The surface protein ActA of the pathogenic bacterium *Listeria monocytogenes* induces actin-driven movement of bacteria in the cytoplasm of infected host cells and serves as a model for actin-based motility in general. We generated and purified soluble recombinant fragments of ActA and assessed their ability to interact with the acidic phospholipids phosphatidylinositol 4,5-bisphosphate and phosphatidylglycerol 3,4,5-trisphosphate, both implicated in the regulation of actin polymerization. Purified ActA consisted of biologically active, elongated molecules with an α-helix and β-sheet content of 11 and 32%, respectively. In the presence of either phosphatidylinositol 4,5-bisphosphate or phosphatidylglycerol 3,4,5-trisphosphate, but not phosphatidylcholine, ActA molecules underwent a structural change that raised the α-helix content to 19% and lowered the β-sheet content to 27%. Co-sedimentation experiments with phosphatidylcholine vesicles containing different acidic phospholipids demonstrated that ActA binds preferentially to D-3 phosphoinositides. The D-3 phosphoinositide binding activity was mapped to a small subregion in the N-terminal domain of ActA. This subregion comprises 19 amino acids and showed homology to cecropins. In addition, we found that amino acids 33 to 74 of ActA mediated actin binding by the whole, folded ActA molecule. These findings shed new light on ActA function.

*Listeria monocytogenes* is a facultative intracellular pathogen capable of invading a wide variety of host cells and spreading directly from cell to cell through bacteria-containing protrusions. One of the mechanisms underlying protrusion formation is directed actin assembly which is initiated by a bacterial surface protein called ActA. With the help of ActA the bacteria induce localized actin assembly at the rear end of the bacterial body thereby generating a force that propels the bacteria forward through the host cell cytoplasm. This characteristic actin-based motility is the reason for *Listeria*’s use as a model system to study actin assembly of eucaryotic cells (for review, see Refs. 1 and 2).

ActA is a 610-amino acid protein with a N-terminal signal sequence of 29 amino acids, which is cleaved off after transport through the bacterial membrane, and a C-terminal membrane-spanning sequence which anchors ActA to the bacterial membrane (3, 4). The ActA protein has been shown to be necessary (4) and sufficient (5, 6) for bacterially induced actin polymerization and movement. ActA deletion mutants are able to invade cells, albeit at a reduced rate, and multiply in the cytoplasm. However, these mutants are unable to induce actin assembly, and therefore cannot move nor spread from cell to cell. Furthermore, in a mouse model of infection these mutants are almost avirulent (4, 7).

The ActA protein has been shown to contain at least three different functional domains: an N-terminal (amino acids 1 to 234), a proline-rich, central (amino acids 235 to 393), and a C-terminal domain (amino acids 394 to 610). When expressed in eucaryotic cells the first two domains exert distinct effects on the cytoskeleton (8), while the C-terminal domain, besides anchoring the protein in the bacterial membrane, serves as a spacer traversing the cell wall. The central domain of ActA harbors a 4-fold repeated proline-rich motif with the amino acid core consensus (E/D)FPFPDPX(D/E). This FP motif is also present in two focal adhesion proteins, zyxin and vinculin, which in this respect can be regarded as eucaryotic homologs of ActA (9, 10). FP motifs represent docking sites for Ena/VASP1 homology 1 domains, carried by proteins of the Ena/VASP family which are constituents of focal adhesions plaques (9). Ena/VASP proteins bind to F-actin (11) and recruit profilin, an actin-binding protein that can promote actin filament elongation (12). Deletion of the proline-rich central domain of ActA strongly reduces but does not completely abolish motility suggesting that this domain acts as an efficient accelerator of actin filament assembly (9, 13, 14).

The N-terminal domain of ActA is sufficient to mediate bacterial motility in cytoplasmic extracts when overexpressed in *Listeria* mutants deleted for actA (13, 15). This domain has been implicated in facilitating actin nucleation by interacting with the Arp2/3 complex, an evolutionarily conserved protein complex consisting of 7 to 8 polypeptides. A mixture of purified ActA and the Arp2/3 complex is an efficient nucleator of actin filament formation, whereas the Arp2/3 complex alone nucleates actin only weakly (16). In addition to its actin nucleation activity, the Arp2/3 complex is responsible for actin filament capping and branching by binding to the pointed ends and to the sides of actin filaments, a property that may account for its occurrence throughout the *Listeria* actin tails (17, 18). ActA alone shows no actin nucleation activity nor has it been possi...
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It is possible to demonstrate that ActA interacts with actin in other ways (16, 19).

The details of the mechanism by which ActA promotes actin polymerization in conjunction with the Arp2/3 complex are not yet clear. It is also not known whether interaction of ActA with the Arp2/3 complex is sufficient for ActA-mediated initiation of actin-based motility in vivo, or whether other factors may be involved. Therefore, in order to improve our understanding of ActA function, we have purified and characterized soluble variants of the ActA molecule. Using these ActA variants we have addressed the question whether ActA might interact with phosphonosidases which have been implicated as central mediators of actin assembly (20) and have reinvestigated whether ActA might directly interact with actin.

**Experimental Procedures**

Molecular cloning, SDS-gel electrophoresis, immunoblotting, and amino acid sequencing were carried out according to standard procedures (21, 22) with enzymes purchased from Amersham Pharmacia Biotech, Promega, or Life Technologies, Inc. Chemicals used were pure grade and purchased from Merck or Fluka unless otherwise specified. Phospholipids or mixtures of PC and acidic phospholipids were resuspended in water and sonicated on ice with a Braun Labsonic U sonicator at maximum power until the turbidity of the solution completely disappeared. negatively charged micelles were removed from the solution by ultrafiltration at −80 °C and rigorously vortexed after thawing. Data processing and figure preparation were carried out using Canvas 3.5 and 5.0, Photoshop 3.0, and Cricket Graph III.

**Vector Construction for ActA Variants and Histidine-tagged GST**

**Histidine-tagged ActA and ActA without Histidine Tag—Fragments of the acta gene were amplified using plasmid pactA3 (4) as template and primer pairs KO6/7 and KO6/71, respectively.** Histidine-tagged N-ActA: a fragment of the acta gene was amplified using pactA3 as template and primer pair KO17/18. The PCR product was restricted with MfeI and ClaI and ligated into the 3.2-kilobase HI fragment of pactA28 into pBluescript II KS+ (Stratagene), resulting in pactA35 and pactA36. Histidine-tagged C-ActA: a fragment of the acta gene was amplified using pactA3 as template and primer pair KO19/20. Histidine-tagged C-ActA: a fragment of the acta gene was amplified using pactA28 as template and primer pair KO15/16. After restriction with Sau96I and ClaI the PCR products for P- or C-ActA and the 0.4-kilobase HindIII/Sau96I fragment of pactA28 were ligated into pBluescript II KS+, resulting in pactA35 and -32, respectively. Histidine-tagged ActA41–56 was constructed by performing two separate PCR reactions with pactA34 as template and primer pairs KO29/33 and KO30/34. The two PCR products were then mixed and used as template for a second PCR with primer pair KO29/30. This fragment was restricted with MfeI and SphI and ligated to the 4517-base pair MfeI/SphI fragment of pactA28, resulting in pactA43. pactA28, -34, -32, -35, -43, and -51 were each restricted with BamHI and SacI and the inserts were ligated into pAT18 containing the promoter sequence of an exoprotease of Streptococcus cremoris in the EcoRI site (see Ref. 5), resulting in pactA30, -38, -36, -39, -46, and -52, respectively. L. monocytogenes LO28actA4 (7), a strain in which an internal fragment of the acta gene corresponding to amino acids 36 to 574 has been exchanged for a glutamine, was transformed with pactA30, -38, -36, -39, and 46 by electroporation as described (13) except that penicillin was used at 0.12 μg/ml.

**Histidine-tagged GST and Histidine-tagged GST-ActA Fusion Proteins—PCR was performed with pactA28 (for histidine-tagged GST) or pactA3 (Ref. 4, for GST-ActA fusion proteins) as a template and primer pairs KO63/64, KO91/92, KO91/94, KO94/96, KO97/98, and KO99/100; the PCR products were restricted with BamHI and EcoRI (histidine-tagged GST-ActA fusion proteins) or HindIII (GST-ActA fusion proteins) and ligated into pGEX-3X-P which corresponds to pGEX-3X (Amersham Pharmacia Biotech) with an enlarged polylinker. The resulting plasmids were pGEX-3X (Amersham Pharmacia Biotech), equilibrated with 50 mM NaCl, 50 mM sodium phosphate, pH 7.5, and sterilized by filtration (Sartorius, Germany). Histidine-tagged ActA fragments: the filtrates were loaded on a Ni-NTA superficol (Qiagen) column (gel volume: 5 ml) equilibrated with loading buffer (300 mM NaCl, 50 mM sodium phosphate, pH 8.0) and eluted in 10 ml with a gradient of 0 to 250 mM imidazole in loading buffer. ActA without the histidine tag: after concentration to 5 ml with Centricon 10 concentrators (Amicon), the protein was applied to a MonoQ HR 5/5 column (Amersham Pharmacia Biotech), equilibrated with 50 mM NaCl, 50 mM sodium phosphate, pH 7.0 and eluted in 10 ml with a gradient of 0 to 250 mM imidazole in loading buffer. ActA without the histidine tag: after concentration to 5 ml with Centricon 10 concentrators (Amicon) and buffer exchange to 50 mM sodium phosphate, pH 7.5, and sterilized with an ultraviolet (UV) lamp, the protein was applied to a MonoQ HR 5/5 column (Amersham Pharmacia Biotech), equilibrated with 50 mM NaCl, 50 mM Tris/HCl, pH 7.5, and eluted with a NaCl gradient over 15 ml from 0.05 to 1 M. All fragments: factions containing ActA fragments were pooled, concentrated using Centricon 10 (Amicon), and applied to a Superose 6HR 30/-column (Amersham Pharmacia Biotech), equilibrated with PBS (137 mM NaCl, 2.7 mM KCl, 18 mM NaHPO4, 1.5 mM K2HPO4, pH 7.2) and CD buffer (100 mM sodium fluoride, 20 mM sodium phosphate, pH 7.1), or 100 mM Tris/HCl, pH 8. Fractions containing ActA were pooled and aliquots stored at −80 °C. The correct processing of ActA, N-ActA, P-ActA, and C-ActA was verified by N-terminal amino acid sequencing after centrifugation onto polyvinyldiene fluoride membranes. GST, histidine-tagged GST, and GST-ActA fusion proteins were expressed in Escherichia coli strain NB42 and purified as described (21, 23). PD10 columns (Amersham Pharmacia Biotech) were used for buffer exchange. Histidine-tagged GST and GST-ActA fusion proteins were purified by metal chelate chromatography as described above. Histidine-tagged GST was additionally purified by affinity chromatography on glutathione-agarose. The purification protocol that GST-ActA was used 184–202 had an increased tendency to precipitate compared with the other GST fusion proteins. Protein concentrations were determined with the Bio-Rad protein assay with bovine γ-globulin as standard. Molar ratios were calculated by assuming proteins to be monomeric.

**Vasp Binding**

Cytoplasmic extracts prepared from non-adherently growing HeLa S3 cells by standard procedures (21) were a gift of Michael Leichter and Gerald Thiel. 1 ml of cytoplasmic extract corresponding to 2.2 × 107 cells was precleared with 100 μl of Ni-NTA Superflow beads (Qiagen).
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were resuspended in 100 mM sodium phosphate, pH 8.0) were loaded with 7.5 µg of the corresponding ActA fragment. The beads were then incubated with 250 µl of the precleared extract for 30 min at 4 °C and after 4 washes with 1 ml of PBS/Tween, bound proteins were eluted with 20 µl of imidazole-elution buffer (500 mM imidazole, 300 mM NaCl, 50 mM sodium phosphate, pH 8.0). Eluate corresponding to 12.5 µl of cell extract was electrophoresed through a 12% SDS-polyacrylamide gel followed by Western blotting. Blots were developed with a mouse monoclonal anti-human VASP antibody (Transduction Laboratories). To control for equal loading of the beads, a total volume of 100 µl of extract was analyzed by SDS-PAGE.

The presence of the respective ActA fragment was confirmed after Coomassie Blue staining.

Actin Polymerization in Cytoplasmic Extracts of Xenopus laevis Eggs

Interphase extracts of Xenopus eggs prepared as described (24) were a gift from Petra Pfeiffer. Extracts were supplemented with 3 µM G-actin-Alexa 488 (Molecular Probes), 1 mM MgATP, and 10 mM creatine phosphate (together less than 15% of total volume). Recombinant soluble forms of ActA were co-precipitated with Ni-NTA Superflow beads as described above. After two washes with 100 µl of Xenopus buffer (100 mM KCl, 1 mM MgCl2, 0.1 mM CaCl2, 50 mM sucrose, 10 mM HEPES, pH 7.7) the supernatant was removed. 1 µl of bead slurry was incubated with 10 µl of Xenopus egg cytosol for 15–30 min at room temperature, the mixture was transferred to a microscope slide and observed under a fluorescence microscope (Axioskop, Zeiss; objective ×10) and photographed on Fuji PROVIA R135 film 400 ASA under identical exposure conditions. Equal loading of Ni-NTA beads was controlled by loading beads as described above followed by control incubation with imidazole-elution buffer. Eluates were analyzed by SDS-PAGE and protein amounts were determined by densitometric scanning of the Coomassie Blue-stained gel.

Direct Actin Binding Assay

G-actin was prepared as described (25). Ni-NTA Superflow beads were loaded with ActA fragments as described above, washed twice with PBS-blocking buffer (PBS supplemented with 3% bovine serum albumin (fraction V), Sigma), and 0.2% teneosan gelatin (Sigma) and the supernatant was removed. Equal loading of the beads was controlled as described above. 1 µl of bead slurry was mixed with 10 µl of PBS blocking buffer supplemented with 1 mM MgATP, G-actin, of which 50% was labeled with Alexa 488 (Molecular Probes) was added to a final concentration of 4 µM and then the mixture immediately transferred to a microscope slide. The samples were observed under a fluorescence microscope (Axioskop, Zeiss; objective ×10) and recorded at 10 or 40) and results recorded within 1 h as described (5) with a CCD camera (Photometrics, Tucson, Arizona) under identical exposure conditions.

F-actin Co-sedimentation and Actin Polymerization Assays

F-actin co-sedimentation assays were carried out as described (25) in a total volume of 100 µl containing 2.4 µM of the respective ActA fragment, 6.75 mM KCl, 150 mM NaCl, 0.1 mM MgCl2, 10 mM sodium phosphate, 40 mM HEPES, pH 7.0. After centrifugation in the presence or absence of 4 µM F-actin for 15 min at 245,000 × g at 4 °C, the pellets were resuspended in 100 µl of 50 mM HEPES, pH 7.0, and equal amounts of supernatants and resuspended pellets were analyzed on a 12% SDS gel. Actin polymerization assays were performed as described (26). Briefly, 2 µM G-actin, of which 5% was labeled with 7-chloro-4-nitro-2,1,3-benzoxadiazol at lysine 373, was polymerized in 100 mM KCl, 20 mM NaCl, 1 mM MgCl2, 0.16 mM CaCl2, 0.4 mM ATP, 2.4 mM NaN3, 6.6 mM sodium phosphate, and 4 mM triethanolamine/HCl, pH 7.2, in the presence or absence of 0.2 or 2 µM ActA. The excitation wavelength was 480 nm. Light emission was recorded every 60 s at 540 nm over a time period of 120 min.

Analytical Gel Filtration and Ultracentrifugation

Gel Filtration—1 to 2 mg/ml protein in 20 µl was loaded onto a Superose 6 PC 3.2/30 column attached to a SMART system (Amersham Pharmacia Biotech).

Analytical Ultracentrifugation—Sedimentation velocity at a rotor speed of 56,000 rpm and sedimentation equilibrium of ActA (0.2 to 2 mg/ml in either PBS, pH 7.2, or CD buffer, pH 7.1, or 0.1 M Tris, pH 8.0) was carried out at 20 °C in a Beckman XLA analytical ultracentrifuge equipped with absorption optics (Ralsdon, 19) in 12-mm double sector cells (Epon). For sedimentation equilibrium runs, cells were filled to 2–3 mm above the FC-43 bottom fluid. Depending on the size of the molecular masses, different rotor speeds were used: first 8,000 or 9,600 rpm for the higher molecular weight species and, after equilibrium was achieved, the rotor speed was raised to 14,000 rpm for determination of extrinsic protein and lipid species. The respective protein and lipid concentrations were calculated using a floating baseline computer program that adjusted the baseline absorbance to obtain the linear fit of ln A versus r² (A, absorbance, r, radial distance). An assumed partial specific volume of 0.73 cm³/g was used. The density and viscosity of the buffer were corrected to 20 °C water using the CRC Handbook of Chemistry and Physics, 70th edition. The partial specific volume (ρ), the sedimentation coefficient (γ), the partial specific volume, and the density; and γ, the viscosity of the solution.

Circular Dichroism Spectra

Spectra were obtained with proteins in CD buffer, pH 7.1, at a concentration of 0.1 to 0.45 mg/ml. Phospholipids were used at a final concentration of 150 µM. Solid guanidine HCl was added to a final concentration of 6 M so that the protein to be tested in 6 M guanidine HCl as well as the control in half-concentrated CD buffer. CD spectra were recorded at 25 °C with a Jasco model 550 spectropolarimeter (HPC) in a thermostatted quartz cell of 1-mm optical path length between 186 and 250 nm or between 212 and 250 nm at 10 nm/min. For each measurement the mean values of 3 spectra were taken to improve the signal to noise ratio. Molar ellipticities [θ] were calculated on the basis of a mean amino acid residue molecular mass of 110 Da. Protein concentrations were determined by UV spectroscopy on the basis of calculated extinction coefficients for denatured proteins, which were corrected for native proteins by measuring the absorbance at 280 nm in the presence and absence of 6 M guanidine HCl (27). To exclude a potential influence of a minor pH change due to phospholipid addition, a CD spectrum of ActA was recorded at pH 6.8 instead of pH 7.1; no significant change of the CD spectrum was observed.

Limited Proteolysis and Western Blotting

Limited proteolysis using chymotrypsin, trypsin, and endoprotease Lys-C, Asp-N, Glu-C, Arg-C, elastase, subtilisin, pepsin, thermolysin, papain, proteinase K, and plasmin was carried out according to standard procedures (22). For proteolysis with chymotrypsin or endoprotease Glu-C, 20 µM ActA was incubated in 0.1 M Tris, pH 8.0, with varying amounts of proteases (sequencing grade; Roche Molecular Biochemicals), the presence or absence of proteinase K, and phospholipid concentrations were determined by densitometric scanning of the Coomassie Blue-stained gel.

Phospholipid Co-sedimentation Assay

Co-sedimentation assays were performed according to Harlan et al. (28) with the following modifications: the samples were in 66 mM sodium fluoride, 13 mM sodium phosphate, pH 7.1. Proteins and fragments were separated through 12% SDS-polyacrylamide gels and transferred to nitrocellulose. The blots were developed using peptide-specific antibodies raised against the 18 N-terminal amino acids of ActA (A18K) (7) or the second FP4 motif in ActA (Y21T) (5) or mouse monoclonal anti-histidine-tag antibody 13435/31 (Dianova). For preparative purposes the limited proteolysis reactions were scaled up to contain 500 µg of ActA. Proteins and fragments corresponding to 500 pmol were blotted or centrifuged onto polyvinylidine fluoride membranes and sequenced using an Applied Biosystems sequencer model 492 according to the manufacturer’s instructions.
perchloracetic acid, samples containing phospholipids in 66 mM sodium fluoride, 13 mM triethanolamine/HCl, pH 7.1, were analyzed before centrifugation and supernatant and pellet fraction of the same samples after centrifugation.

RESULTS

Soluble ActA Binds VASP and Retains Actin Polymerization Activity—

Multicopy vectors encoding soluble, histidine-tagged ActA fragments (Fig. 1A) were transfected into a L. monocytogenes strain carrying a deletion of the actA gene. The ActA fragments were purified from the supernatants of Listeria cultures yielding about 2 mg of pure protein per liter (except for N-ActA which repeatedly yielded 5 to 10 times less). In agreement with previous observations of membrane-bound ActA fragments (4, 13), the purified proteins migrated aberrantly in SDS gels (Fig. 1B). For reasons that remained unclear, P-ActA migrated as a triplet and C-ActA as a doublet band.

To verify the functional activity of our purified material, selected ActA fragments were tested for their ability to recruit VASP from cytoplasmic extracts. As expected, nickel beads coated with ActA or P-ActA, both harboring FP4 motifs, pulled VASP out of cytoplasmic extracts (Fig. 1C, lanes 1 and 2). Nickel beads coated with C-ActA, which does not contain FP4 motifs and uncoated nickel beads did not precipitate VASP (Fig. 1C, lanes 3 and 4).

To test the soluble ActA fragments for their ability to induce actin assembly we used cytoplasmic Xenopus egg extracts which are known to support the actin-based motility of L. monocytogenes (5, 29). Equal amounts of ActA fragments were coated onto nickel beads and incubated in extract supplemented with Alexa-488-labeled G-actin. Bright fluorescent staining indicated strong accumulation of actin on beads coated with ActA (Fig. 1D, first, fifth, and seventh panel). Given the relatively large size of the beads (about 60 to 160 μm in diameter) compared with bacteria (1.5 to 2 μm long) and the presumably uniform ActA distribution on the bead surface, one does not expect these beads to move, since asymmetric distribution of ActA was shown to be a prerequisite for actin-based motility for this kind of particle (6, 30). However, we observed fluorescent protrusions, indicating large amounts of polymerized actin, on 50 to 80% of the beads (arrowheads, Fig. 1D) which could correspond to the characteristic actin tails that are generated by moving bacteria (5, 29). N-ActA-coated beads also showed bright actin staining, but actin-containing protrusions were much smaller and rare (10 to 15% of beads; Fig. 1D, second panel). While C-ActA-beads did not emit fluorescence (Fig. 1D, fourth panel) and were indistinguishable from uncoated beads, P-ActA-coated beads, unexpectedly, showed a weak albeit clearly detectable staining that varied in intensity from bead to bead (Fig. 1D, third panel). Taken together with the VASP binding data, these results confirmed that our puri-

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**Fig. 1.** Soluble, recombinant fragments of ActA show predicted biological activities. A, schematic drawing of mature, secreted, recombinant ActA proteins. All proteins contain amino acids 1 to 30 of the mature ActA molecule and lack the membrane anchor (amino acids 585 to 610). Thin lines with Δ correspond to deleted regions. Histidine tags are depicted as black and FP4 motifs as gray boxes. The membrane anchor is replaced by arginine and 6 histidines in all constructs except for N-ActA, where glutamine and arginine precede 6 histidines. ActA without the histidine tag ends with amino acid 584. Amino acids are numbered according to Kocks et al. (4). B, purified ActA molecules on Coomassie Blue-stained 8% (left) or 12% (right) polyacrylamide SDS gels. C, recruitment of VASP from cytoplasmic extracts of HeLa cells. Nickel beads coated with equal amounts of ActA, P-ActA, or C-ActA and an uncoated bead sample were incubated in pre-cleared extract. Bound proteins were eluted from the beads and eluates were used for SDS-PAGE followed by Western blotting with a monoclonal antibody against VASP. VASP is detected as a doublet band corresponding to phosphorylated and non-phosphorylated forms. D, ActA-coated nickel beads induce actin assembly in Xenopus egg cytoplasmic extracts. Nickel beads coated with equal amounts of the indicated ActA fragments were incubated in Xenopus egg cytoplasmic extract containing 2 μM G-actin-Alexa 488. Upper panel, fluorescence; lower panel, corresponding phase-contrast images. Arrowheads indicate actin-rich protrusions.
fied ActA fragments possessed the expected biological activities.

Direct Binding of ActA to Actin—To determine whether the actin accumulation on ActA-coated beads as seen in Xenopus egg cytoplasmic extracts was exclusively indirect (owing to recruitment of cellular factors) or reflected to some extent a direct interaction between ActA and actin, ActA-coated nickel beads were incubated in PBS blocking buffer supplemented with a mixture of unlabeled and fluorescently labeled G-actin. ActA-coated beads were clearly fluorescent suggesting direct binding of ActA to actin (Fig. 2A, first panel). The overall signal intensity was weaker than in the Xenopus egg cytoplasmic extract assay, and no actin-containing protrusions indicative of large amounts of polymerized actin were observed. Beads coated with histidine-tagged GST control protein were completely negative (data not shown) and ActA-coated nickel beads showed no fluorescence when incubated with Alexa 488-coupled goat immunoglobulin G (data not shown). The ActA-actin interaction seemed to occur with low affinity, since repeated washing of ActA beads resulted in a drastic decrease in signal intensity (not shown). Consistent with this, using ActA-expressing bacteria instead of beads in the same assay, a faint signal could only be detected when a Listeria strain strongly overexpressing ActA was used (data not shown).

We used different ActA fragments to determine which ActA domain was responsible for actin binding. N-ActA-coated beads showed clear fluorescence, albeit slightly less intense than ActA-coated beads (Fig. 2A, second panel). Beads coated with P-ActA were completely negative (Fig. 2A, third panel) while C-ActA-coated beads showed a weak background fluorescence (Fig. 2A, fourth panel). These results indicated that the N terminus of ActA contains direct actin binding activity, while the actin recruitment by the proline-rich central domain in the cytoplasmic extracts (see Fig. 1D, third panel) was indirect. The latter finding could be explained by the recruitment of F-actin (11) or profilin-actin complexes (9) through Ena/VASP family proteins that bind to the FP1 motifs in the proline-rich domain of ActA.

A synthetic peptide corresponding to amino acids 33 to 74 of ActA has previously been shown to interact with G- and F-actin as a synthetic peptide in vitro (15). Therefore, to further characterize the direct actin binding by the N-terminal ActA domain, we used a mutant ActA molecule, ActAΔ41–56 (see Fig. 1A), lacking amino acids 41 to 56 that are conserved between ActA and iActa, an ActA homolog from Listeria ivanovii, a Listeria species pathogenic for animals (7, 15). As assessed by circular dichroism, the mutant ActAΔ41–56 molecule had the same secondary structure as wild type ActA (data not shown; see also below). ActAΔ41–56-coated nickel beads induced actin accumulation in the cytoplasmic extract assay (Fig. 1D, sixth panel) although they lacked the actin-rich protrusions that emanated from the bead surface of ActA-coated beads (Fig. 1D, compare fifth to sixth panel). However, binding of fluorescently labeled actin to nickel beads coated with ActAΔ41–56 was impaired (fifth panel in Fig. 2A) and reached only background levels similar to that observed with the C-terminal domain (see Fig. 2A, fourth panel). Since the location of an actin-binding site within amino acids 33 to 74 of ActA has been proposed by Lasla et al. (15), we tested a histidine-tagged GST fusion protein comprising amino acids 33 to 74 of ActA for binding of actin-Alexa488. While nickel beads coated with GST-ActA33–74 were inactive, indicating sterical or conformational constraints, bright fluorescence was observed with glutathione beads coated with GST-ActA33–74 (Fig. 2A, sixth panel). Glutathione beads coated with histidine-tagged GST (Fig. 2A, seventh panel) or with GST-ActA41–56 (data not shown) were completely negative.

F-actin co-sedimentation assays were used to determine whether ActA interacts with filamentous actin. In low salt buffer containing 30 mM NaCl, a weak, possibly nonspecific co-sedimentation of ActA and N-ActA with F-actin was detected, while C-ActA showed even weaker activity and P-ActA was negative (data not shown). However, under physiological salt conditions none of these ActA fragments co-sedimented with F-actin (Fig. 2B). In actin polymerization assays, at a 1:1 molar ratio of ActA to G-actin, soluble ActA had a small inhibitory effect on actin polymerization kinetics (Fig. 2C) which was never observed with P-ActA nor C-ActA nor GST (data not shown). However, in agreement with previous observations (16), ActA had no detectable effect on actin polymerization at a 1:10 molar ratio of ActA to actin (data not shown). Thus, while the bead assay clearly indicated actin binding by ActA, no F-actin binding activity could be detected under physiological conditions in a co-sedimentation assay.
Soluble ActA Molecules Are Elongated and Low in Secondary Structure—Cross-linking experiments suggest that membrane-bound ActA molecules on the bacterial surface can form dimers (31). We used gel filtration and analytical ultracentrifugation to determine the molecular weight and shape of native, soluble ActA. Using gel filtration ActA eluted as single peak between molecular mass standards ferritin (440 kDa) and thyroglobulin (669 kDa) corresponding to a molecular mass of about 650 kDa and to a Stokes radius of 75 Å. However, sedimentation velocity and sedimentation equilibrium centrifugation revealed that soluble ActA consisted of a mixture of at least three different molecule species (Table I). Two molecule species had very similar sedimentation coefficients of 2.5 S and molecular masses of 68 and 138 kDa, respectively. Since the calculated molecular mass of ActA is 65 kDa and it is known that shape can compensate mass for elongated molecules (see, for example, Ref. 32), we concluded that these molecule species may correspond to monomers and dimers. The third molecule species had a sedimentation coefficient of about 6.0 S and a molecular mass of approximately 250 kDa that was difficult to determine experimentally. This molecule species may thus correspond to a tetramer or higher order aggregates, or a mixture of both. The ratios of the two 2.5 S and the 6.0 S molecule species varied in different runs between 10 to 1 and 1 to 10. Varying protein concentration, buffer salts and pH caused no conclusive change in the ratio of the 2.5 S and the 6.0 S species. The frictional ratios for all three molecule species were even higher than the frictional ratio for the highly elongated protein fibrinogen ($f/f_s = 2.34$). Thus, soluble ActA consisted of a mixture of monomers, dimers, and one or several higher molecular weight forms, all of which were significantly elongated.

CD spectra in the far-UV region were obtained to determine the secondary structure of ActA. Fig. 3A shows representative CD spectra of ActA, and of N-ActA, P-ActA, and C-ActA. The spectra had a prominent minimum either at 199 nm (C-ActA) or 200 nm (ActA, N-ActA and P-ActA). Shoulders at 222 nm that are characteristic for the presence of $\alpha$-helices were observed in all spectra. Addition of 6 M guanidine hydrochloride destroyed this secondary structure (Fig. 3B). $\alpha$-Helix and $\beta$-sheet conformations are evenly distributed over the ActA molecule and are not concentrated in one of the three ActA domains.

**Interaction with Phosphoinositides Leads to Changes in Secondary Structure of ActA—**Actin-binding proteins such as vinculin, profilin, and gelsolin bind to and are regulated by phosphoinositides (20, 34). Moreover, profilin and a peptide from gelsolin undergo conformational changes in the presence of such phospholipids (35–37). To assess whether ActA might interact with phospholipids in a similar way, we measured the circular dichroism of ActA in the presence of PC, PI(4,5)P$_2$, and PI(3,4,5)P$_3$ (Fig. 3C). Addition of PC vesicles had no effect on the CD spectrum of ActA. However, addition of PI(4,5)P$_2$ and PI(3,4,5)P$_3$ micelles resulted in clear and similar conformational changes. In contrast to this, 1D-myoinositol 1,4,5-trisphosphate had no significant effect indicating that the lipid part of the molecule was crucial for the observed conformational change (data not shown). In the presence of PI(4,5)P$_2$ or

| Molecular mass | $[x_{20,0}]$ | Stokes radius | $f/f_s$ |
|----------------|-------------|---------------|----------|
| 68 ± 10        | 2.5         | 63            | 2.38     |
| 138 ± 10       | 2.5         | 127           | 3.79     |
| ~250           | 6.0         | 106           | 2.51     |

**Fig. 3.** CD spectra of recombinant forms of ActA change in the presence of phosphoinositides. CD spectra in the far UV (185 to 250 nm) were recorded at 25 °C in a thermostatted quartz cell of 1-mm optical path length. A, CD spectra of indicated ActA fragments. The spectrum of ActA is representative of five independent experiments using four different ActA preparations. B, CD spectrum of ActA in the presence and absence of 6 M guanidine hydrochloride. C and D, CD spectra of the indicated ActA fragments in the presence and absence of the indicated phospholipids. Phospholipids were added to a final molar ratio of phospholipid to protein of 50:1.
PI(3,4,5)P_2 the minimum of the CD spectrum shifted from 200 to 203.5 nm and the CD signal at 222 nm decreased approximately 2-fold. This corresponds to an increase in α-helical conformation from 11 to 19% and a decrease in β-sheet from 32 to 27% (see Table II). Similar results were obtained when soluble ActA without a histidine tag (see Fig. 1, A and B) was used (data not shown).

To determine which region of ActA was responsible for the secondary structure changes, circular dichroism of N-ActA, P-ActA, and C-ActA was measured in the presence and absence of PI(4,5)P_2. As shown in Fig. 3D, a clear conformational change was observed with N-ActA while the CD spectrum of P-ActA did not change significantly. The spectrum of C-ActA also changed in the presence of PI(4,5)P_2, albeit to a lesser extent. These results indicated that the observed secondary structure change upon interaction with PI(4,5)P_2 or PI(3,4,5)P_3 is mainly due to a conformational change in the N-terminal part of ActA. This view was supported by the calculated values (see Table II).

To further investigate the apparent structural change of ActA upon phosphoinositide binding, limited proteolysis of ActA under nondenaturating conditions was performed in the presence of PI(4,5)P_2. As shown in Fig. 3D, a clear conformational change was observed with N-ActA while the CD spectrum of P-ActA did not change significantly. The spectrum of C-ActA also changed in the presence of PI(4,5)P_2, albeit to a lesser extent. These results indicated that the observed secondary structure change upon interaction with PI(4,5)P_2 or PI(3,4,5)P_3 is mainly due to a conformational change in the N-terminal part of ActA. This view was supported by the calculated values (see Table II).

TABLE II

| Protein | Without phospholipid | +PI(4,5)P_2 |
|---------|----------------------|------------|
|         | α-Helix | β-Sheet | β-Turn + remainder | α-Helix | β-Sheet | β-Turn + remainder |
| ActA    | 11      | 32      | 58                  | 19     | 27      | 55                  |
| N-ActA  | 10      | 34      | 57                  | 23     | 22      | 54                  |
| P-ActA  | 10      | 28      | 67                  | 12     | 26      | 66                  |
| C-ActA  | 11      | 25      | 64                  | 17     | 24      | 59                  |

Upon cleavage of ActA by chymotrypsin in the presence or absence of PI(4,5)P_2 a striking difference of the ActA cleavage pattern was observed (Fig. 4C, upper compared with lower panels). Cleavage in the presence of PI(3,4,5)P_3 led to a banding pattern indistinguishable from cleavage in the presence of PI(4,5)P_2, while PC had no effect (data not shown). It seems unlikely that this effect is due to interaction of phosphoinositides with chymotrypsin, since similar results were obtained with endoproteinase Glu-C (data not shown). Western blotting revealed that in the presence of PI(4,5)P_2 the N-terminal and the proline-rich central domain of ActA were protected against proteolysis by chymotrypsin (Fig. 4C, second and third upper panels compared with second and third lower panels) suggesting that interaction with PI(4,5)P_2 led to a masking of one or several potential cleavage sites in these domains. The C terminus of ActA, which undergoes a minor conformational change in the presence of PI(4,5)P_2, was not protected against proteolysis (Fig. 4C, upper compared with lower forth panel).

ActA Has a Binding Preference for D-3 Phosphoinositides

FIG. 4. Proteolytic cleavage of ActA changes in the presence of PI(4,5)P_2. A, Coomassie Blue-stained SDS-polyacrylamide 8–25% gradient gel showing the time course of limited chymotrypsin cleavage of ActA. The molar enzyme to substrate (E/S) ratio is indicated. The two major proteolysis products are indicated by arrows. B, schematic depiction of the preferential chymotrypsin cleavage site as determined by N-terminal sequencing and Western blotting of the 30.5- and 64-kDa bands. C, ActA was incubated in 0.1 M Tris, pH 8.0, with the indicated amounts of chymotrypsin for 1 h at 25 °C in the absence (upper panel) or presence (lower panel) of PI(4,5)P_2. Molar enzyme to substrate ratios are indicated above the lanes. Left, Coomassie Blue-stained SDS-polyacrylamide 8–25% gradient gels. Right, Western blots of selected E/S ratios. Blots were developed with antibodies against amino acids 1 to 18 in the N terminus of ActA (anti-N), against the second of the 4-fold repeated FP4 motifs (anti-P), and against the C-terminal histidine tag (anti-C-His). The position of the peptides used for immunization is indicated by gray boxes in B.
Phosphoinositide and Actin Binding by Bacterial Protein ActA

That Maps to Amino Acids 184 to 202 in the N Terminus—We used a lipid co-sedimentation assay previously described for pleckstrin homology domains (28) to characterize the phospholipid binding specificity of ActA and to identify the region in ActA that is responsible for phospholipid binding. As shown in Fig. 5A, ActA exhibited the highest affinity for PC vesicles containing PI(3,4,5)P_3, PI(3,4)P_2, or PI(3,5)P_2, while weaker binding was seen to PI(4,5)P_2 and very little binding to any other lipid tested. In control experiments, less than 0.3% of the lipids were detected in the supernatants indicating that all vesicles contained full PC/PI(3,4,5)P_3 binding activity compared with ActA and Fig. 4C. A to C, each column corresponds to the mean of two independent measurements.

![Fig. 5. Phospholipid specificity and identification of the phosphoinositide-binding region. A and B, co-sedimentation of ActA (A) or GST-ActA184–202 (B) with PC vesicles containing 9% (mol/mol) of different acidic phospholipids. PA, phosphatic acid; PS, phosphatidylserine; PI, phosphatidylinositol; PI(4P), phosphatidylinositol 4-phosphate. C, co-sedimentation of different ActA fragments or control proteins with PC-vesicles containing 9% (mol/mol) PI(3,4,5)P_3, BSA, bovine serum albumin. 0% corresponds to the background sedimentation in the absence of phospholipid (A) or in the presence of PC (B and C). A to C, each column corresponds to the mean of two independent measurements.](image)

The significance of this region for lipid binding was supported by a data base search with the program BLOCKS (38) using the N-terminal domain of ActA which revealed significant homology of amino acids 185 to 201 of ActA with the N terminus of cecropins (Fig. 6B), positively charged peptide antibiotics of animals that act by killing bacteria through membrane lysis (39). The N-terminal part of cecropins has a high propensity to form an amphipathic \( \alpha \)-helix (Fig. 6C) that is believed to associate with membranes (40). Amino acids 185 to 199 of ActA are also predicted to have a high probability to form an amphipathic \( \alpha \)-helix (Fig. 6C). This stretch of amino acids is rich in spaced basic and hydrophobic residues consistent with known phosphoinositide binding motifs in various cytoskeleton proteins (34). In addition, it shows similarity to putative PI(3,4,5)P_3 binding motifs of small Rho-like GTPases and other proteins (41). In order to test the importance of this motif for ActA-phosphoinositide interaction, we used histidine-tagged GST fusion proteins containing amino acids 136 to 235, 184 to 235, and 184 to 202 of ActA. As shown in Fig. 5C, all three fusion proteins displayed full PI(3,4,5)P_3 binding activity. In contrast to this, histidine-tagged GST, the actin-binding fusion protein GST-ActA33–74 and GST-ActA41–56 showed no binding (Fig. 5C and data not shown). The binding properties of different ActA fragments are summarized in Fig. 6A. The binding specificity of two of the GST-ActA fusion proteins (GST-ActA184–202 and GST-ActA136–235) was addressed by comparing binding of PI(3,4)P_2 and PI(4,5)P_2. The binding specificity, i.e., preferred binding of D-3 phosphoinositides was similar to the binding specificity of the whole ActA molecule (Fig. 5, A and B, and data not shown).

**DISCUSSION**

The physicochemical characterization of soluble, secreted ActA revealed that ActA molecules are strongly elongated. However, despite its monomer mass of 65 kDa and its partial oligomerization into dimers and possibly tetramers or higher-order aggregates, we were unable to reveal a distinct morphology of the ActA molecule by electron microscopy neither by glycerol spraying/low-angle rotary metal shadowing nor by negative staining. The CD spectra of ActA indicated that ActA has a relatively low secondary structure content providing a possible explanation for our finding that native, soluble ActA is highly susceptible to proteolytic attack. Taken together, these data suggest that ActA may act as a long, flexible scaffolding protein whose main function consists in providing an assembly of docking sites for host cell factors.

Actin binding by ActA has been a matter of debate since Lasa et al. (15) showed that a synthetic peptide corresponding to amino acids 33 to 74 of ActA interacts directly with F- and G-actin in vitro. Here, using ActA coated beads and fluorescently labeled G-actin, we found that the whole, folded protein molecule binds actin and could attribute this binding activity to the N-terminal domain of ActA. Moreover, actin binding by ActA was abolished by deletion of amino acid residues 41 to 56 and, consistent with the peptide data (15), amino acids 33 to 74 exhibit actin binding activity in our assay. At a 1:1 molar ratio

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2 U. Aebi, M. Häner, G. Cicchetti, and C. Kocks, unpublished observations.

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**FIG. 5.** Phospholipid specificity and identification of the phosphoinositide-binding region. A and B, co-sedimentation of ActA (A) or GST-ActA184–202 (B) with PC vesicles containing 9% (mol/mol) of different acidic phospholipids. PA, phosphatic acid; PS, phosphatidylserine; PI, phosphatidylinositol; PI(4P), phosphatidylinositol 4-phosphate. C, co-sedimentation of different ActA fragments or control proteins with PC-vesicles containing 9% (mol/mol) PI(3,4,5)P_3, BSA, bovine serum albumin. 0% corresponds to the background sedimentation in the absence of phospholipid (A) or in the presence of PC (B and C). A to C, each column corresponds to the mean of two independent measurements.**
of ActA to actin, ActA exhibited a slight inhibitory effect on actin polymerization kinetics, but neither ActA nor the N-terminal, proline-rich central or C-terminal domain showed F-actin binding under physiological salt conditions. Therefore, in contrast to the ActA peptide, the whole ActA molecule may bind only to G-actin.

Lasa et al. (15) described an in vivo phenotype for a mutant Listeria bacterium expressing an ActA molecule lacking a region comprising the actin-binding site (amino acids 21 to 97). This mutant showed discontinuous actin-based movement (“stalling and going”). Consistent with this, we found that soluble ActA carrying a deletion of amino acids 41 to 56, and thus unable to bind to actin, was impaired in the accumulation of actin-rich protrusions on beads incubated in cytoplasmic extracts (see Fig. 1D, fifth and sixth panel). We suggest that those phenotypes may be explained by an impaired ability to recruit G-actin, a limiting factor for actin nucleation. The N-terminal domain of the ActA protein has been shown to interact with the Arp2/3 complex and this interaction leads to a dramatic increase in actin filament nucleation activity (16). N-WASP, WASP, and Scar1, homologous cytoskeleton proteins involved in dynamic remodeling of the actin cytoskeleton, share a conserved C-terminal domain that contains an arrangement of binding sites for the Arp2/3 complex and for G-actin that has been shown to facilitate actin nucleation (42–44). We propose that the same applies to the ActA protein (see Fig. 6D).

Interestingly, part of the Arp2/3 complex binding fragment of Scar1 (amino acids 534 to 555) and the corresponding region in the C terminus of WASP (amino acids 473 to 495) share homology with amino acids 116 to 137 of ActA (45). These sequences all contain a basic amino acid cluster next to an acidic region. Thus, with respect to correct positioning of the Arp2/3 complex and G-actin molecules in order to facilitate actin filament nucleus formation, ActA may mimic the C-terminal domains of WASP and Scar1. In order to facilitate nucleation, G-actin binding to ActA should occur transiently with a relatively low affinity. This prediction is in agreement with our data and may explain why actin binding by ActA has not been found in previous experiments with intact bacteria where the ActA concentration may have been limiting (17). The functional relevance of this actin-binding site will have to be addressed by a detailed mutational analysis in vivo and by in vitro assays examining the ability of different ActA mutants to stimulate the nucleation activity of the Arp2/3 complex.

Phosphorylated phosphoinositides represent a novel class of ligand for ActA. We showed that upon binding of PI(4,5)P2 or PI(3,4,5)P3, ActA underwent a secondary structure change which led to an increase in $\alpha$-helix and a decrease in $\beta$-sheet content. Since our ActA preparations consisted of a heterogeneous mixture of monomer, dimer, and higher order aggregates, it is unclear at present whether this secondary structure change upon lipid binding occurs in all molecules or in a distinct molecule species, or leads to a change in the multimerization state. The identical CD spectra and limited proteolysis profiles of ActA in the presence of PI(4,5)P2 or PI(3,4,5)P3 micelles suggest that ActA binds both lipids through the same binding site. Co-sedimentation of the ActA protein with PC vesicles containing various acidic phospholipids revealed a higher binding affinity of ActA to D-3 phosphoinositides compared with the respective 4- or 4,5-phosphorylated isomers. This result suggests that the environment of the phosphoinositide head group influences the binding specificity. It remains open, however, whether phosphoinositides are in vivo ligands of ActA.

3 G. Cicchetti and C. Kocks, unpublished data.
Using various recombinant ActA fragments, two ActA fragments generated by limited proteolysis and GST-ActA fusion proteins, we were able to localize the PC/P(3,4,5)P$_3$ binding activity of ActA to amino acids 184 to 202. These amino acids show homology to known binding sites for phosphoinositides (34) and to consensus motifs for P(3,4,5)P$_3$-binding sites (41), and they display homology to the N-terminal part of cecropins. The latter forms an amphipathic α-helix in hydrophobic environments in vitro and is believed to laterally associate with membranes by adopting an in-plane orientation in the membrane (40). Also amino acids 185 to 199 of ActA are predicted to have a high tendency of forming an amphipathic α-helix with mostly positively charged amino acid residues on one side (Fig. 6C) which could interact with the negatively charged phosphates of the phosphoinositide head group. Ligand-induced formation of this α-helix could thus contribute to the observed increase in α-helical conformation in the N terminus of ActA after phosphoinositide binding.

Based on these findings, we propose a model (Fig. 6D), in which ActA brings together G-actin and the Arp2/3 complex in an orientation that would favor actin filament nucleus formation and would thereby stimulate the nucleation activity of the Arp2/3 complex. Phosphoinositides are not necessary for ActA-stimulated nucleation by the Arp2/3 complex in vitro (16), but phosphoinositide binding may regulate the ActA-Arp2/3 interaction in vivo, for example, by regulating the docking of the complex onto or its release from ActA. This regulation could involve a structural change in ActA or a change in dimerization state, or both. (Preliminary data show that phosphoinositide binding by ActA does not interfere with actin binding.) Phosphoinositide binding by ActA could also serve to generate secondary docking sites for phosphoinositide-binding proteins involved in actin assembly. Some of those have been implicated in Listeria motility such as the small GTPases Rho or Rac, profilin, capZ, or α-actinin (see Refs. 46 and 47 and reviewed in Ref. 1). For example, ActA could sequester profilin in order to enhance rapid filament elongation by promoting the dissociation of profilin from barbed filament ends (48) or the dissociation of profilactin itself (36). Another possibility would be the sequestration of capping proteins such as capZ which are thought to compete with efficient barbed end elongation of actin filaments by profilactin through the Ena/VASP-profilin system. Indeed, the local maintenance of a pool of uncapped actin filaments is thought to play a role in Listeria motility (49). Interestingly, recent results show interference of ActA with P(1,4,5)P$_3$-mediated filament uncapping in vitro. On the other hand, Listeria overexpressing mutant ActA molecules carrying a deletion of amino acids 158 to 231, thus comprising the phosphoinositide-binding region (amino acids 184 to 202), and mutation of the phospholipid-binding amino acids in ActA will be required to find out what role phospholipid binding by ActA may play in regulating actin assembly or some other aspect of cellular infection by Listeria.

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