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Identification of cofilin, coronin, Rac and capZ in actin tails using a *Listeria* affinity approach

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SUMMARY

Actin assembly is involved in cell motility and intracellular movement of *Listeria monocytogenes*. Induction of *Listeria* actin tails is mediated by the surface protein ActA. The N-terminal domain of ActA is sufficient for this function. Cell components known to play a role in the actin-based motility of *Listeria* are VASP (vasodilatator-stimulated phosphoprotein), the multiprotein Arp2/3 complex and cofilin. VASP interacts with the central domain of ActA. Proteins interacting with the N-terminal domain of ActA have not been identified. To identify novel host cell components of ActA-induced actin tails, we used bovine brain extracts and an affinity approach with *Listeria* as matrix. Several known components of *Listeria* tails were isolated including VASP, Arp3 and cofilin. Cofilin was identified by peptide sequencing, and cofilin recruitment and *Listeria* tail length were found to be pH-dependent, in agreement with its recently reported role in enhancing actin filament turnover. In addition, three proteins not previously known to be associated with *Listeria* tails, coronin, Rac and capZ, were identified in our affinity approach. In infected cells, the localization of the identified proteins was studied by immunofluorescence. Our findings suggest that these latter proteins, which are known to play critical roles in cellular actin rearrangements, may also be involved in the dynamics of *Listeria*-induced actin assembly.

Key words: ActA, Actin polymerization, CapZ, Cofilin, Coronin, *Listeria*, Rac

INTRODUCTION

The study of the actin dynamics involved in the movement of the intracellular bacterium *Listeria monocytogenes* in the cytoplasm of infected cells has revealed similarities with localized actin assembly in eukaryotic cells, suggesting that *Listeria* could be a useful tool to identify cellular components present at the leading edge of moving cells (Lasa et al., 1998). Directional actin assembly is initiated at the surface of the bacterium, and requires expression of the bacterial surface protein ActA and subsequent recruitment of host cell factors (Kocks et al., 1992; Tilney et al., 1992; Theriot et al., 1994; Smith et al., 1995). ActA consists of an N-terminal domain (amino acids 1-234), a central proline-rich repeat domain (235-395) and a C-terminal domain (396-609), through which the protein is anchored to the membrane. The central proline-rich domain of ActA is needed for VASP (or its homolog Mena) and profilin recruitment to the bacterial surface (Chakraborty et al., 1995; Reinhard et al., 1995; Gertler et al., 1996). We have previously shown that the N-terminal domain of ActA is necessary and sufficient to confer actin-based motility in *Xenopus* egg extracts (Lasa et al., 1995, 1997). Host cell ligands able to interact with the N-terminal domain have not yet been identified. Recently, a mammalian multiprotein complex similar to a previously reported profilin-binding complex in *Acanthamoeba* (Machesky et al., 1994), was shown to stimulate actin polymerization at the surface of *Listeria* (Welch et al., 1997b). This so-called Arp2/3 complex, purified from human platelet extracts, contained two actin-related proteins, Arp2 and Arp3, and five novel proteins, p41-Arc, p34-Arc, p21-Arc, p20-Arc and p16-Arc (Welch et al., 1997a,b). Whether this complex interacts directly with ActA is unknown. In addition to VASP and the Arp2/3 complex, actin-depolymerizing factor (ADF)/cofilin was recently shown to participate in the dynamics of *Listeria* movement (Carlier et al., 1997; Rosenblatt et al., 1997).

In this report, we describe a *Listeria* affinity approach with bovine brain extracts for identifying protein components in ActA-induced actin tails. Using this method, we have isolated the known components, cofilin, VASP and Arp3, as well as three proteins not previously known to be present in the *Listeria* actin tails.

MATERIALS AND METHODS

Brain extracts

A fresh bovine brain was cut into pieces and ground by 15 passages in a Dounce homogenizer in an ice-cold extraction buffer (20 mM

Hepes, pH 7.5, 100 mM potassium acetate, 1 mM magnesium acetate, 1 mM EGTA, 0.2 mM $CaCl_2$, 0.5 mM ATP, 1 mM DTT, 10 μg/ml each of leupeptin, aprotinin, pepstatin-A and chymostatin, 1 mM PMSF) with a volume of 1 ml/g wet brain tissue. Nuclei were removed by centrifugation at 2,000 ***g***. The crude extracts were obtained by centrifugation of the homogenate at 10,000 ***g*** and were quick-frozen for storage at −80°C. Before being used, crude extracts were supplemented with 0.1% Triton X-100 and clarified by centrifugation at 150,000 ***g*** for 1 hour at 4°C. Concentration of proteins in clarified extracts was evaluated to 10 mg/ml by Bradford assay using albumin as a standard.

Platelet extracts

High-salt soluble extracts obtained from the Triton-insoluble cytoskeleton by treatment with 0.6 M KCl were prepared from human platelets as described in Welch et al. (1997b), depleted in some actin (Schaier et al., 1992), desalted and quick-frozen for storage in 20 mM Pipes, pH 7.25, 50 mM KCl, 1 mM $MgCl_2$, 0.5 mM ATP, 2 mM EGTA, 0.5 mM DTT, 80 mM sucrose, at −80°C.

Bacteria

Two *L. monocytogenes* strains were used: the *actA* deletion mutant (LO28Δ*actA*) (Gouin et al., 1995) as a control, and LO28Δ*actA* transformed with a multicopy plasmid encoding *actA* (pactA3) (Lasa et al., 1995). Bacteria were grown at 37°C in brain heart infusion medium supplemented with 10 μg/ml chloramphenicol. Overnight cultures were diluted 20-fold in fresh medium and grown to stationary phase; samples of the culture were frozen down and stored at −80°C in 20% glycerol. Bacteria were pelleted and resuspended in a 10 mM Pipes, pH 7.25, 40 mM KCl buffer before being added to the extracts.

Assay for visualization of *Listeria* actin tails

0.5 μl of a bacterial suspension (10^7 bacteria) were incubated with 10 μl of clarified extracts supplemented with 3 μM rhodamine-actin (from Cytoskeleton), 1 mM ATP and 30 mM creatine phosphate, at room temperature for 15 minutes, and 2.5 μl were squashed between a glass slide and a coverslip. The samples were observed after a 30 minute incubation, with a Zeiss Axiovert microscope.

Gel electrophoresis of brain extract proteins isolated using *L. monocytogenes*

Bacteria (10^9 bacteria in a volume of 20 μl/ml extract) were incubated in clarified extracts supplemented with ATP-regenerating mix for 1 hour at room temperature, washed three times in a low-salt buffer (10 mM Pipes, pH 7.25, 40 mM KCl, 5 mM ATP-$MgCl_2$) by low-speed centrifugation. Washed bacteria were resuspended in a high-salt buffer (10 mM Pipes, pH 7.25, 0.6 M KCl, 5 mM ATP-$MgCl_2$) and then, after an additional centrifugation, in SDS-sample buffer. Polypeptides eluted with KCl or solubilized with SDS were recovered by centrifugation, resolved by 12% SDS-PAGE and visualized by silver staining or immunoblotting with the indicated antibodies.

Peptide sequencing

KCl-eluted proteins were precipitated with trichloroacetic acid, washed with cold acetone, solubilized with SDS-sample buffer and separated by 12% SDS-PAGE. Polypeptides were visualized by staining with 0.03% amido black and the corresponding gel slices were treated with trypsin. Resulting peptides were separated by HPLC, and amino acid sequencing was carried out with an Applied Biosystems 473A sequencer. The obtained sequence was compared with protein sequence data bases using the BLITZ program.

Immunoblotting and immunofluorescence

The ActA-specific affinity-purified polyclonal antibodies were produced against a peptide comprising part of the first proline-rich repeat (Kocks et al., 1993). Polyclonal anti-peptide antibodies that recognize cofilin, VASP and Arp3, were obtained by immunizing rabbits with the following peptides: CTLAEKLGGSAVISLEGKPL, which is derived from the conserved C-terminal sequence of cofilin; KTPKDESANQEEPEA, which is derived from the human sequence of VASP; and CYEEIGPSIVRHNPVFGVMS, which was the same as used by Welch et al. (1997a) and is derived from the bovine C-terminal sequence of Arp3. Polyclonal antibodies specific for coronin were obtained using a recombinant human protein (Grogan et al., 1997). Affinity purification of the antibodies was done as described in Friederich et al. (1995). Antibodies specific for capZ were generously provided by K. Barkalow and J. A. Cooper (Schafer et al., 1996). mAbs specific for β-actin and Rac1 were from Sigma and Upstate biotechnology, respectively. Immunoblotting was performed with the indicated antibodies using ECL detection reagents (Amersham). For immunofluorescence staining, Vero fibroblast-like monkey cells or J774 macrophage-like murine cells were infected for 4-6 hours with *L. monocytogenes* strain LO28 (Vicente et al., 1985). Infected cells were permeabilized with 0.2% Triton X-100 in 50 mM KCl, 3 mM $MgCl_2$, 10 mM EGTA, 2 M glycerol, 10 mM Pipes, pH 6.8, and then fixed in 3.7% paraformaldehyde in PBS, or cells were fixed in 3% paraformaldehyde and then permeabilized either with 0.4% Triton in PBS or methanol. Immunofluorescence was performed using mAbs to actin and Rac1, or affinity-purified rabbit primary antibodies to cofilin, coronin, capZβ, and ActA, and Texas Red-conjugated or FITC-conjugated secondary antibodies (Vector laboratories); FITC-conjugated phalloidin was used to stain filamentous actin. Preparations were observed with a laser scanning confocal Wild Leitz microscope.

RESULTS

Strategy for identification of putative host factors involved in *Listeria*-induced actin assembly

In order to identify putative host factors involved in *Listeria*-directed actin assembly, we designed an assay to detect proteins in cell-free extracts that bind to ActA-expressing *Listeria* ($ActA^+$ bacteria), using Δ*actA Listeria* ($ActA^-$ bacteria) as a negative control. We prepared high-speed Triton-soluble extracts (clarified extracts) from bovine brain tissue, which is rich in cytoskeletal proteins, and is easily obtained in large amounts. When complemented with an ATP-regenerating system, these clarified extracts allowed *Listeria*-stimulated actin polymerization within a 20 minute incubation. In this system, the tail length generally increased for 1 hour and remained constant (average size 30 μm) for an additional hour (see below, Fig. 3B). Thus, clarified bovine brain extracts support *Listeria*-induced actin tail formation and provide a suitable system with which to analyse cytoskeletal proteins recruited by ActA-expressing *Listeria*. In our approach, bacteria were incubated with extracts, washed and centrifuged at low speed, and bound proteins were subsequently eluted and analyzed by SDS-PAGE.

Actin and a 19 kDa polypeptide are two major proteins associated with *Listeria* in clarified brain extracts

$ActA^+$ or $ActA^-$ bacteria were incubated in clarified brain extracts complemented with an ATP-regenerating system. After a 1 hour incubation, bacteria were washed twice in low-salt buffer, and bound proteins were eluted with high-salt (KCl). Remaining proteins were solubilized with SDS-sample

buffer. KCl-eluted or SDS-solubilized proteins were separated by SDS-PAGE and visualized after silver staining. Several polypeptides specifically associated with ActA$^+$ bacteria were detected. Among the KCl-eluted proteins, two major species of 43 kDa and 19 kDa (p19) were intensively stained (Fig. 1). The 43 kDa species present in both KCl-eluted and SDS-solubilized proteins, was identified as actin by immunoblotting (shown in Fig. 5). p19 was further analyzed (see below).

Cofilin is associated with ActA-expressing *Listeria*, and its recruitment is pH-dependent

Since most of the silver-stained p19 polypeptide was detected in the high-salt eluate of the ActA$^+$ bacteria, this eluate was used to identify it. KCl-eluted proteins were precipitated by TCA, separated by 12% SDS-PAGE, and stained with amido black. The excised band corresponding to p19 was digested with trypsin and the 18-amino-acid sequence of one tryptic peptide allowed the identification of p19 as the actin-binding protein cofilin (100% identity with amino acids 54-71 of porcine brain cofilin) (Fig. 2).

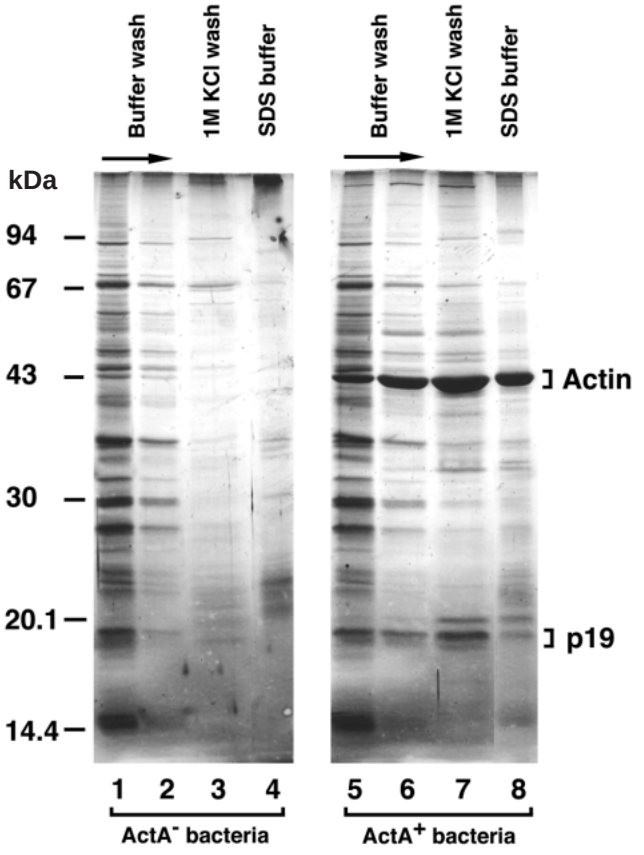


Fig. 1. Comparative SDS-PAGE analysis of proteins from bovine brain extracts associated with ActA$^+$ and ActA$^-$ *Listeria*. ActA$^-$ or ActA$^+$ bacteria were incubated in clarified brain extracts for 1 hour. After washing with low-salt buffer, bound proteins were eluted with high-salt buffer and the remaining proteins were subsequently solubilized with SDS-sample buffer. Proteins eluted with low-salt buffer wash (lanes 1 and 2, or 5 and 6), with 1 M KCl buffer (lanes 3 or 7), or solubilized with SDS (lanes 4 or 8), were resolved on 12% SDS-polyacrylamide gels under reducing conditions and silver stained. The positions of the two major species of 43 kDa (actin) and 19 kDa (p19) are indicated. Additional species with apparent mobilities of 56, 50, 48, 39, 34, 32, 26 and 20 kDa, were detected among the proteins eluted with high-salt or SDS buffers associated with ActA$^+$, but not ActA$^-$, bacteria. The positions of molecular mass markers are shown.

Since it was previously shown that increases in pH above 7.3 decrease the amount of filament-bound cofilin and increase the actin-depolymerizing activity of cofilin (Yonezawa et al., 1985; Hawkins et al., 1993), we tested the effect of increasing the pH in bovine brain extracts on cofilin association and actin tail length. Cofilin associated with ActA$^+$ bacteria in brain extracts buffered at pH 7.4-7.8 was barely detected by immunoblotting compared to extracts buffered at pH 6.6-7.2 (Fig. 3A). Note that pH 6.9 corresponds to the conditions used in initial experiments (Fig. 1). Furthermore, *Listeria* tails observed after a 1 hour incubation in brain extracts buffered at the higher pH values (pH 7.7-8.3) were shorter compared to tails in extracts buffered at the lower pH values (pH 6.50-7.25), consistent with an increase in cofilin actin-depolymerizing activity at pH values above 7.3 (Fig. 3B). There may be other interpretations of these observations, since changing the pH in extracts may affect other actin-binding protein properties. Of note are the changes in morphology of *Listeria* tails with respect to pH. Within a 15 minute incubation of bacteria in extracts, actin tail formation started in phase-contrast dense areas at any tested pH value (6.5-8.5) (not shown). Later, tails often appeared to originate together from actin-rich 'clumps' at pH values below 7.3, whereas individual tails were observed at a pH above 7.3 (Fig. 3B). These observations suggest that actin and/or other factors playing a critical role in ActA-induced actin assembly may be initially associated with membrane components, and released at the higher pH values.

Since cofilin is known to be an abundant actin-binding protein in brain, it was of interest to test whether similar results were found with extracts having lower levels of cofilin. Human platelet extracts (pH 7.25) were used to isolate proteins associated with actin tails. KCl-eluted and SDS-solubilized polypeptides from either ActA$^+$ or ActA$^-$ bacteria were separated by SDS-PAGE. Silver staining allowed visualization of several polypeptides (Fig. 4). By western blot analysis of KCl-eluted proteins, the 20 kDa and the 43 kDa species were identified as cofilin and actin, respectively (not shown).

Thus, the *Listeria* affinity approach allowed detection of several polypeptides associated with *Listeria* that are assembling actin. Our data show that cofilin is one of these proteins, and that cofilin recruitment is pH-dependent.

Isolation of known components of *Listeria* tails

To identify other proteins that associate with ActA$^+$ *Listeria* in brain extracts, western blot analysis of the KCl-eluted and

p19 peptide	**EILVGDVGQTVDDPYATF**
Porcine cofilin (54-71)	**EILVGDVGQTVDDPYATF**
Porcine destrin (54-71)	**EILVGDVG**VT**I**T**DP**FKH**F**

Fig. 2. Sequence of an internal peptide obtained from the 19 kDa polypeptide. The KCl-eluted 19 kDa polypeptide (p19) was identified by amino-acid sequencing of a peptide obtained by trypsin digestion of the corresponding excised gel slice (see Fig. 1). The sequence is aligned with homologous sequences of porcine brain cofilin (Matsuzaki et al., 1988) and porcine brain destrin, another member of the ADF/cofilin family.

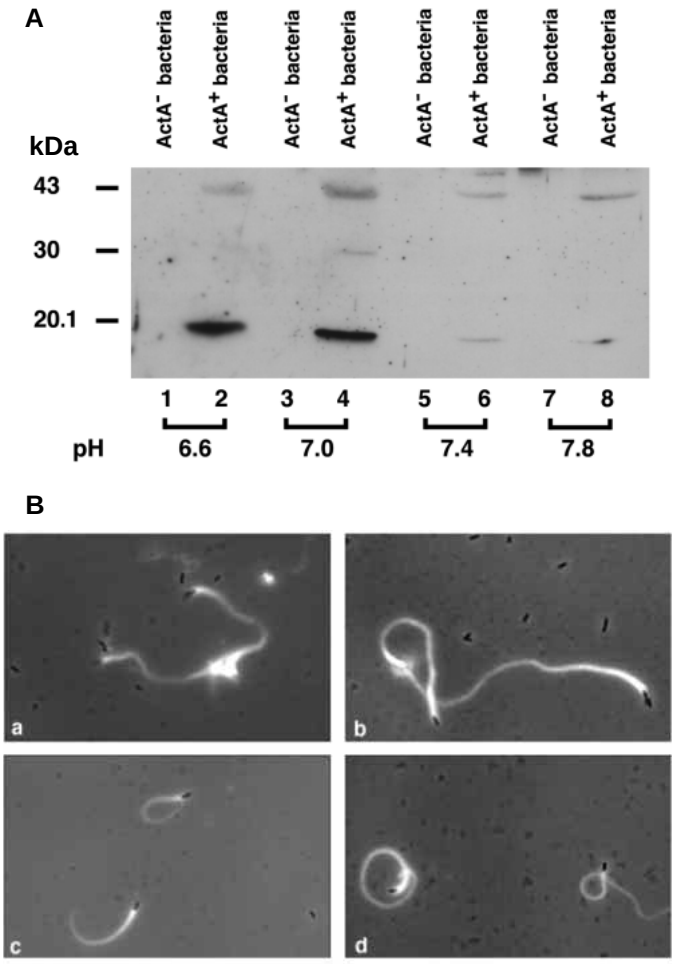


Fig. 3. Recruitment of cofilin and actin tail length at various pH values. (A) Before adding either ActA$^-$ or ActA$^+$ bacteria, the pH in brain extracts was adjusted to 6.6 (lanes 1 and 2), 7.0 (lanes 3 and 4), 7.4 (lanes 5 and 6) or 7.8 (lanes 7 and 8). After washing with low-salt buffer, bound proteins eluted with high-salt buffer were resolved on 12% SDS-polyacrylamide gels under reducing conditions and immunoblotted with polyclonal antibodies to cofilin. The antibodies to cofilin cross-reacted with an additional 43 kDa species. The positions of molecular mass markers are shown. For immunofluorescence analysis (reported in Fig. 6), these anti-peptide antibodies were further affinity-purified (western blot in Fig. 6A). (B) Before adding ActA$^+$ bacteria, the pH in brain extracts was adjusted to 6.50 (a), 7.25 (b), 7.70 (c) or 8.3 (d). After a 1 hour incubation in extracts supplemented with rhodamine-actin and ATP regenerating mix, the average length of actin tails was 30 μm at pH <7.3 (a,b) and 15 μm at pH >7.3 (c,d) (20 measurements per sample). Both types of experiments were performed independently several times at various pH values and the most representative results are presented.

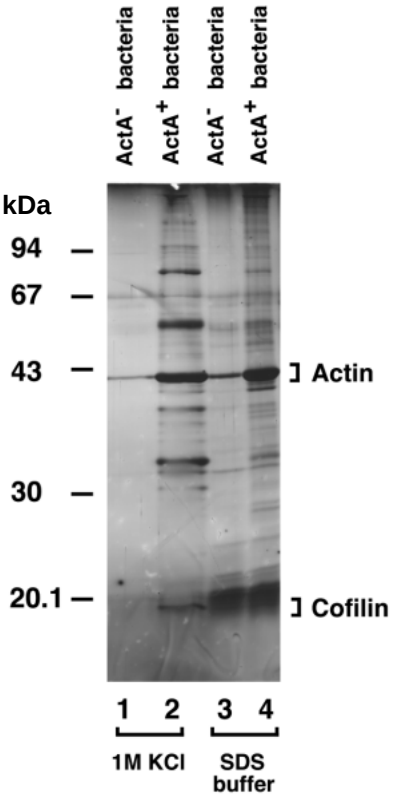


Fig. 4. Comparative SDS-PAGE analysis of proteins from human platelet extracts associated with ActA$^+$ and ActA$^-$ *Listeria.* ActA$^-$ or ActA$^+$ bacteria were incubated in platelet extracts for 1 hour. After washing with low-salt buffer, bound proteins were eluted with high-salt buffer and subsequently solubilized with SDS-sample buffer. KCl-eluted proteins (lanes 1 and 2), and SDS-solubilized proteins (lanes 3 and 4) were resolved on 12% SDS SDS-polyacrylamide gels under reducing conditions. Silver staining allowed detection of several proteins specifically associated with ActA$^+$ bacteria, with apparent mobilities of 80, 56, 50, 43, 41, 39, 34, 32, 29, 28 and 20 kDa. Actin and cofilin were identified by immunoblotting with a β actin mAb and with anti-cofilin polyclonal antibodies, respectively (not shown). The positions of molecular mass markers are shown.

SDS-solubilized proteins from ActA$^+$ or ActA$^-$ bacteria was performed using antibodies to several actin-binding proteins. The results of the western blots are shown in Fig. 5.

Using this method we first analyzed cell protein components known to be involved in *Listeria* actin tail assembly. As expected, actin, VASP, and Arp3 were detected in the KCl-eluted and SDS-solubilized proteins from ActA$^+$ but not ActA$^-$ bacteria (Fig. 5A-C). As shown in Fig. 5D, cofilin recruited by ActA$^+$ bacteria was present in the KCl eluate, and this protein was not detected in the subsequent SDS solubilization step. It is interesting to note that ActA was not extracted with KCl but was solubilized with SDS from the bacterial surface (Fig. 5E). The identification of the proteins known to be implicated in *Listeria*-stimulated actin polymerization suggested that our method is a useful tool for identifying novel protein components.

Identification of coronin, Rac and capZ as novel components of *Listeria* tails

Listeria-associated proteins from brain extracts were immunoblotted using antibodies to several candidate proteins. Coronin is an actin-binding protein involved in motility and phagocytosis in *Dictyostelium* (de Hostos et al., 1991), and was recently reported to be partly co-purified with the Arp2/3 complex from human neutrophils (Machesky et al., 1997). Probing with anti-coronin antibodies revealed that coronin was present as a 57 kDa species in the KCl-eluted and SDS-solubilized proteins from ActA$^+$ bacteria (Fig. 5F). Coronin has

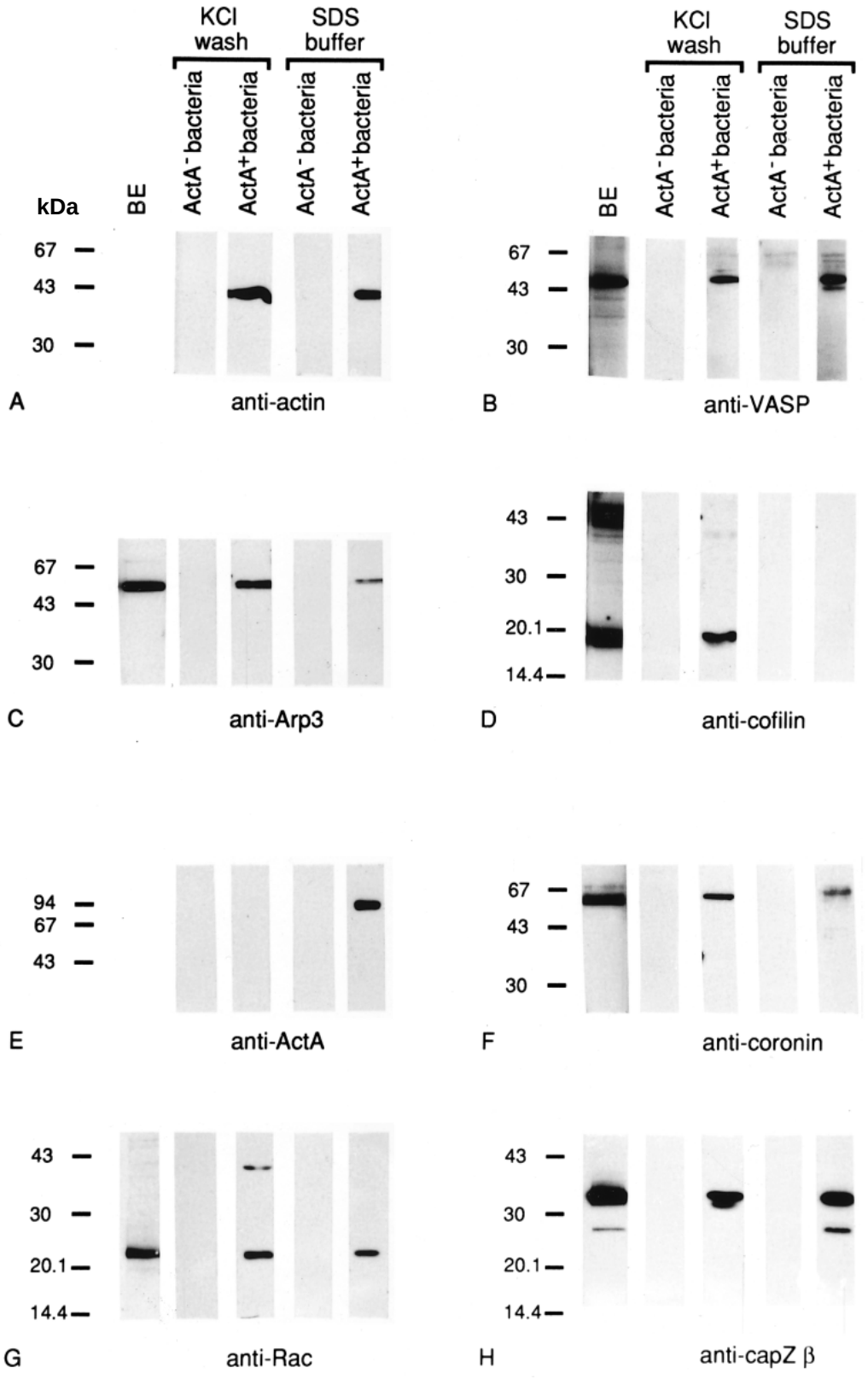


Fig. 5. Western blot analysis of proteins associated with ActA$^+$ bacteria. After 1 hour incubation of ActA$^-$ or ActA$^+$ bacteria in bovine brain extracts, bacteria were recovered as described in Fig. 1, and KCl-eluted proteins or SDS-solubilized proteins were separated by SDS-PAGE and immunoblotted with: (A) mAb specific for β-actin, (B) polyclonal anti-peptide antibodies to VASP, (C) affinity-purified polyclonal anti-peptide antibodies to Arp3, (D) polyclonal anti-peptide antibodies to cofilin, (E) affinity-purified antibodies to ActA, (F) polyclonal antibodies to coronin, (G) mAb specific for Rac1 and (H) mAb specific for capZβ. Reactivity of the antibodies in clarified brain extracts is shown in lanes marked BE (except in A and E). The positions of molecular mass markers are shown.

been reported to interact with components of the neutrophil NADPH oxidase complex like p21rac (Grogan et al., 1997), a small GTPase implicated in membrane ruffling in Swiss 3T3 cells (Ridley et al., 1992). Probing with a Rac1-specific mAb showed that Rac was associated with the ActA$^+$ bacteria (Fig. 5G). Finally, since our previous results suggested that ActA may be involved in maintaining actin filaments uncapped at the bacterial surface (Lasa et al., 1997), we tested whether the

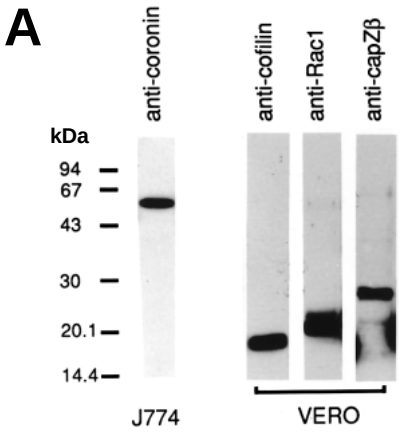
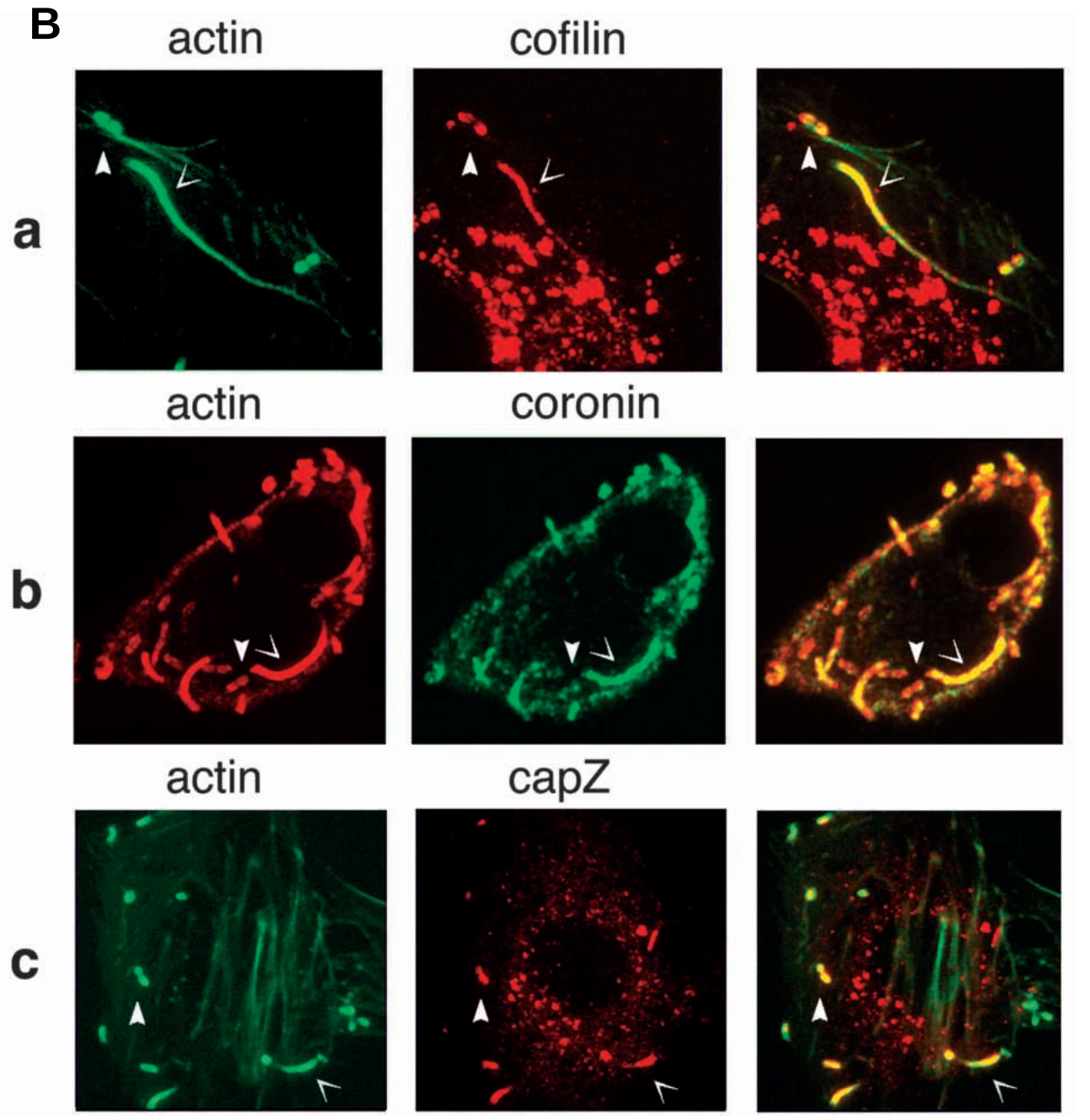


Fig. 6. Localization of cofilin, coronin and capZ in infected cells. (A) Immunoblots of Vero cell extracts with affinity-purified polyclonal rabbit antibodies to cofilin and capZβ, and mAb to Rac1, or J774 cell extracts with affinity-purified polyclonal antibodies to coronin. The positions of molecular mass markers are shown. (B) Confocal laser scanning images of Vero cells (a,c) or J774 cells (b). (a) Localization of cofilin visualized by immunofluorescence and of actin stained with FITC-phalloidin in the same cell; (b) localization of coronin and of actin visualized by immunofluorescence; (c) localization of capZ visualized by immunofluorescence and of actin stained with FITC-phalloidin. The right part of each panel was generated by superimposing the images of red and green fluorescence. An overlap of red and green appears yellow and indicates co-localization. Open arrowheads indicate actin tails, and solid arrowheads indicate bacteria surrounded by actin.



capping protein capZ was present in the proteins associated with *Listeria*. Probing with a mAb specific for the 32-kDa β subunit of capZ led to the identification of capZ in the KCl-eluted and SDS-solubilized proteins (Fig. 5H). CapZ is an heterodimer which binds to barbed ends of actin filaments (Barkalow et al., 1996). We verified that the 34 kDa α subunit was also present by immunoblotting (not shown). These data suggest that capZ was associated with ActA$^+$ bacteria as a dimer.

To demonstrate that the proteins specifically recruited by ActA$^+$ bacteria are present in ActA-induced tails, we performed immunofluorescence staining of infected cells. The antibodies to cofilin, coronin, capZβ and Rac1 used to localise these proteins in infected cells, reacted with a single protein species in cell lysates (Fig. 6A). Cofilin, coronin and capZ co-localized with actin at the surface of stationary bacteria and to *Listeria* tails (Fig. 6B). Rac staining was diffuse in the cytosol and did not allow any conclusion on Rac localization to *Listeria* tails (not shown). Cofilin also localized to cell nuclei (not shown).

DISCUSSION

Using *Listeria* as matrix and bovine brain extracts, we designed an affinity approach for identifying protein components of *Listeria* actin tails. Bound proteins were eluted from the bacteria with high-salt in order to detect mammalian proteins with minimal contamination by bacterial proteins. Remaining proteins associated with bacteria were subsequently solubilized with SDS. KCl-eluted and SDS-solubilized proteins were further analyzed by peptide sequencing or by western blotting using several antibodies. As expected, our approach allowed the isolation of three known components of *Listeria* tails: VASP, which binds to ActA (Chakraborty et al., 1995), Arp3, a protein of the Arp2/3 complex, which is involved in stimulation of actin polymerization at the bacterial surface (Welch et al., 1997a,b), and cofilin, which was recently shown to enhance filament turnover in *Listeria* tails (Carlier et al., 1997; Rosenblatt et al., 1997) (see below). These results indicate that the method was suitable for identifying novel proteins involved in *Listeria*-induced actin assembly.

We identified cofilin by peptide sequencing. Cofilin was eluted in the high-salt step and was not associated with ActA$^+$ bacteria after addition of cytochalasin D to the extracts (data not shown), suggesting that this protein is recruited by *Listeria* via actin filaments. Increasing the pH of extracts resulted in shorter *Listeria* tails and decreased recruitment of cofilin to *Listeria* tails. Both the decrease in length of actin tails and the decrease in associated cofilin could be due to an increase in the actin-depolymerizing activity of cofilin, which has been documented to be pH-dependent (Yonezawa at al., 1985; Hawkins et al., 1993). These results are consistent with cofilin playing a role in depolymerization of actin filaments in the tails and are in agreement with recent reports by two other groups showing, in the course of this study, that cofilin enhances actin filament turnover in *Listeria* tails in immunodepletions and add-back experiments in cell-free extracts (Carlier et al., 1997; Rosenblatt et al., 1997). By immunofluorescence we found that cofilin localized to the surface of stationary bacteria and to the actin tails, as reported in *Xenopus* tissue culture cells (Rosenblatt et al., 1997). Interestingly, cofilin also localized to the nuclei of the cells, suggesting that this protein may be present with actin, as already described in the case of the so-called actin/cofilin rods in nuclei of heat-shocked cells (Nishida et al., 1987; Ohta et al., 1989).

In addition to actin and cofilin, we identified three novel proteins which were not previously known to be present in *Listeria* tails: the actin-binding protein coronin, the GTPase Rac, and the barbed end-capping protein capZ. The localization of coronin and capZ to *Listeria* tails was confirmed by immunofluorescence analysis of infected cells. The proteins identified using bovine brain extracts, i.e. cofilin, coronin, Rac and capZ, were also found using human platelet extracts (not shown).

Coronin is an actin-binding protein involved in phagocytosis, motility and cytokinesis in *Dictyostelium* (de Hostos et al., 1991). Rac is important for activity of the NADPH oxidase (Abo et al., 1992), membrane ruffling and lamellipodial extension in fibroblasts (Ridley et al., 1992), and regulation of uncapping of actin filaments in permeabilized platelets (Hartwig et al., 1995). Both coronin and Rac were identified as proteins associated with *Listeria*. Whether coronin binds to actin filaments or is recruited by the Arp2/3 complex to the tails, is not known. Whether Rac recruitment depends on the presence of coronin is not also known. Alternatively, it is tempting to speculate that Rac might be recruited by VASP, a proline-rich protein sharing conserved domains with two GTPase-binding proteins, p140mDia, which binds to Rho (Watanabe et al., 1997), and the Wiskott-Aldrich syndrome protein (WASP), which binds to Cdc42 (Symons et al., 1996). Although co-localization of Rac to actin assembled by *Listeria* could not be shown by immunofluorescence of infected cells, identification of Rac by immunoblotting of *Listeria*-associated proteins in bovine brain extracts strongly suggests that Rac is recruited to *Listeria* tails. We are in the process of examining the role of Rac in signaling leading to bacterial-induced actin assembly. The role of filament capping in the actin-based motility is still unclear. Recently, the capping protein gelsolin was shown to localise to *Listeria* tails in *Xenopus* tissue culture cells but depletions in gelsolin had no effect on bacterial movement in egg extracts containing EGTA (Rosenblatt et al., 1997). Our data demonstrate that capZ is present in the *Listeria* tails, suggesting that this protein might be critical for filament capping. Interestingly, capZ has a calcium-insensitive actin filament barbed-end capping activity, whereas activity of the barbed-end capping and severing protein gelsolin is calcium-dependent. Whether gelsolin is associated with *Listeria* in our assay is currently unknown. All reported studies support the view that actin assembly is stimulated at filament barbed ends at the bacterial surface, whereas the actin tail depolymerizes at pointed ends (Lasa et al., 1998). Whether ActA protects the filament barbed ends from capping by capZ is under investigation.

In conclusion the data indicate that coronin, Rac and capZ are recruited by *Listeria*-induced actin tails, suggesting that these proteins may participate in the dynamics of the bacterial induced actin polymerization. Neither vinculin nor profilin was detected in the proteins associated with *Listeria* (not shown). Note that *Listeria* tails were found to be somewhat dismantled after washes and centrifugations and that some of their components might have been lost. We are in the process of identifying additional polypeptides isolated in our approach

and studying the function of these proteins in bacterial movement.

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