The bacterial actin nucleator protein ActA of *Listeria monocytogenes* contains multiple binding sites for host microfilament proteins

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Background: Several intracellular pathogens, including *Listeria monocytogenes*, use components of the host actin-based cytoskeleton for intracellular movement and for cell-to-cell spread. These bacterial systems provide relatively simple model systems with which to study actin-based motility. Genetic analysis of *L. monocytogenes* led to the identification of the 90 kD surface-bound ActA polypeptide as the sole bacterial factor required for the initiation of recruitment of host actin filaments. Numerous host actin-binding proteins have been localized within the actin-based cytoskeleton that surrounds *Listeria* once it is inside a mammalian cell, including α-actinin, fimbrin, filamin, villin, ezrin/radixin, profilin and the vasodilator-stimulated phosphoprotein, VASP. Only VASP is known to bind directly to ActA. We sought to determine which regions of the ActA molecule interact with VASP and other components of the host microfilament system.

Results: We used the previously developed mitochondrial targeting assay to determine regions of the ActA protein that are involved in the recruitment of the host actin-based cytoskeleton. By examining amino-terminally truncated ActA derivatives for their ability to recruit cytoskeletal proteins, an essential element for actin filament nucleation was identified between amino acids 128 and 151 of ActA. An ActA derivative from which the central proline-rich repeats were deleted retained its ability to recruit filamentous actin, albeit poorly, but was unable to bind VASP.

Conclusions: Our studies reveal the initial interactions that take place between invading *Listeria* and host microfilament proteins. The listerial ActA polypeptide contains at least two essential sites that are required for efficient microfilament assembly: an amino-terminal 23 amino-acid region for actin filament nucleation, and VASP-binding proline-rich repeats. Hence, ActA represents a prototype actin filament nucleator. We suggest that host cell analogues of ActA exist and are important components of structures involved in cell motility.

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Background

A common property of pathogenic bacteria that multiply directly in the cytoplasm of infected cells is their ability to move intracellularly and to spread from cell to cell using components of the host actin-based cytoskeleton. These bacteria, from such diverse species as *Listeria* [1,2], *Rickettsia* [3] and *Shigella* [4], recruit proteins of the host microfilament system for their intracellular movement. The non-spore-forming Gram-positive intracellular bacterium *Listeria monocytogenes* is the best characterized of these intracytoplasmic pathogenic bacteria. Once inside the host cell cytoplasm, actin 'clouds' accumulate around the bacterial cells. These are subsequently reorganized into a 'comet tail', located at one pole of the bacterial surface (reviewed in [5,6]). Movement is generated by continuous polymerization of actin at the surface of one listerial pole, thus propelling the bacterium forwards [7,8].

Characterization of *Listeria* mutants that are unable to accumulate actin filaments led to the identification of the 90 kD surface-bound ActA polypeptide [9,10]. ActA has several prominent features: a highly charged amino-terminal domain, a central region containing four copies of a repeated motif rich in proline residues, and a hydrophobic carboxy-terminal end that anchors the protein to the bacterial surface (Fig. 1a). Immunofluorescence studies with polyclonal and monoclonal anti-ActA antibodies demonstrated that ActA is uniformly present on the bacterial surface [11,12], but is not released into the actin comet tails [11,13]. The direct expression of the ActA polypeptide from a eukaryotic vector demonstrated that it is the sole listerial factor required for the initiation of recruitment of host actin filaments around intracellular bacteria [14].

When expressed in eukaryotic cells, ActA is spontaneously targeted to mitochondria, where it induces the assembly of mini-cytoskeletons around these subcellular organelles. This ability to associate with mitochondria has been located to the 26 carboxy-terminal amino acids of the ActA protein, which resemble motifs characteristic of mitochondrial targeting signals (see Fig. 1a) [14]. As the actin-based cytoskeleton thus accumulates at a novel site in the cell, this approach provided us with a simple model with which to probe different regions of the ActA

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molecule for their interactions with host microfilament proteins. Mitochondrially targeted expression of ActA largely simulates the initial phase of actin recruitment, namely the formation of actin clouds, which normally occurs around intracytoplasmic *Listeria*.

Examination of the protein composition of the actin comet tails created by intracellularly motile *Listeria* has revealed numerous host actin-binding proteins. These include α-actinin [15], filamin [16], fimbrin [17], villin and ezrin/radixin [18]. These proteins are associated with the most distal actin filaments in the tails, suggesting that none of them interacts directly with the bacterial surface. An exception is profilin [19], which appears to be concentrated around the rear half of intracellularly motile *Listeria*, where it is most probably required for polarized polymerization of host-cell actin. However, purified profilin does not bind to *Listeria* grown outside the host cell, despite the presence of surface-bound ActA on such *Listeria* [19], indicating that an additional host factor is required to mediate the ActA–profilin interaction.

Recently, it has been shown that the vasodilator-stimulated phosphoprotein, VASP, is involved in the actin-based motility of intracellular pathogens such as *L. monocytogenes*, *L. ivanovii* and *Shigella flexneri* [12]. This protein is associated with microfilaments and focal adhesion plaques, is a substrate of cAMP- and cGMP-dependent

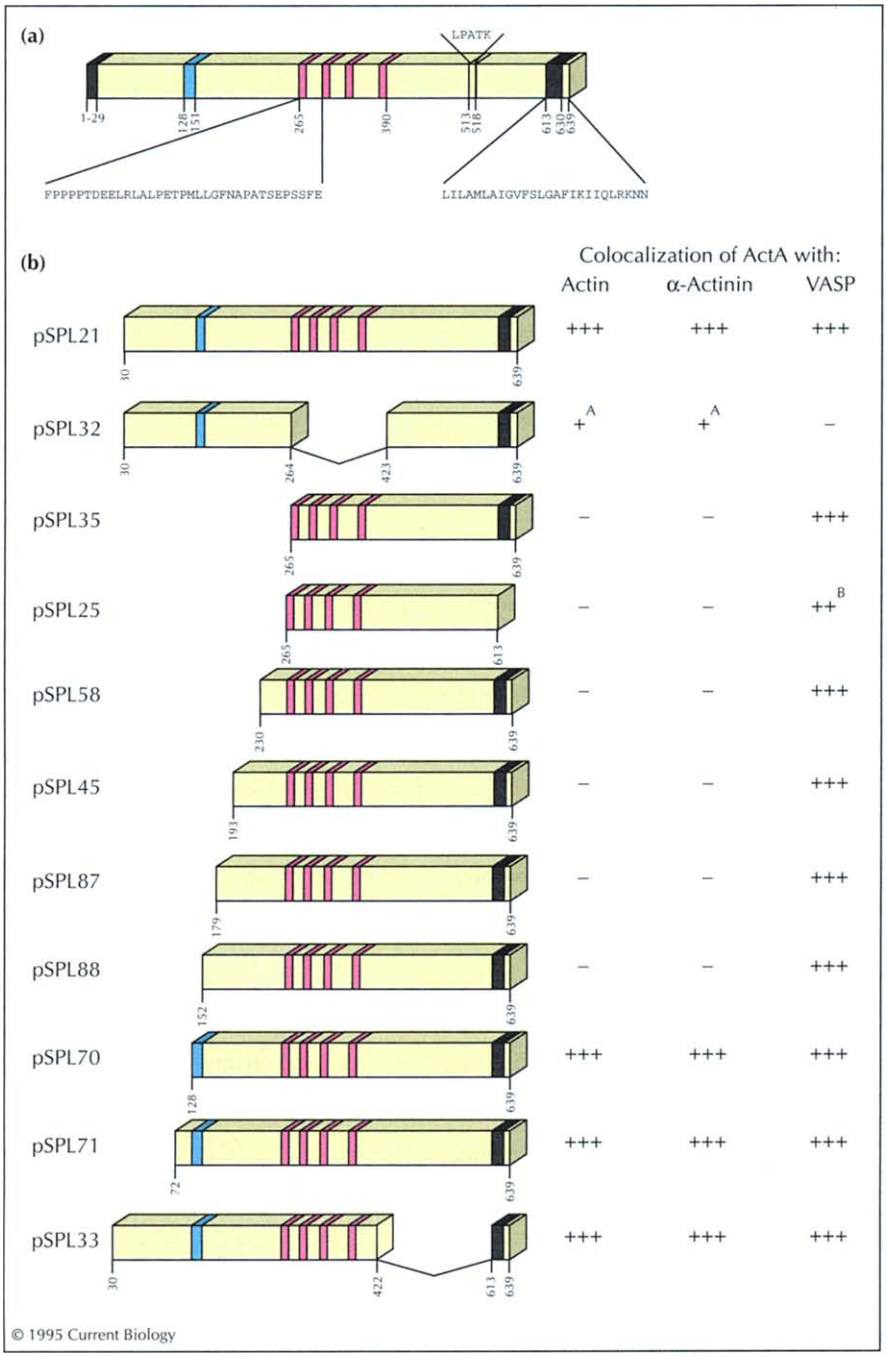

Fig. 1. **(a)** Schematic representation of the ActA polypeptide. The positions of the various functional motifs [9] are indicated. The dark grey boxes denote the amino-terminal bacterial signal peptide (amino acids 1–29) and the hydrophobic carboxy-terminal bacterial membrane anchor (amino acids 613–639). The site identified in this study as being involved in F-actin recruitment (amino acids 128–151) is shown in blue, and the proline-rich repeat region (amino acids 265–390) in pink; the LPATK motif (amino acids 513–518) typical of cell-wall proteins of Gram-positive bacteria is also indicated. The proline-rich core sequence $FPP^{I}/_{P}PT^{D}/_{E}{}^{E}/_{D}EL$, shown in pink, is repeated four times within the molecule. The membrane anchor has motifs reminiscent of mitochondrial targeting signals and directs ActA to mitochondria when expressed in eukaryotic cells [14]. **(b)** Schematic representation of the various derivatives of ActA used in this study. All derivatives were targeted to mitochondria, with the exception of that expressed by pSPL25, which was diffusely distributed in the cytoplasm. [A] Only detectable in cells expressing high levels of truncated ActA; [B] detectable as increased diffuse cytoplasmic labelling depleted from focal contacts.

protein kinases [20,21], and has been detected around intracytoplasmic *Listeria* prior to the accumulation of clouds of filamentous (F-) actin. VASP remains associated with the surface of highly motile *Listeria*, where it is located between one extremity of the bacterial surface and the front of the actin comet tail [12]. As the polymerization of actin filaments occurs only at the very front of the tail [7,8], VASP has the properties required of a host protein that promotes actin polymerization. Purified VASP binds directly to the ActA polypeptide *in vitro* [12], suggesting that it serves as a connector protein between ActA and profilin, thereby contributing to F-actin nucleation and/or polymerization.

In this study, we have investigated the regions of the ActA molecule that are necessary and sufficient to support the association of this protein with components of the host microfilament system. We have also examined the association of actin and other cytoskeletal proteins with mitochondrially bound ActA by double immunofluorescence microscopy, using antibodies specific to ActA and other cytoskeleton proteins.

Results

Mitochondrially targeted ActA recruits host cytoskeletal proteins to these subcellular organelles

Recently, various microfilament-associated proteins have been identified in the cytoskeletal structures recruited by *Listeria* [1,12,15–18]. The ability of full-length ActA targeted to mitochondria of transiently transfected PtK_2 (potoroo kidney epithelial) cells to recruit cytoskeletal proteins was assessed by indirect immunofluoresence microscopy. In addition to actin and α-actinin [14], ezrin/radixin and VASP were recruited to mitochondrially targeted ActA (Fig. 2a–d), indicating that the 'mini-cytoskeletons' formed on the mitochondrial surface closely resemble the actin cytoskeleton which surrounds intracellularly motile *Listeria*.

F-actin and VASP interact with distinct domains of ActA

In a previous study using fluorescent phalloidin to assay for F-actin accumulation, analysis of an ActA derivative lacking the proline-rich repeats (pSPL32; Fig. 1b) gave ambiguous results [14]. Re-examination of PtK_2 cells transfected with this ActA derivative, using a monoclonal anti-actin antibody, indicated that the accumulation of actin on mitochondria did occur (Fig. 3b), as in cells transfected with full-length ActA (Fig. 2b). However, the level of actin was less than that recruited by full-length ActA (Fig. 2a,b), even though a very high level of the ActA derivative was detectable on mitochondria (Fig. 3a). This suggested that F-actin accumulation takes places at a region of the ActA molecule that is distant to the proline-rich repeat region. Examination of this ActA derivative for recruitment of other microfilament-associated proteins showed that α-actinin and ezrin/radixin were recruited to the mitochondrial surface (Fig. 3c,f), whereas VASP was not detectable around mitochondria (Fig. 3d,e). Therefore, ActA seems to have at least two distinct sites for interacting with cytoskeletal proteins: the proline-rich region for binding to VASP and a separate region for interaction with other microfilament proteins.

To delineate these regions of ActA further, we analyzed a derivative lacking the 264 residues amino-terminal to the proline-rich region (pSPL35) for its ability to recruit F-actin and VASP to the mitochondrial surface. This derivative was unable to accumulate F-actin, even though mitochondria stained intensively with anti-VASP antibodies (Fig. 4a,e). Thus, the domain required for F-actin accumulation is located within this amino-terminal region of ActA. As this derivative was as efficient as full-length ActA at recruiting VASP, it can be deduced that

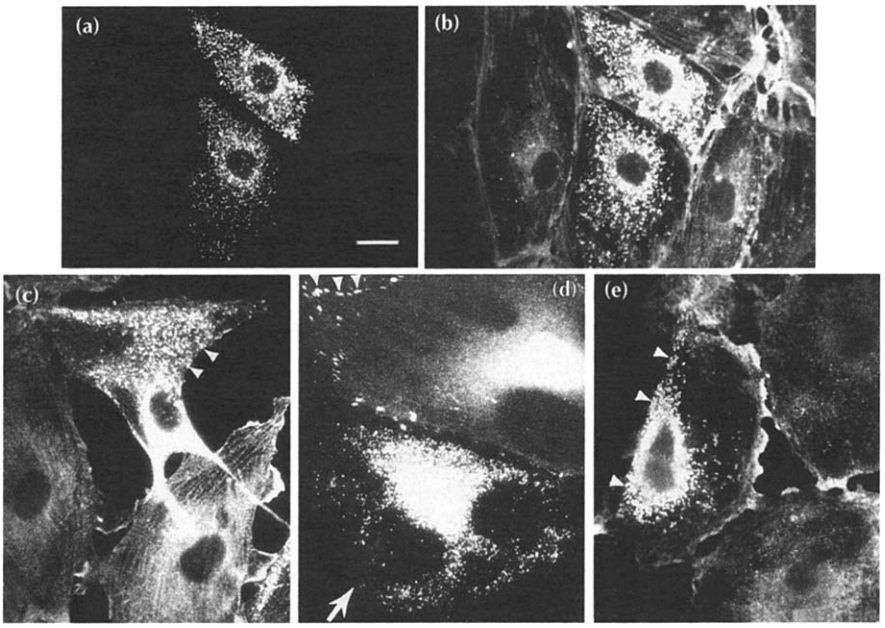

Fig. 2. Colocalization of microfilament-associated proteins with mitochondrially targeted ActA in PtK2 cells transiently transfected with the full length ActA (pSPL21; see Fig. 1b). **(a,b)** Transfected cells were processed for double immunofluorescence microscopy using (a) polyclonal anti-ActA, and (b) monoclonal anti-actin antibodies: ActA is targeted to mitochondria (a) and recruits actin (b) to the mitochondrial surface in the transfected cells. **(c–e)** Transfected cells were analyzed with the following primary antibodies: (c) monoclonal anti-α-actinin antibodies, (d) polyclonal anti-VASP antibodies, (e) monoclonal anti-ezrin/radixin antibodies. All the microfilament-associated proteins tested are recruited to mitochondria by the full-length ActA polypeptide. The arrow in (d) indicates the transfected cell; the arrowheads indicate the focal contacts of the adjacent non-transfected cell. Arrowheads in (c) and (e): mitochondria decorated with α-actinin (c) or ezrin/radixin (e) in transfected cells. Bar = 20 μm.

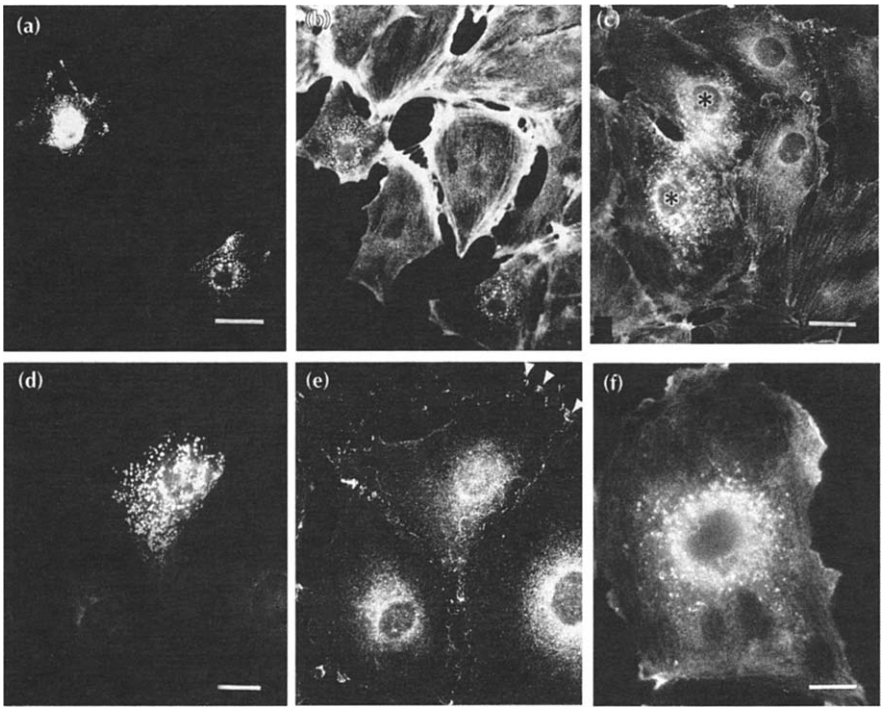

Fig. 3. Immunofluorescent localization of microfilament-associated proteins in PtK_2 cells transiently transfected with the ActA derivative lacking the proline-rich repeats (pSPL32; see Fig. 1b). Transfected cells were processed as follows: for double immunofluorescence microscopy in **(a,b)** and **(d,e)**, polyclonal anti-ActA antibodies (a) or a mixture of N20 and N81 monoclonal anti-ActA antibodies (d) were used together with monoclonal actin antibodies (b) or polyclonal anti-VASP antibodies (e). The same cells are shown in (a) and (b), (d) and (e). **(c)** Monoclonal anti-α-actinin antibodies. **(f)** Monoclonal anti-ezrin/radixin antibodies. Note that the pSPL32 ActA derivative, which lacks the proline-rich repeats, accumulates (b) F-actin, (c) α-actinin and (f) ezrin/radixin around mitochondria, but not (e) VASP (compare with the ActA labelling in d). F-actin recruitment was significantly weaker than that mediated by full-length ActA (see Fig. 2b). Both micrographs (Figs 2b and 3b) were taken with the same exposure times. Asterisks in (c) indicate transfected cells, arrowheads in (e) indicate unaltered VASP localization in focal contacts of the transfected cell. Bars represent (a,b) 30 μm; (c) 25 μm; (d,e) 15 μm; and (f) 10 μm.

VASP binds to the proline-rich repeat domain of ActA and that VASP-binding is not sufficient to induce F-actin recruitment.

In order to localize the potential site(s) involved in F-actin accumulation, we constructed further ActA derivatives with successively truncated amino-terminal regions (see Fig. 1b: pSPL71, 70, 88, 87, 45, 58). All derivatives that lacked more than the 152 amino-terminal residues showed no actin labelling, despite intense ActA and VASP staining of targeted mitochondria (see Figs 1b and 4b,f for pSPL88). However, PtK_2 cells expressing an ActA derivative starting with amino-acid 128 (pSPL70) did show actin accumulation, indicating that an essential element for actin filament recruitment is located between amino acids 128 and 151 (see Fig. 4c,g for pSPL70). The carboxy-terminal region of ActA following the proline-rich repeat region (from amino acid 422) was required for neither efficient F-actin recruitment nor VASP-binding. This was demonstrated by an ActA derivative with an internal deletion of 191 amino acids (pSPL33), from the end of the repeat region up to the membrane anchor (amino acid 613). Expression of this derivative produced identical patterns of accumulation for both actin (Fig. 4h) and VASP (data not shown) as was seen with the full-length ActA polypeptide.

Transfected constructs express stable ActA polypeptides

The results described above could be ascribed to altered stabilities of the differently truncated ActA proteins in transfected cells. To ensure that the expressed ActA polypeptides were of the predicted size, we analyzed total lysates of transiently transfected HeLa cells by immunoblotting, using specific affinity-purified polyclonal anti-ActA antibodies. Although HeLa cells do not lend themselves easily to immunofluorescence studies, unlike PtK_2 cells, they can be transfected at higher rates, and produce detectable amounts of truncated ActA derivatives for immunoblotting analyses. Immunoblots of ActA constructs, transiently expressed in HeLa cells, are shown in Figure 5. The constructs used in this study produced ActA derivatives of the predicted molecular weights, with little degradation. The exception is the full length ActA derivative (pSPL21, Fig. 5 lane 2), which is associated with a protein of lower molecular weight, presumably a proteolytic protein fragment.

Redistribution of VASP by ActA and a synthetic ActA proline-rich peptide analogue

VASP is normally associated with microfilaments, highly dynamic membrane regions and focal adhesions (see below; Fig. 7a) [20]. Recruitment of VASP to the ActA associated with mitochondria was so efficient that it led to the depletion of VASP from the focal contacts of the transfected cells (Fig. 6). This dramatic effect was not restricted to mitochondrially bound ActA (Fig. 6b,d) — a derivative that was deleted for both the amino-terminal actin-recruiting region and the mitochondrial-targeting sequence, and as a consequence was diffusely distributed in the cytoplasm, also efficiently depleted VASP from focal contacts (pSPL25; Fig. 6a,b).

In order to investigate the binding sites for VASP within ActA, we capitalized on the observation described above. We hypothesized that the microinjection of a peptide representative of a proline-rich repeat into tissue culture cell lines would result in a redistribution of VASP, particularly from lamellopodia and focal adhesion sites. Following microinjection of cultured human fibroblasts with

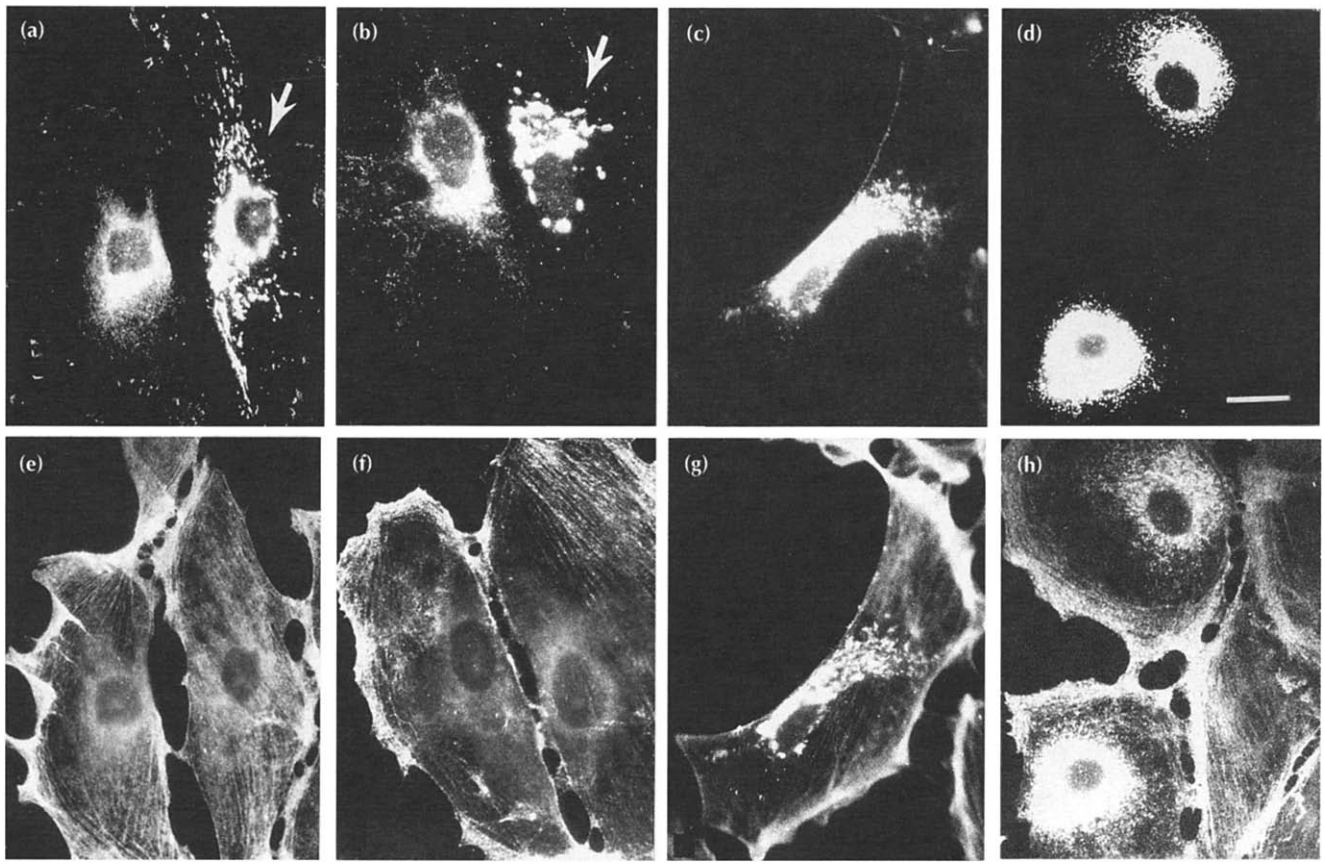

Fig. 4. Distribution of actin in PtK_2 cells transiently transfected with different truncated ActA derivatives. Cells were analyzed by double immunofluorescence microscopy using **(a–c)** polyclonal anti-VASP or **(d)** polyclonal anti-ActA antibodies together with monoclonal anti-actin antibodies **(e–h)**; the same cells are shown in (a) and (e), (b) and (f), (c) and (g), (d) and (h). Cells were transfected with the following plasmids: (a,e) pSPL35, (b,f) pSPL88, (c,g) pSPL70, (d,h) pSPL33. The ActA derivatives starting with amino acids 265 (pSPL35) or 152 (pSPL88) recruit VASP (a,b) to mitochondria (arrows), but do not recruit F-actin (e,f), whereas the ActA construct starting with amino acid 128 (pSPL70) is able to recruit F-actin (g). The ActA derivative with an internal deletion at the carboxyl terminus (amino acids 423–612, pSPL33) behaved just as the full-length ActA in recruiting actin (h). Bar = 20 μm.

the synthetic peptide (C)264-DFPPPPTDEELRL-276 (single-letter amino-acid code), derived from the indicated residues of the primary ActA sequence, cells were processed for indirect immunofluorescence microscopy using anti-actin and anti-VASP antibodies (Fig. 7). This synthetic ActA-repeat analogue caused complete depletion of VASP from lamellopodia and focal adhesion sites, resulting in a diffuse VASP distribution (Fig. 7b,c). In addition, lamellopodial structures in the periphery of injected cells were no longer detectable by actin labelling (Fig. 7d). That these effects were specific was demonstrated by the microinjection of an unrelated ActA-derived synthetic peptide, (C)41–DEWEEEKTEEQPSE–54, which failed to reproduce the effects observed above (data not shown).

Discussion

We have used an assay that targets the *Listeria* ActA polypeptide to mitochondria in transiently transfected PtK_2 cells, to examine regions of this protein required for recruitment of an actin-based mini-cytoskeleton. The microfilament-based structures formed around mitochondria are reminiscent of the actin clouds recruited by *Listeria* in the host cytoplasm, following their escape from the phago-lysosomal compartment. We have identified a region, located between amino acids 128–151 of the primary ActA sequence, as an element essential for the accumulation of actin filaments. In addition, the host focal adhesion and microfilament-associated protein VASP was found to interact with the proline-rich repeats of ActA. Deletion of the region required for VASP interaction reduces, but does not abolish, F-actin accumulation. On the other hand, truncated ActA derivatives, unable to recruit F-actin, still bind to VASP. The carboxy-terminal region of ActA which follows the proline-rich repeats is not required for interaction with host microfilament-associated proteins. Within the bacterial cell, it may form a spacer region that exposes the amino-terminal residues, including the proline-rich repeats, above the murein sheath around the bacterium. Microinjection of a synthetic peptide comprising one of the proline-rich repeats depleted lamellopodia and focal adhesion plaques of VASP, suggesting that eukaryotic proteins carrying similar or identical motifs serve to restrict this microfilament-associated protein to these specialized regions of the cell.

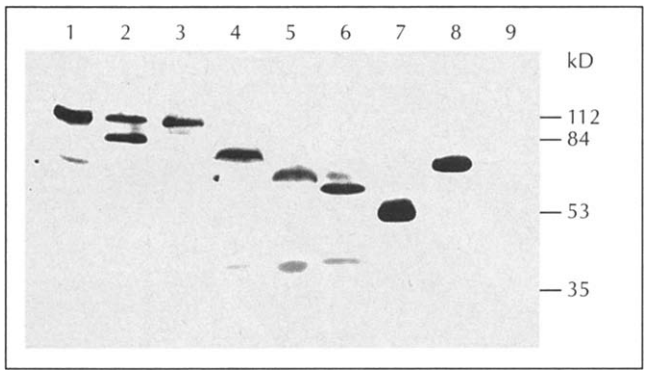

Fig. 5. Immunoblot analysis of HeLa-tTA cells transiently expressing various ActA derivatives. HeLa-tTA cells were transfected with the different *actA* constructs. Total lysates of transfected cell batches were separated by SDS–PAGE and immunoblotted with affinity-purified polyclonal anti-ActA antibodies. The calculated weights of the individual ActA derivatives are given in parenthesis. Lane 1, SDS-extract of *L. monocytogenes* EGD grown in broth culture (70.2 kD); lanes 2–8, lysates of Hela-tTA cells transfected with the following *actA* constructs: lane 2, pSPL21 (70.2 kD); lane 3, pSPL71 (65 kD); lane 4, pSPL70 (59 kD); lane 5, pSPL88 (56 kD); lane 6, pSPL45 (51 kD); lane 7, pSPL35 (43 kD); lane 8, pSPL32 (51.8 kD). Lane 9: lysate of non-transfected HeLa-tTA cells. The lower bands in lanes 1 and 2 probably represent proteolytic ActA fragments. All ActA polypeptides migrate aberrantly in SDS gels (see also [9]). Molecular weight markers are shown on the right.

Examination of the region required for F-actin accumulation revealed a highly positively charged sequence motif, 146–KKRRK–150, that could be involved in F-actin nucleation. A similar sequence, KKEK, found in the carboxyl terminus of villin, has been reported to be essential for villin function *in vivo*. This sequence not only binds actin, but also appears to promote polymerization of monomeric actin [22]. It has previously been noted that the ActA sequence 92–LKEKAEK–99, which shares homology with the presumed actin-binding site of caldesmon, LKEKQ [23], could serve as an actin-nucleating sequence in ActA [13]. Our results do not support an essential role for this sequence in F-actin nucleation, as ActA derivatives lacking this sequence (such as that encoded by pSPL70) are still able to induce F-actin accumulation. The specific amino acids involved in this interaction could be identified by creating 'in frame' deletions or point mutations within the region identified in this study, and assaying the resulting mutant ActA derivatives for their ability to recruit F-actin.

Deletion of the four proline-rich motifs of ActA abolished its interaction with the host microfilament-associated protein VASP. Furthermore, the presence of this region on untargeted, cytoplasmically located ActA derivatives, such as pSLP25, depleted focal adhesions of VASP. Binding of VASP to the repeat region of ActA was observed in the absence of the amino-terminal region that is required for F-actin recruitment. Therefore, VASP binds to ActA independently of F-actin accumulation, and is by itself not capable of recruiting F-actin.

Microinjection studies showed that a peptide comprising a single proline-rich repeat was highly effective in depleting VASP from focal adhesions, suggesting that VASP binds directly to the sequence DFPPPPTDEELRL. The actual binding sequence may be slightly smaller, as the sequence that is conserved amongst the various repeats corresponds to a core sequence, $^{D}/_{E}FPP^{P}/_{I}PT^{D}/_{E}{}^{E}/_{D}EL$. Hence, there seem to be at least four potential binding sites for VASP on a single ActA molecule. It has recently been reported that the microinjection of *Listeria*-infected

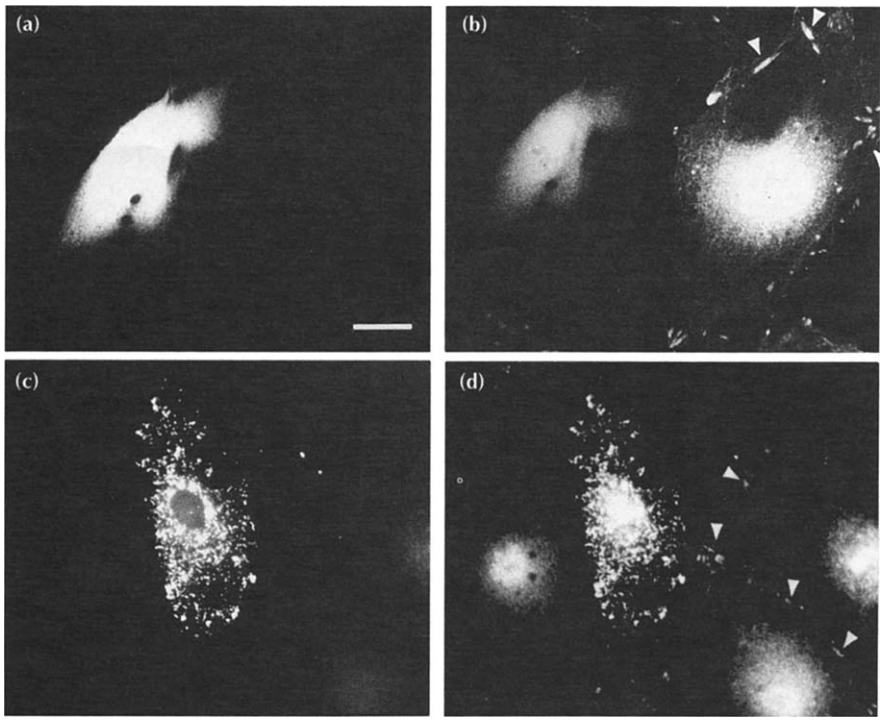

Fig. 6. Depletion of VASP from focal contacts in cells expressing ActA. PtK_2 cells were transiently transfected with **(a,b)** pSPL25 or **(c,d)** pSPL21, and processed for double immunofluorescence microscopy using a mixture of N4 and S119 monoclonal anti-ActA antibodies (a,c) and polyclonal anti-VASP antibodies (b,d). Both ActA derivatives deplete VASP from focal contacts in the transfected cells. Association of VASP with focal contacts in a non-transfected cell is indicated by arrowheads in (b) and (d). Bar = 20 μm.

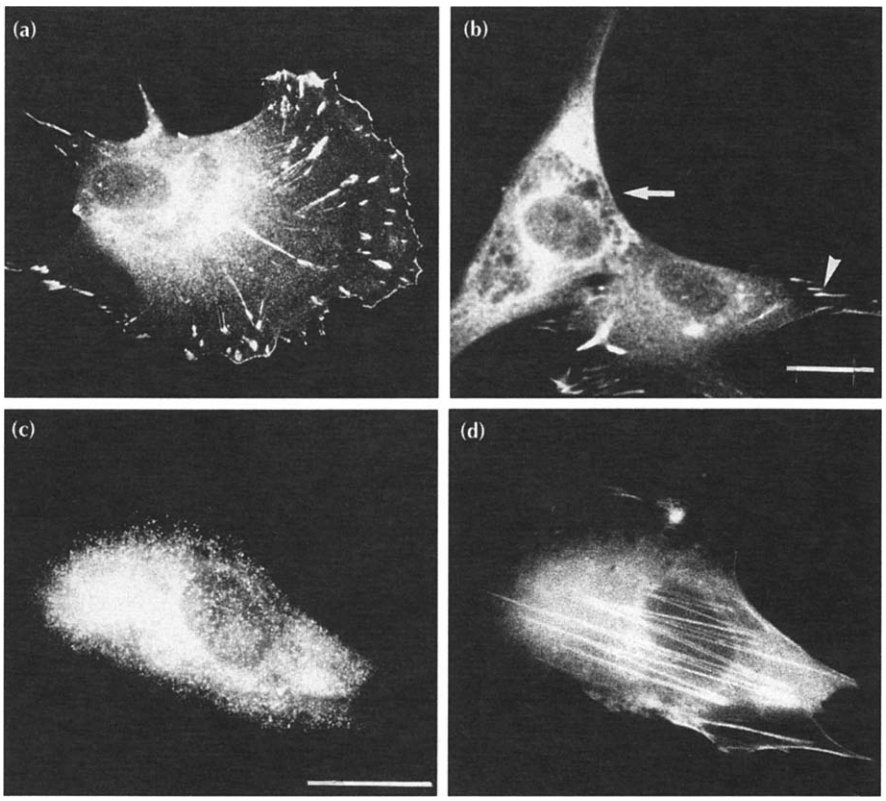

Fig. 7. Redistribution of VASP in fibroblast cells microinjected with a synthetic peptide analogue of ActA comprising a single proline-rich repeat. **(a)** Distribution of VASP in an uninjected human skin fibroblast. VASP is concentrated in focal adhesion plaques and in the periphery of the leading lamellapodium. **(b)** Diffuse distribution of VASP in a cell that had previously been injected with the ActA-derived peptide (arrow); in the adjacent uninjected cell, VASP is associated with focal contacts (arrowhead). **(c,d)** Double fluorescence images of a microinjected human fibroblast labelled with polyclonal anti-VASP antibodies (c) and fluorescent phalloidin (d). In addition to the diffuse distribution of VASP (c) lamellopodial structures are not detectable by actin labelling (d). The bar in (b), valid for both (a) and (b), and the bar in (c), represent 20 μm.

PtK_2 cells with the peptide CFEFPPPPTDE induced arrest of bacterial movement and reduction of dynamic activities in the cortical regions of the microinjected cells [24], a phenotype that might be predicted if the peptide interacts directly with VASP. Therefore, VASP is not only involved in bacterial movement but appears also to participate in initiating and maintaining dynamic changes in the actin-based cytoskeleton of eukaryotic cells. These results, in turn, suggest that host cell proteins harbouring such proline-rich motifs exist and that they directly interact with VASP.

We have shown previously that purified VASP directly binds to ActA [12]. As VASP is also a ligand for profilin [25], and as profilin itself does not bind ActA [19], we suggest that VASP recruits monomeric actin in the form of profilactin to the bacterial surface. Support for this hypothesis stems from immunolocalization studies of VASP [12] and profilin [19] in *Listeria*-infected cells. Both polypeptides colocalize on motile *Listeria* at one bacterial pole, between the bacterial surface and the front of the actin comet tail, where continuous actin polymerization occurs. Moreover, in contrast to the other microfilament-associated proteins such as α-actinin, fimbrin, filamin, villin and ezrin/radixin, VASP and profilin remain associated with the bacterial surface of motile *Listeria* — they are not detectable within actin comet tails. Thus, VASP-binding could increase the local concentration of monomeric actin in close proximity to the site of F-actin nucleation on the same ActA molecule, by recruiting profilactin and thereby promoting efficient actin filament formation. In the absence of VASP, F-actin accumulation would be much less effective, as was seen with the ActA derivative lacking proline-rich repeats. In addition, binding of VASP to ActA could lead to conformational changes in ActA that favour F-actin nucleation.

Although we have delineated the regions on ActA that are required for the interaction with cytoskeletal proteins, important questions remain to be addressed. For instance, it is not known whether actin associates directly with ActA in the absence of other host proteins; the precise binding site for VASP on ActA remains to be determined. Similarly, as the primary sequence of VASP has recently been determined [26], the ActA-binding region on VASP can now be examined in detail. Finally, listerial strains harbouring mutated ActA derivatives, with deletions or mutations in the regions delineated here, can be assessed for their ability to induce actin-tail formation and intracellular motility.

Conclusions

We have demonstrated that the ActA polypeptide of *L. monocytogenes* harbours at least two functional domains involved in actin filament recruitment: the proline-rich repeats that interact directly with VASP, and an amino-terminal region that is highly positively charged and that is involved in F-actin nucleation. Cooperative interactions between these two domains are required for efficient actin filament recruitment. Our results provide missing details and extend current models of actin accumulation by ActA [6,19]. Furthermore, as VASP also binds profilin [25], our results implicate VASP as an important component of dynamic actin-based structures in cell

motility, and suggest that host cell analogues of ActA are important components of structures involved in motility.

Materials and methods

Reagents

Restriction endonucleases, *Taq* polymerase, sequencing reagents and ligase were purchased from Gibco BRL (Eggenstein, Germany), Biolabs (Schwalbach, Germany), Perkin–Elmer (Ueberlingen, Germany), Applied Biosystems (Weiterstadt, Germany) and Boehringer Mannheim (Germany), and were used following the manufacturers' instructions. All other chemicals, including fluorescein-labelled phalloidin, were purchased from Sigma (Deisenhofen, Germany), unless otherwise indicated.

Cloning and expression of actA derivatives

The structure of the ActA polypeptide is schematically depicted in Fig. 1a. The *act*A gene of *L. monocytogenes* strain EGD [9] was inserted into the eukaryotic expression vector pMPSVHE under the control of the constitutively expressing promoter of the Myeloma proliferative sarcoma virus (MPSV) [27], as described recently [14]. Sequences 5′ to the *act*A gene were modified to allow optimal expression in eukaryotic cells [28], and were cloned into the pMPSVHE vector using unique *Hind*III and *Bam*HI restriction sites introduced into regions flanking the gene by specific PCR primers.

Transient expression of *act*A derivatives in PtK_2 and HeLa cells was performed as described earlier [14]. The different ActA derivatives are shown in Fig. 1b. *Act*A sequences were amplified from genomic DNA of *L. monocytogenes* EGD by PCR, using specific oligonucleotide primers (below). Restriction sites at the 5′ end (*Hind*III) and 3′ end (*Bam*HI) are indicated in bold; nucleotides deviating from the published *act*A sequence are indicated in italic; the pSPL21 and pSPL32 constructs were described earlier [14]. To construct pSPL35, the following primer pair (5′-end/3′-end) was used:

5′–TAA***A***AGCTTCGGACATGCCGCCACCAC–3′;

5′–TTTGAATTTC**GGATCC**TTCACCTCACTTT–3′.

For pSPL25, the same 5′ primer was used as for pSPL35, and for the 3′ end, 5′–CATTGCAAG*TT*ATA**GGATCC**TATGGTTCCC–3′ was used. For the other amino-terminal truncated constructs, the 3′ end was derived by the same oligonucleotide as used for pSPL35; primer sequences for the different 5'-ends were:

5′–AAA***A***AGCTTACGTGATAAAATGGACGAAAATC	pSPL58;
5′–GTGACT***A***AGCTTCTAGCATGCAGTC	pSPL45;
5′–TAAGAAAA**AAGCTT**CGAAAATGTCAGTTG	pSPL87;
5′–TTAAAAA**AAGCTT**GAAAGCCATGGCATCATC	pSPL88;
5′–GAGCC***A***AGCTTCCAGCTATGCAAGTGGAGC	pSPL70;
5′–TGAAGT**AAGCTT***A*CGTGATATGAAAGAACTAG	pSPL71.

pSPL33 consisted of a fragment encoding the amino-terminal part (the *Hind*III/*Sau*3A fragment from pSPL21) fused to the proposed membrane anchor sequence; it was synthesized by PCR using the primers: 5′–AGGG**GATC**ATACGACGTTAACTCTTGCA and 5′–TTTGAATTTCATA**GAATTC**ACCTCACT, with *Bam*HI and *Eco*RI cloning sites. All inserts and fusion areas of the constructs were verified by sequencing using *Taq* Dye Deoxy™Terminator cycle sequencing (Applied Biosystems, Weiterstadt, Germany) and analysed on an Applied Biosystems 373 A automated DNA Sequencer.

Antibodies

The affinity-purified polyclonal rabbit antibodies and the N20 and N81 monoclonal anti-ActA antibodies have been described previously [11], as have the polyclonal rabbit anti-VASP antibodies [20]. The N4 and S119 monoclonal anti-ActA antibodies were provided by K. Niebuhr (unpublished). The monoclonal antibodies against chicken brain ezrin/radixin 13H9 [29] were a generous gift of F. Solomon and B. Winkler. Immunostaining of actin with a specific monoclonal antibody was found to be more sensitive than fluorescent phalloidin. The monoclonal anti-actin antibody (C4) was purchased from Boehringer, Mannheim (Germany), and the monoclonal anti-α-actinin antibody (A 5044) was from Sigma.

Immunofluorescence microscopy of transfected cells

For transfection, PtK_2 cells (ATCC CCL56) were grown as monolayers on coverslips in 24-well dishes in MEM (Gibco), supplemented with glutamine, nonessential amino acids and 10 % fetal calf serum (FCS). HeLa–tTA cells [30] were grown in DMEM (Gibco) supplemented with glutamine and 10 % FCS; subconfluent cultures were transfected as described previously [14]. After 48 h from the start of transfection, cells were washed once with PBS and processed for immunofluorescence microscopy. For the localization of α-actinin, cells were fixed and permeabilized with cold (–20 °C) methanol; otherwise cells were fixed in 3.7 % formaldehyde in PBS and permeabilized with 0.2 % (v/v) Triton X–100 in PBS. After incubation with primary antibodies, coverslips were further processed for double immunofluorescence microscopy using fluorescein- or rhodamine-labelled goat anti-rabbit antibodies and fluorescein- or rhodamine-labelled goat anti-mouse antibodies (Dianova, Hamburg, Germany). Samples were examined with a Zeiss Axiophot microscope equipped with epifluorescence. Photographs were taken with Kodak T-MAX 400 film.

Immunoblot analysis of transfected cells

ActA derivatives were subcloned as *Hind*III/*Bam*HI fragments into vector pSBCtTA (a derivative of pUHC13 kindly provided by H. Hauser, GBF, Braunschweig) and transiently transfected into HeLa-tTA cells [30] grown in DMEM (Gibco) supplemented with 10 % FCS and glutamine in 10 cm dishes. 24 h from the start of transfection, cells were washed twice with PBS and harvested with a rubber policeman by adding 50 µl eight-fold concentrated boiling SDS sample buffer to a final volume of 400 µl. After heating at 95 °C for 5 min, probes were aliquotted, frozen in liquid nitrogen and stored at –80 °C. Samples were separated by 10 % SDS–PAGE and transferred onto PVDF membranes (Millipore) as described [11]. Filters

were incubated with affinity-purified polyclonal anti-ActA antibodies and secondary peroxidase-conjugated goat anti-rabbit antibodies (Dianova). Immunoblots were developed with the ECL system (Amersham). Cell-wall extracts of *L. monocytogenes* EGD were isolated and processed for SDS–PAGE as described [11].

Microinjection experiments

Peptides corresponding to amino acids 41–54 and 264–276 of the ActA sequence were synthesized with an amino-terminal cysteine. As a result of the reduced solubility of the proline-rich peptide at high concentrations, both peptides were cross-linked to ovalbumin using sulpho-MBS (Pierce) as described previously [9], following the procedure recommended by the manufacturer. After overnight reaction, the MBS–ovalbumin peptide mixtures were extensively dialyzed against PBS and concentrated (Centricon 30; Amicon). Human skin fibroblasts [20] were grown in DMEM supplemented with 10 % FCS and glutamine on CELLocate coverslips (Eppendorf, Hamburg, Germany), and microinjected with the ovalbumin–peptide conjugates as described previously [31], using a concentration of 10 mg ml^{-1}. Cells were fixed and processed for immunofluorescence microscopy 3 h after the injection.

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