins such as profilin, which are associated with ActA in cell cytoplasm. Bound actin monomer would complete a nucleation site, whose formation would be unfavorable in the absence of ActA, because the Arp2/3 complex alone is thought to nucleate by binding rare and unstable actin dimers (Mullins et al., 1998). Elongation of newly formed filaments at the bacterial surface may be facilitated by profilin and VASP, which enhance bacterial motility in a system of purified proteins (Loisel et al., 1999). The similarities between ActA and WASP family proteins suggest that they represent a fascinating example of convergent evolution. Interestingly, the unrelated bacterial pathogen Shigella flexneri initiates actin-based motility by recruiting and activating N-WASP at its surface rather than mimicking its activity (Suzuki et al., 1998; Egile et al., 1999). Further understanding of the mechanism of ActA-Arp2/3-mediated actin nucleation will lead us to a greater understanding of cell motility and the mechanisms of microbial pathogenesis.

We are extremely grateful to Deena Yarar and Bruce Goode, and Peter Lauer for stimulating conversations during the course of this work and for sharing reagents, protocols, and unpublished observations. We would like to thank Zhongjie Yao, for assistance in the generation of the complete ActA deletion and Rebecca Heald, and David Drubin for their advice in preparation of this manuscript.

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In contrast to the loss of activity in vitro, mutant L. monocytogenes expressing the monomer-binding deletion still associate with F-actin, are motile, and can spread from cell to cell, although each of these parameters is slightly impaired compared with the wild type. Thus, actin binding by ActA may serve a redundant function in cells, perhaps because other ActA-associated proteins in host cytosol bind actin monomer in a manner that facilitates nucleation (Fig. 10). One candidate monomer-binding protein is profilin, which localizes to the surface of L. monocytogenes (Theriot et al., 1994) through interactions with host Ena/ VASP family proteins (R einhard et al., 1995; G erlert et al., 1996; K ang et al., 1997) that bind directly to the proline-rich repeats of ActA (Chakraborty et al., 1995; Pistor et al., 1995; Smith et al., 1996; N iebehr et al., 1997).

The Role of Actin Nucleation by ActA and the Arp2/3 Complex in L. monocytogenes Motility

We propose a model in which actin nucleation at the L. monocytogenes surface results from an interaction between monomeric actin and the Arp2/3 complex with at least three regions within the NH2-terminal domain of ActA (Fig. 10). Interaction of the Arp2/3 complex with the cofilin homology sequence and the acidic stretch may induce a conformational change in the complex that promotes nucleation. Nucleation may also require correct positioning of an actin monomer in close proximity to the Arp2/3 complex, a function provided by the actin-binding region in ActA or, alternatively, by host proteins such as profilin, which are associated with ActA in cell cytoplasm. Bound actin monomer would complete a nucleation site, whose formation would be unfavorable in the absence of ActA, because the Arp2/3 complex alone is thought to nucleate by binding rare and unstable actin dimers (Mullins et al., 1998). Elongation of newly formed filaments at the bacterial surface may be facilitated by profilin and VASP, which enhance bacterial motility in a system of purified proteins (Loisel et al., 1999). The similarities between ActA and WASP family proteins suggest that they represent a fascinating example of convergent evolution. Interestingly, the unrelated bacterial pathogen Shigella flexneri initiates actin-based motility by recruiting and activating N-WASP at its surface rather than mimicking its activity (Suzuki et al., 1998; Egile et al., 1999). Further understanding of the mechanism of ActA-Arp2/3-mediated actin nucleation will lead us to a greater understanding of cell motility and the mechanisms of microbial pathogenesis.

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Figure 10. Model for actin nucleation by ActA and the Arp2/3 complex at the L. monocytogenes surface. The NH2-terminal domain of ActA interacts directly with the Arp2/3 complex. The acidic stretch (A) and cofilin homology sequence (C) of ActA both contribute to Arp2/3 complex activation, whereas the actin-binding region (AB) recruits and presents the actin monomer to both contribute to Arp2/3 complex activation, whereas the actin-binding region (AB) recruits and presents the actin monomer to...
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