Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 11890–11894, December 1993
Microbiology

Expression and phosphorylation of the *Listeria monocytogenes* ActA protein in mammalian cells

(microbial pathogenesis/actin/okadaic acid)

RODNEY A. BRUNDAGE*, GREGORY A. SMITH†, ANDREW CAMILLI‡, JULIE A. THERIOT§¶, AND DANIEL A. PORTNOY†||

†Department of Microbiology, University of Pennsylvania, School of Medicine, Philadelphia, PA 19104-6076; and §Departments of Biochemistry and Biophysics and Pharmacology, University of California, San Francisco, CA 94143

Communicated by Emil C. Gotschlich, September 3, 1993

ABSTRACT **Movement of *Listeria monocytogenes* within infected eukaryotic cells provides a simple model system to study the mechanism of actin-based motility in nonmuscle cells. The *actA* gene of *L. monocytogenes* is required to induce the polymerization of host actin filaments [Kocks, C., Gouin, E., Tabouret, M., Berche, P., Ohayon, H. & Cossart, P. (1990) *Cell* 68, 521–531; Domann, E., Wehland, J., Rohde, M., Pistor, S., Hartl, M., Goebel, W., Leimeister-Wachter, M., Wuenscher, M. & Chakraborty, T. (1992) *EMBO J.* 11, 1981–1990]. In this study, an in-frame deletion mutation within the *actA* gene was constructed and introduced into the *L. monocytogenes* chromosome by allelic exchange. This mutation resulted in a decrease (3 orders of magnitude) in virulence for mice. In tissue culture cells, the *actA* mutant was absolutely defective for the nucleation of actin filaments and consequently was impaired in cell-to-cell spread. Antiserum raised to a synthetic peptide encompassing the proline-rich repeat (DFPPPPTDEEL) of ActA was used to characterize the expression of the ActA protein. The ActA protein derived from extracellular bacteria migrated as a 97-kDa polypeptide upon SDS/PAGE, whereas the protein from infected cells migrated as three distinct polypeptides, one that comigrated with the 97-kDa extracellular form and two slightly larger species. Treatment of infected cells with okadaic acid resulted in decreased amounts of all forms of ActA and the appearance of a larger species of ActA. Phosphatase treatment of ActA immunoprecipitated from intracellular bacteria resulted in conversion of the larger two species to the 97-kDa form. Labeling of infected cells with $^{32}P_i$ followed by immunoprecipitation showed that the largest molecular form of ActA was phosphorylated. Taken together, these data indicate that ActA is phosphorylated during intracellular growth. The significance of the intracellular modification of ActA is not known, but we speculate that it may modulate the intracellular activity of ActA.**

Listeria monocytogenes is a facultative intracellular Gram-positive bacterium that has been studied for decades as a model for the study of host–pathogen interactions. There is an excellent mouse model of infection in which immunity is exclusively cell mediated (1, 2). Also, tissue culture models of infection have been developed that facilitate quantitation of intracellular growth and cell-to-cell spread (3–7). The intracellular phase of the *L. monocytogenes* life cycle can be divided broadly into four stages: (*i*) internalization (8), (*ii*) escape from the host vacuole (7, 9, 10), (*iii*) multiplication in the cytosol (11), and (*iv*) cell-to-cell spread without an extracellular phase (9, 10). Cell-to-cell spread is initiated by using components of the host cell's actin cytoskeleton that propel the bacteria through the cytoplasm at rates up to 1.5 μm/sec (9, 10, 12–14). Next, the bacteria are often seen in long projections extending from the cell, which may facilitate cell-to-cell spread. The ability of *L. monocytogenes* to nucleate host actin filaments is similar to the unrelated Gram-negative pathogens *Shigellae* (15) and *Rickettsiae* (16, 32).

Several genetically linked genes encoding *L. monocytogenes* determinants of pathogenicity have been described and recently reviewed (17). These include a hemolysin (listeriolysin O), two distinct phospholipases C, a metalloprotease, and the focus of this report, ActA (18, 19). The precise functions of these determinants of pathogenicity have been difficult to assign due to the polycistronic organization of the genes, the interdependence of the gene products, and their redundancy in function (17). Nevertheless, two recent reports have shown that insertion mutations within *actA* result in *L. monocytogenes* mutants unable to mediate the polymerization of host actin filaments (18, 19). The interpretation of the precise effect of these insertion mutations on the virulence of the organism was complicated by the polar effect on the downstream genes *plcB*, which encodes a broad-range phospholipase C that is also required for pathogenicity, and three open reading frames of unknown function (20). Thus, it is unclear whether the effect of these mutations on virulence was strictly due to the loss of the ActA protein. However, the defect in actin polymerization was complemented by *actA* cloned on a plasmid (18). Therefore, it is clear that ActA is essential for the bacteria-mediated polymerization of host actin filaments.

The *actA* gene predicts a protein of 610 amino acids, which contains a signal sequence, a region of four proline-rich repeats, and a hydrophobic stretch of amino acids at its C terminus, which may serve the role of membrane anchor (18, 19). The protein has a surface-associated polarized distribution (21, 22) and can be removed from the bacterial surface with SDS under conditions that do not result in lysis of the bacteria (18). Lastly, although the *actA* gene predicts a mature protein with a molecular mass of 67 kDa, it migrates on SDS/PAGE at an apparent molecular mass of >90 kDa.

The capacity of *L. monocytogenes* to enter cells and use a host system for actin-based motility has made it a very attractive model to dissect the host components required for this complex process. In this report, genetic evidence is provided that ActA is absolutely required by the bacteria for nucleation of actin filaments and for virulence. Furthermore,

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

*Present address: Institute for Cancer Research, Fox Chase Cancer Research Center, Philadelphia, PA 19111.
‡Present address: Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, MA 02115.
¶Present address: Whitehead Institute for Biomedical Research, 9 Cambridge Center, Cambridge, MA 02142.
||To whom reprint requests should be addressed.

we show that ActA is phosphorylated during growth in mammalian cells.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. Three strains of *L. monocytogenes* were used in this study. The wild-type strain was 10403S, which has an LD_{50} for BALB/c mice of $\approx 2 \times 10^4$ (6) and expresses very low levels of ActA after growth in bacteriological medium. DP-L1942 was derived from 10403S and has an in-frame deletion mutation in the *actA* gene. The construction of DP-L1942 is described below. DP-L1470 was derived from 10403S and harbors a plasmid, pAM401::*plcA prfA*, as described by Camilli *et al.* (23). This strain expresses easily detectable levels of ActA after *in vitro* cultivation due to the presence of *prfA* present on a multicopy plasmid.

All strains were propagated in brain/heart infusion agar and broth (BHI; Difco). All stock cultures were stored as suspensions of cells at −70°C in 50% (vol/vol) glycerol. For routine use, bacteria were stored on BHI agar. DP-L1470 was propagated in the presence of chloramphenicol (15 μg/ml).

Tissue Culture, Infection of Cells, and Mice. The primary cell line used for most of these studies was the J774 macrophage-like cell line. J774 cells were maintained at 37°C in a 50-ml spinner flask in Dulbecco's modified Eagle's medium (DMEM) containing glucose (4500 mg/liter), 10% fetal bovine serum, 100 units of penicillin per ml, 10 μg of streptomycin per ml, and 2 mM L-glutamine. Bone marrow-derived macrophages were isolated as described (6). The potoroo kidney epithelial (PtK2) cell was propagated as described (14).

Infection of J774 cells and bone marrow-derived macrophages was performed as described (6). The concentration of gentamicin, which is bactericidal for extracellular bacteria but has no measurable effect on intracellular bacteria (6), varied depending on the experiment. Infection of PtK2 cells, use of rhodamine/phalloidin, and fluorescence microscopy were performed as described (14).

The LD_{50} for BALB/c mice injected intravenously was done as described (6).

Generation of an In-Frame Deletion in *actA*. The basic strategy for the generation of in-frame deletions in *L. monocytogenes* has been described (23). A 1.77-kb fragment of the *actA* gene was amplified from *L. monocytogenes* strain EGD (19) DNA by PCR and ligated into pKSV7 (24) using *Eco*RI. The *Eco*RI restriction sites of the amplified product were generated as noncomplementary ends of the amplification primers. The PCR products derived from 10403S and EGD had the same molecular mass, but the allele of *actA* derived from 10403S lacked one of the *Hpa* II sites useful for generation of the in-frame deletion. Primers used were based on the DNA sequence of Vasquez-Boland *et al.* (20) and consisted of 5′-GGAATTCATTACTGCCAATTG-CATTA-3′ and 5′-GGAATTCGTCTTCTGCACTTT-TAGC-3′. A 903-bp in-frame deletion corresponding to ≈50% of the *actA* coding sequence was generated by digestion with *Hpa* II and the plasmid was circularized with T4 ligase. The resulting plasmid was transformed into 10403S by electroporation and allelic exchange was performed as described (23).

Generation of Antiserum Specific for ActA. The peptide DFPPPPTDEEL derived as a consensus sequence for the ActA proline-rich repeat (18, 19) was synthesized at the Biomolecular Resource Center at the University of California (San Francisco). To facilitate sulfhydryl coupling to carrier protein and agarose beads, an N-terminal cysteine was added to the peptide. The peptide was coupled to keyhole limpet hemocyanin (Sigma) and antiserum was raised in rabbits as described (25). For immunofluorescence, antiserum was affinity purified on peptide coupled to Affi-Gel 10 (Bio-Rad) as described (25) and used at 10 μg/ml. For Western blots, antiserum was used at a dilution of 1:4000.

Labeling of Bacterial Proteins in J774 Cells. The method of *in vivo* labeling was adapted from that of Headley and Payne for *Shigella flexneri* (26). J774 cells (10^6) were grown overnight on 35-mm Petri dishes in 2 ml of DMEM without antibiotics. The cells were infected with 10^7 bacteria grown overnight in BHI at 30°C. After 30 min, the cells were washed three times with phosphate-buffered saline (PBS) at 37°C and DMEM at 37°C was added. After a further 30 min, gentamicin sulfate was added to a final concentration of 50 μg/ml. At 3.5 h after infection, the monolayer was washed three times with PBS at 37°C and the medium was replaced with 1 ml of methionine-free DMEM/10% dialyzed fetal calf serum/225 μg of cycloheximide per ml (Sigma)/30 μg of anisomycin per ml (Sigma). After 30 min, the medium was replaced with 100 μl of the same medium containing 90 μCi of [^{35}S]methionine (1 Ci = 37 GBq) (Tran^{35}S-label; ICN), and 30 min later the monolayer was washed three times in PBS and lysed in 100 μl of 2× final sample buffer (FSB) (2% SDS/0.06 M Tris·HCl, pH 6.8/10% glycerol/5% 2-mercaptoethanol/0.05% bromophenol blue) and boiled immediately for 5 min. In a parallel experiment, the number of bacteria per monolayer was determined by including coverslips in a Petri dish as described (6). Approximately 25% of the sample was subjected to SDS/PAGE (8% polyacrylamide) as described (6), followed by fluorography (EN^{3}HANCE; DuPont). Okadaic acid (GIBCO) was used at a final concentration of 1 μM and was present for 3 h prior to the addition of [^{35}S]methionine. For labeling with ^{32}P, infected cells were incubated in phosphate-free DMEM plus 1 mCi of $^{32}P_i$ per ml for 1 h.

Immunoprecipitation of *in Vivo* Labeled ActA and Phosphatase Treatment. Monolayers of J774 cells were washed once with PBS at 4°C and lysed by gently rocking at 4°C for 30 min in 0.5 ml of RIPA buffer (150 mM NaCl/50 mM Tris·HCl/10 mM EDTA/1.0% Nonidet P-40/0.5% deoxycholate/0.1% SDS/250 μM phenylmethylsulfonyl fluoride, pH 8.0). The lysate was further solubilized by passage through a 26-gauge needle and subjected to centrifugation in a microcentrifuge for 10 min at 4°C. Ten microliters of ActA antiserum was added to the supernatant fluid and the sample was rocked for 30 min at 4°C, followed by addition of 0.5 ml of RIPA buffer containing 10 mg of protein A-Sepharose (Sigma). After rocking at 4°C for 1 h, the immunoprecipitated material was washed three times in RIPA buffer, suspended in 80 μl of 2× FSB, and boiled for 5 min before SDS/PAGE (8% polyacrylamide).

Phosphatase treatment was performed on immunoprecipitated material (see above) after washing an additional three times in water. The immunoprecipitate was treated with 20 units of calf intestinal phosphatase (Boehringer Mannheim) in buffer supplied by the manufacturer.

Western Blotting. ActA from extracellular bacteria was extracted from *L. monocytogenes* DP-L1470 grown to stationary phase in BHI. One milliliter of culture was subjected to centrifugation in a microcentrifuge, washed once in PBS, suspended in 100 μl of 2× FSB, and boiled for 5 min. The bacteria were removed by centrifugation and the supernatant fluid [containing SDS-extractable material including ActA (18)] was diluted in 1× FSB, and the equivalent of 2×10^7 bacteria was subjected to electrophoresis. J774 cells or infected J774 cells as described above were lysed in 2× FSB and subjected to SDS/PAGE (8% polyacrylamide). Approximately 2×10^7 bacteria and 4×10^5 J774 cells were applied per lane. Western blotting was performed as described (27). The primary antibody was the rabbit anti-peptide anti-serum described above and used at a dilution of 1:4000 followed by ^{125}I-labeled protein A.

RESULTS

Construction of an In-Frame Deletion in *actA*. The *actA* gene was cloned in *Escherichia coli* and a 903-bp *Hpa* II fragment was deleted, resulting in the deletion of ≈50% of the *actA* coding sequence including the entire region containing the proline-rich repeats. The deleted *actA* allele was introduced into the chromosome of *L. monocytogenes* by allelic exchange. Since the deletion was in-frame, it was predicted to have no effect on the expression of downstream genes, including *plcB*, which encodes the broad spectrum phospholipase C (20, 28, 29). Indeed, the ActA mutant expressed normal levels of the *plcB* gene product in infected J774 cells (H. Goldfine, C. Knob, and D.A.P., unpublished observation). The strain containing the deleted form of *actA* (DP-L1942) had an LD_{50} in BALB/c mice of 2×10^7 compared to 2×10^4 for the parental wild-type strain.

Role of *actA* in Tissue Culture Cell Models. The ability of wild-type *L. monocytogenes* and the *actA* mutant to nucleate actin filaments was first examined in the potoroo kidney epithelial cell line PtK2. In these extremely flat cells, intracellular bacteria and their actin-based tails can be seen by phase-contrast microscopy (Fig. 1 *1* and *4*). The bacteria were also stained with antiserum specific for the proline-rich repeat in ActA, which had been deleted in the *actA* mutant (Fig. 1 *2* and *5*) and with rhodamine/phalloidin to label F-actin (Fig. 1 *3* and *6*). It is clear that each wild-type bacterium had ActA on its surface and some of the bacteria had actin-based tails, indicating that they were in the process of actin-based motility (14). As would be predicted, the *actA* mutant did not react with the antiserum and showed no evidence for rhodamine/phalloidin staining, indicating that the mutant was defective in the induction of actin filaments. Consequently, the *actA* mutant was absolutely defective for intracellular movement when examined by video microscopy (40).

Visual inspection of infected cells suggested that the ActA mutant grew normally but was defective in intracellular and intercellular spread. This was further examined by monitoring the growth of the bacteria in J774 cells over a 14-h period (Fig. 2). The cells were infected so that ≈1 in 20 macrophages contained a single bacterium after 30 min, and the subsequent

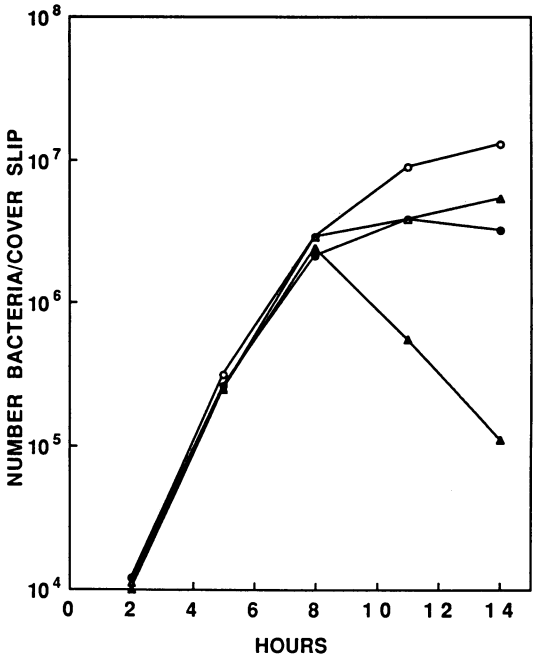

FIG. 2. Intracellular growth of *L. monocytogenes* strains in the mouse macrophage-like cell line J774. Data represent average number of colony-forming units determined from three coverslips for each time point. ○, 10403S in the presence of gentamicin (5 μg/ml); ●, 10403S in the presence of gentamicin (50 μg/ml); △, DP-L1942, the *actA* mutant, in the presence of gentamicin (5 μg/ml); ▲, DP-L1942 in the presence of gentamicin (50 μg/ml).

intracellular growth of the bacteria was monitored by examination of colony-forming units. After 1 h of infection, gentamicin was added to a final concentration of either 5 or 50 μg/ml. Both concentrations of gentamicin inhibit extracellular growth, but the higher concentration is rapidly bactericidal, while the lower dose is slowly bactericidal (data not shown). The wild-type and the *actA* mutant had identical growth rates during the first 8 h of infection in both concen-

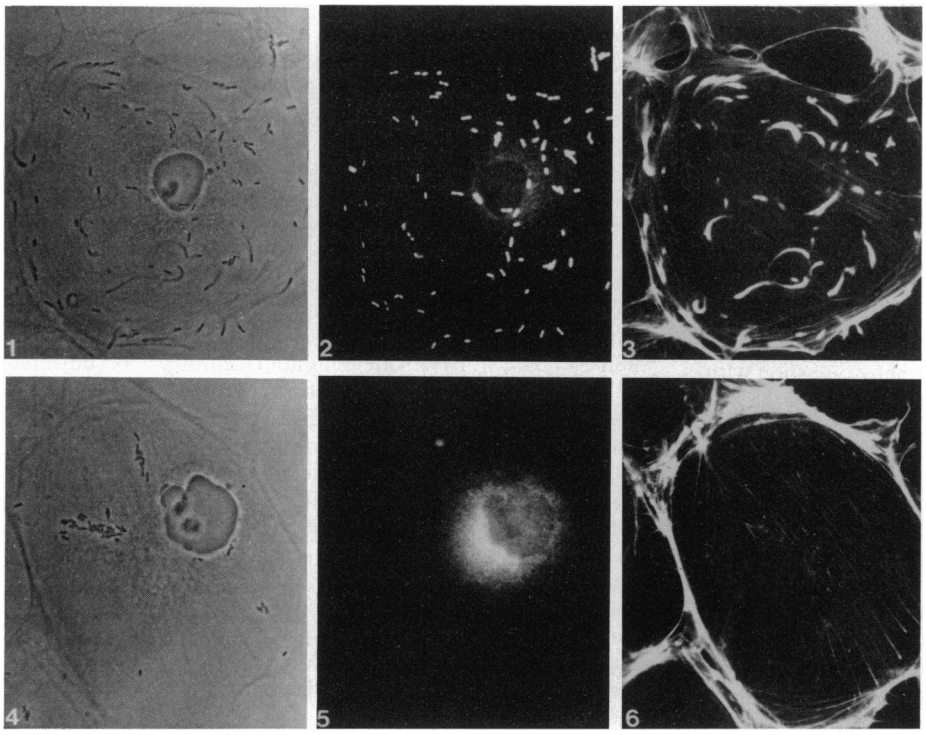

FIG. 1. Phase-contrast and fluorescence micrographs of *L. monocytogenes* strains after 5 h of growth in PtK2 cells. (*1–3*) The same cell infected with the wild-type strain 10403S. (*4–6*) Another cell infected with the *actA* mutant DP-L1942. *1* and *4*, Phase-contrast micrographs; *2* and *5*, stained with anti-ActA antiserum specific to the proline-rich repeat, followed by fluorescein isothiocyanate-labeled goat anti-rabbit anti-serum; *3* and *6*, stained with rhodamine/phalloidin, which stains F-actin.

trations of gentamicin (average doubling time, 51 min from $T = 2$ to $T = 8$) (Fig. 2). However, after 8 h, the *actA* mutant showed a dramatic loss of colony-forming units in the presence of a high gentamicin concentration. These data are consistent with the visual observation that the *actA* mutant is unable to spread. Thus, after 8 h and in the absence of cell-to-cell spread, a single cell infected with one bacterium would be expected to harbor between 250 and 500 bacteria. Further bacterial growth likely resulted in lysis of the host cell and subsequent killing of the bacteria by extracellular gentamicin, accounting for the sharp decrease in colony-forming units. In contrast, the wild-type bacteria showed no net decrease in colony-forming units at high gentamicin concentrations, presumably because of their ability to spread from infected cells into uninfected cells. In the presence of low concentrations of gentamicin, the *actA* mutant likely infected neighboring cells via an extracellular route and continued to propagate in newly infected cells.

Expression of ActA in Mammalian Cells. The expression of ActA in J774 cells was first examined by Western blotting of infected cells. In addition to the 97-kDa polypeptide seen after extracellular growth (Fig. 3*A*, lane 1), two new molecular species appeared of higher molecular mass (lane 3). The three species of ActA were the major SDS-extractable bacterial proteins observed in [^{35}S]methionine-labeled infected cells (Fig. 3*B*). These results indicated that *actA* was expressed at high levels in cells and that the ActA protein was modified during intracellular growth so that it migrated as a triplet of bands. ActA also migrated as a triplet in infected PtK2 cells and in bone marrow-derived macrophages (data not shown). Examination of both laboratory and clinical isolates of *L. monocytogenes* showed that 9 of 10 strains expressed multiple forms of ActA after intracellular growth (L. Rong and D.A.P., unpublished observation).

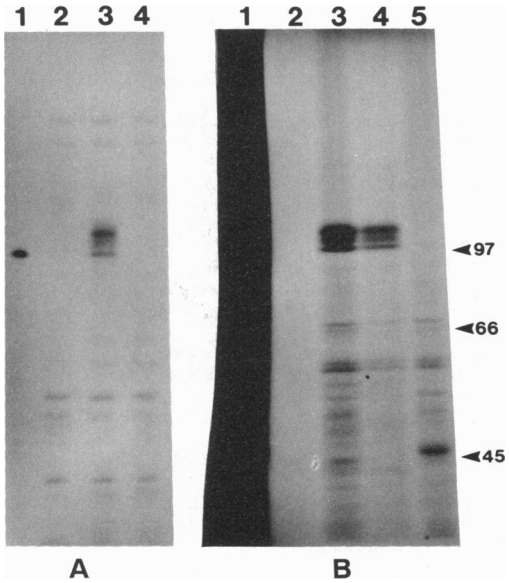

FIG. 3. Intracellular expression of ActA by *L. monocytogenes* in J774 cells. (*A*) Western blot reacted with antiserum specific to the ActA proline-rich repeat region. Lanes: 1, SDS-extractable proteins of DP-L1470 grown extracellularly (note that ActA appears as a single band of ≈97 kDa); 2, uninfected J774 cells (this represents background); 3, J774 cells infected with the wild-type strain 10403S (note that ActA migrates as a triplet); 4, J774 cells infected with the DP-L1942 (the *actA* mutant). (*B*) Autoradiogram of [^{35}S]methionine-labeled proteins. Lanes: 1, uninfected J774 cells labeled without eukaryotic protein synthesis inhibitors; 2, uninfected J774 cells labeled in the presence of eukaryotic protein synthesis inhibitors; 3, J774 cells infected with wild-type strain 10403S (ActA triplet is the most abundant of the labeled bacterial proteins); 4, J774 cells infected with DP-L1470; 5, J774 cells infected with the *actA* mutant DP-L1942. The truncated ActA polypeptide migrates at ≈47 kDa. Cycloheximide and anisomycin were added to the cells in lanes 2–5 for 30 min before addition of [^{35}S]methionine and during the 30-min labeling period. Numbers on right are kDa.

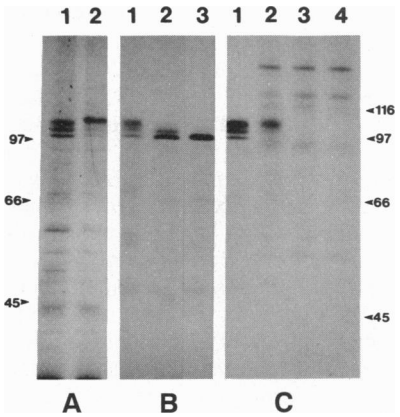

FIG. 4. Phosphorylation of ActA in J774 cells. (*A*) [^{35}S]Methionine-labeled bacterial proteins produced during intracellular growth of *L. monocytogenes* in J774 cells. Lanes: 1, strain 10403S; 2, strain 10403S infected in the presence of okadaic acid, an inhibitor of host phosphatases. (*B*) Bacterial proteins immunoprecipitated with ActA antiserum after [^{35}S]methionine labeling of infected cells in the presence of eukaryotic protein synthesis inhibitors. Lanes: 1, after incubation of the immunoprecipitated material in phosphatase buffer for 22 h; 2, calf intestinal phosphatase treatment for 2 h; 3, phosphatase treatment for 22 h. (*C*) Lanes: 1, [^{35}S]methionine-labeled bacterial proteins from strain 10403S showing the three forms of ActA; 2, proteins *in vivo* labeled with ^{32}P during infection by the wild-type strain followed by immunoprecipitation with ActA antiserum (the largest two forms of ActA are phosphorylated); 3, same as lane 1 but with cells infected with DP-L1942, the *actA* mutant; 4, same as lanes 2 and 3 but with uninfected cells (this represents the background). Numbers on left and right are kDa.

Labeling of bacterial proteins with [^{35}S]methionine during intracellular growth in the presence of okadaic acid, a potent inhibitor of host protein-serine/threonine phosphatases 1 and 2A (30), resulted in a considerable decrease in the amount of all three forms of ActA and the appearance of a new, slightly higher molecular mass form (Fig. 4*A*). This suggested that multiple sites of phosphorylation on ActA might be responsible for the higher molecular mass forms seen exclusively during intracellular growth. To test this hypothesis, ActA labeled with [^{35}S]methionine during intracellular growth was immunoprecipitated and incubated *in vitro* with calf intestinal phosphatase (Fig. 4*B*). Phosphatase treatment resulted in conversion of the three forms to a single form that comigrated with the 97-kDa form made by extracellular bacteria. Lastly, infected cells were labeled with $^{32}P_i$, and immunoprecipitation of ActA revealed that the largest molecular mass forms of ActA were phosphorylated (Fig. 4*C*). Taken together, these data indicate that ActA is phosphorylated in mammalian cells and suggest that this results in altered migration upon SDS/PAGE.

DISCUSSION

The results of this study confirm and extend the work of others (18, 19) showing that ActA is an essential determinant of *L. monocytogenes* pathogenesis, which is required for the induction of actin polymerization and cell-to-cell spread. An in-frame *actA* deletion mutant was absolutely defective for actin-based motility and cell-to-cell spread, and it was 3 orders of magnitude less virulent in mice. These results strongly support the premise that actin-based motility and cell-to-cell spread are essential for the pathogenicity of *L. monocytogenes*.

The results of this study also demonstrated that the *L. monocytogenes* ActA protein is a major bacterial protein expressed during intracellular growth. Furthermore, ActA is phosphorylated exclusively during intracellular growth in all cell types so far examined. Interestingly, phosphorylation resulted in two new forms of ActA, which migrated more slowly on SDS/polyacrylamide gels. Although we have not yet established the actual extent of protein phosphorylation, we favor a model in which there are two sites of phosphorylation per ActA molecule that result in the observed changes in apparent molecular mass. Consistent with this model, treatment of ActA with phosphatase resulted first in loss of the largest double-phosphorylated band followed by conversion of the single-phosphorylated middle band to the unphosphorylated 97-kDa form. A dramatic shift in apparent molecular mass due to a single phosphorylation event has also been observed in a number of other systems—notably, the microtubule-associated protein tau (31).

The ability of *L. monocytogenes* to mediate actin polymerization in mammalian cells is shared by other bacterial species (15, 16, 32). *S. flexneri*, an enteric bacterial pathogen, uses a host system of actin-based motility that is probably similar to that used by *L. monocytogenes* (15, 33, 34). An outer membrane protein of ≈120 kDa, IcsA (VirG), is required by *S. flexneri* for actin nucleation and cell-to-cell spread and may serve the analogous function of ActA (15, 34–36). d'Hauteville and Sansonetti (37) have reported that the IcsA protein contains a consensus site for phosphorylation by cAMP-dependent protein kinase A and can be phosphorylated *in vitro* at that site. Removal of the kinase recognition site led to an increased capacity of the bacteria to spread from cell to cell during the first 3 h of infection in a HeLa cell infection assay. However, intracellular phosphorylation of IcsA was not examined. ActA lacks the consensus protein kinase A phosphorylation site found in IcsA but, like IcsA, has numerous potential sites of phosphorylation by other host kinases. For example, ActA has multiple consensus sites for phosphorylation by casein kinase II (21). However, the precise sites of ActA that are phosphorylated intracellularly remain to be identified. Elimination of the sites of phosphorylation by mutation should lead us to the precise role of ActA phosphorylation that could be required for its activity or, conversely, as suggested for *S. flexneri* (37), that could inhibit its activity. It is as yet unclear whether a host kinase is responsible for phosphorylation of ActA, although phosphorylation was observed only after growth in mammalian cells.

ActA is clearly required by *L. monocytogenes* to mediate actin polymerization, but its precise function is not understood. Since *L. monocytogenes* grown outside of host cells do not efficiently nucleate polymerization of pure actin (38), it is possible that ActA functions by providing binding sites for host cell actin-associated proteins that promote polymerization. The repeated sequence, whose consensus is DFPPPPTDEEL, is found four times in the middle of the ActA polypeptide and could be such a binding site. One candidate for a host protein that might bind to ActA is the actin monomer binding protein profilin, which binds strongly and specifically to polyproline *in vitro* (39). Profilin does in fact localize at the interface of *L. monocytogenes* and its actin-based tail during intracellular movement and *in vitro* after exposure to cytoplasmic extracts (40). However, there is no direct evidence that profilin binds directly to ActA, and efforts to demonstrate profilin binding *in vitro* have been unsuccessful. Thus, if ActA is indeed the bacterial protein that binds profilin, it must be processed or modified in the host cell before binding can occur. It is tempting to speculate that phosphorylation of ActA modulates some feature of its activity, which in addition to nucleation of actin filaments may be to bind profilin and/or other host proteins.

We thank Archie Bouwer and David Hinrichs for performing the animal studies, Lew Tilney for his advice and enthusiasm and for suggesting we examine the affect of okadaic acid, and Jorge Galan for suggesting the use of phosphatase. This work was supported by National Institutes of Health Grant AI-26919 (D.A.P.). R.A.B. was supported by National Institutes of Health Grant HD14474 (L. G. Tilney). J.A.T. was a Howard Hughes Medical Institute Predoctoral Fellow.

1. Kaufmann, S. H. E. (1993) *Annu. Rev. Immunol.* **11**, 129–163.
2. Portnoy, D. A. (1992) *Curr. Opin. Immunol.* **4**, 20–24.
3. Kuhn, M., Kathariou, S. & Goebel, W. (1988) *Infect. Immun.* **56**, 79–82.
4. Havell, E. A. (1986) *Infect. Immun.* **54**, 787–792.
5. Sun, A. N., Camilli, A. & Portnoy, D. A. (1990) *Infect. Immun.* **58**, 3770–3778.
6. Portnoy, D. A., Jacks, P. S. & Hinrichs, D. J. (1988) *J. Exp. Med.* **167**, 1459–1471.
7. Gaillard, J. L., Berche, P., Mounier, J., Richard, S. & Sansonetti, P. (1987) *Infect. Immun.* **55**, 2822–2829.
8. Gaillard, J. L., Berche, P., Frehel, C., Gouin, E. & Cossart, P. (1991) *Cell* **65**, 1127–1141.
9. Mounier, J., Ryter, A., Coquis-Rondon, M. & Sansonetti, P. J. (1990) *Infect. Immun.* **58**, 1048–1058.
10. Tilney, L. G. & Portnoy, D. A. (1989) *J. Cell Biol.* **109**, 1597–1608.
11. Marquis, H., Bouwer, H. G. A., Hinrichs, D. J. & Portnoy, D. A. (1993) *Infect. Immun.* **61**, 3756–3760.
12. Dabiri, G. A., Sanger, J. M., Portnoy, D. A. & Southwick, F. S. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 6068–6072.
13. Sanger, J. M., Sanger, J. W. & Southwick, F. S. (1992) *Infect. Immun.* **60**, 3609–3619.
14. Theriot, J. A., Mitchison, T. J., Tilney, L. G. & Portnoy, D. A. (1992) *Nature (London)* **357**, 257–260.
15. Bernardini, M. L., Mounier, J., d'Hauteville, H., Coquis-Rondon, M. & Sansonetti, P. J. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 3867–3871.
16. Teysseire, N., Chiche-Portiche, C. & Raoult, D. (1992) *Res. Microbiol.* **143**, 821–829.
17. Portnoy, A. D., Chakraborty, T., Goebel, W. & Cossart, P. (1992) *Infect. Immun.* **60**, 1263–1267.
18. Kocks, C., Gouin, E., Tabouret, M., Berche, P., Ohayon, H. & Cossart, P. (1992) *Cell* **68**, 521–531.
19. Domann, E., Wehland, J., Rohde, M., Pistor, S., Hartl, M., Goebel, W., Leimeister-Wachter, M., Wuenscher, M. & Chakraborty, T. (1992) *EMBO J.* **11**, 1981–1990.
20. Vazquez-Boland, J.-A., Kocks, C., Dramsi, S., Ohayon, H., Geoffroy, C., Mengaud, J. & Cossart, P. (1992) *Infect. Immun.* **60**, 219–230.
21. Kocks, C., Hellio, R., Gounon, P., Ohayon, H. & Cossart, P. (1993) *J. Cell Sci.* **105**, 699–710.
22. Niebuhr, K., Chakraborty, T., Rohde, M., Gazlig, T., Jansen, B., Kollner, P. & Wehland, J. (1993) *Infect. Immun.* **61**, 2793–2802.
23. Camilli, A., Tilney, L. G. & Portnoy, D. A. (1993) *Mol. Microbiol.* **8**, 143–157.
24. Smith, K. & Youngman, P. (1992) *Biochimie* **74**, 705–711.
25. Sawin, K. E., Mitchison, T. J. & Wordeman, L. G. (1992) *J. Cell Sci.* **101**, 303–313.
26. Headley, V. L. & Payne, S. M. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 4179–4183.
27. Portnoy, D. A., Tweten, R. K., Kehoe, M. & Bielecki, J. (1992) *Infect. Immun.* **60**, 2710–2717.
28. Geoffroy, C., Raveneau, J., Beretti, J., Lecroisey, A., Vazques-Boland, J.-A., Alouf, J. E. & Berche, P. (1991) *Infect. Immun.* **59**, 2382–2388.
29. Goldfine, H., Johnston, N. C. & Knob, C. (1993) *J. Bacteriol.* **175**, 4298–4306.
30. Cohen, P., Holmes, C. F. B. & Tsukitani, Y. (1990) *Trends Biochem. Sci.* **15**, 98–102.
31. Baudier, J. & Cole, R. D. (1987) *J. Biol. Chem.* **262**, 17577–17583.
32. Heinzen, R. A., Hayes, S. F., Peacock, M. G. & Hackstadt, T. (1993) *Infect. Immun.* **61**, 1926–1935.
33. Kadurugamuwa, J. L., Rohde, M., Wehland, J. & Timmis, K. N. (1991) *Infect. Immun.* **59**, 3463–3471.
34. Makino, S., Sasakawa, C., Kamata, K., Kurata, T. & Yoshikawa, M. (1986) *Cell* **46**, 551–555.
35. Pal, T., Newland, J. W., Tall, B. D., Formal, S. B. & Hale, T. L. (1989) *Infect. Immun.* **57**, 477–486.
36. Goldberg, M. B., Barzu, O., Parsot, C. & Sansonetti, P. J. (1993) *J. Bacteriol.* **175**, 2189–2196.
37. d'Hauteville, H. & Sansonetti, P. J. (1992) *Mol. Microbiol.* **6**, 833–841.
38. Tilney, L. G., DeRosier, D. J., Weber, A. & Tilney, M. S. (1992) *J. Cell Biol.* **118**, 83–93.
39. Theriot, J. A. & Mitchison, T. J. (1992) *Trends Cell Biol.* **2**, 219–222.
39. Tanaka, M. & Shibata, H. (1985) *Eur. J. Biochem.* **151**, 291–297.
40. Theriot, J. A., Rosenblatt, J., Portnoy, D. A., Goldschmidt-Clermont, P. J. & Mitchison, T. J. (1994) *Cell*, in press.