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The ActA protein of *Listeria monocytogenes* acts as a nucleator inducing reorganization of the actin cytoskeleton

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***Listeria monocytogenes*, a facultative intracellular pathogen, employs actin and other microfilament-associated proteins to move through the host cell cytoplasm. Isogenic mutants of *L.monocytogenes* lacking the surface-bound ActA polypeptide no longer interact with cytoskeletal elements and are, as a consequence, non-motile (Domann *et al.*, 1992, *EMBO J.*, 11, 1981–1990; Kocks *et al.*, 1992, *Cell*, 68, 521–531). To investigate the interaction of ActA with the microfilament system in the absence of other bacterial factors, the listerial *act*A gene was expressed in eukaryotic cells. Immunofluorescence studies revealed that the complete ActA, including its C-terminally located bacterial membrane anchor, colocalized with mitochondria in transfected cells. When targeted to mitochondria, the ActA polypeptide recruited actin and α-actinin to these cellular organelles with concomitant reorganization of the microfilament system. Removal of the internal proline-rich repeat region of ActA completely abrogated interaction with cytoskeletal components. Our results identify the ActA polypeptide as a nucleator of the actin cytoskeleton and provide the first insights into the molecular nature of such controlling elements in microfilament organization.**

Key words: actin nucleating protein/cytoskeleton/intracellular movement/SH3 domains/virulence factor

Introduction

Listeria monocytogenes is a facultative intracellular bacterium that is capable of intracytoplasmic growth in infected host cells following escape from a phagolysosomal compartment. A key virulence mechanism of *L.monocytogenes* is its ability to harness the host cell cytoskeletal elements for intra- and intercellular movement (see Tilney and Tilney, 1993). The cytoplasmically located bacteria induce the accumulation of short actin filaments on their surface which are subsequently released and interconnected to each other by other cytoskeletal proteins such as α-actinin (Tilney and Portnoy, 1989; Dabiri *et al.*, 1990; Mounier *et al.*, 1990). These physically cross-linked actin filaments lead to the formation of tail-like structures at one bacterial pole and it is this actin polymerization that provides the locomotive force in intracellular bacterial motility (Sanger *et al.*, 1992; Theriot *et al.*, 1992). Bacteria in the vicinity of the host cell plasma membrane are able to induce pseudopod-like structures or protrusions which can be internalized by neighbouring cells, allowing effective spread of bacteria from one cell to another (Tilney and Portnoy, 1989).

The analysis of mutants that cannot accumulate actin in infected cells allowed the identification of the *act*A gene locus. Its gene product encodes a bacterial surface protein with an apparent molecular mass of 90 kDa, which is involved in the interaction with cytoskeletal elements of the host cell (Domann *et al.*, 1992; Kocks *et al.*, 1992).

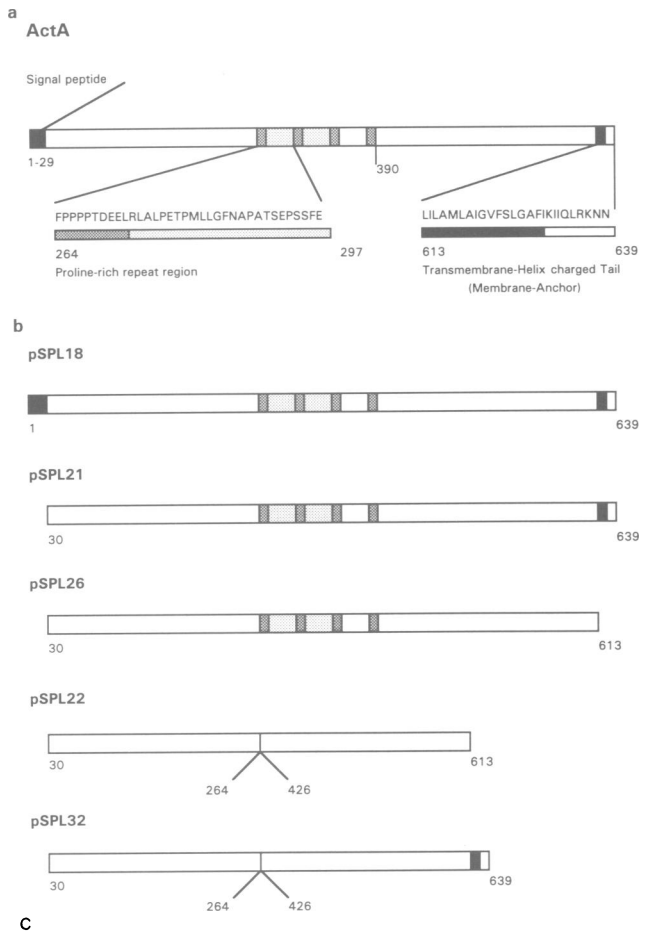
The highly elaborate mechanism of microfilament-based bacterial movement has made *Listeria* an attractive model system for the analysis of microfilament function in living cells. Even though specific antibodies against ActA revealed that this polypeptide is associated with the cell wall and is exposed on the bacterial surface (Kocks *et al.*, 1993; Niebuhr *et al.*, 1993), it is not clear whether the ActA protein alone can induce the accumulation of actin filaments or whether other listerial factors are participating in this process.

We have expressed the *act*A gene in eukaryotic cells to examine its role in inducing actin-cytoskeleton reorganization and furthermore to analyse the interaction of ActA with the microfilament system in the absence of other listerial factors. The ActA polypeptide was directed to the mitochondria where it recruited actin microfilaments to these cellular organelles. This unexpected finding enabled us to initiate the functional analysis of this peculiar bacterial virulence factor.

Results

Molecular cloning of the actA gene in a eukaryotic expression vector

The structure of the ActA polypeptide is schematically depicted in Figure 1a. Although the ActA polypeptide has been implicated in microfilament reorganization, direct evidence for its interaction with actin is lacking (Domann *et al.*, 1992; Kocks *et al.*, 1992). To test whether the ActA polypeptide is itself capable of interacting with host cell cytoskeletal components in the absence of other listerial factors, we expressed the ActA polypeptide in eukaryotic cells susceptible to *Listeria* infection. The *act*A gene of *L.monocytogenes* was inserted into the eukaryotic expression vector pMPSVHE under the control of the constitutively expressing promoter of the myeloma proliferative sarcoma virus (MPSV) (Artelt *et al.*, 1988) and transfected into the PtK_2 potoroo kidney epithelial cell line. Sequences 5′ to the *act*A gene were modified to allow optimal expression in eukaryotic cells (Kozak, 1991) and cloned into the pMPSVHE vector using unique *Hin*dIII and *Bam*HI restriction sites, introduced into regions flanking the gene by specific PCR primers (see Materials and methods). Recombinant plasmids used in this study were identical with respect to sequences flanking the *act*A gene; this was verified for each plasmid by DNA sequencing. Expression of the



c

Construct	Subcellular localization of ActA	Colocalization of ActA with	
		Actin	α-Actinin
pSPL18	mitochondria	+	+
pSPL21	mitochondria	+	+
pSPL26	cytoplasm/ nucleus	+[a]	+[a]
		−	−
pSPL22	nucleus	−	−
pSPL32	mitochondria	−	−

[a]Detectable as increased diffuse cytoplasmic labeling (data not shown).

Fig. 1. Expression and differential location of ActA polypeptides in transfected PtK_2 cells. (**a**) Schematic representation of the ActA polypeptide indicating the positions and sizes of the various structural motifs. The proline-rich internal repeats consist of two completely identical repeats, as depicted above, and the motif $FPP^{I}/_{P}PT^{D}/_{E}^{E}/_{D}EL$ that occurs four times. (**b**) Schematic representation of ActA polypeptides encoded by the different plasmids used for transfection. (**c**) Subcellular locations and properties expressed by different ActA polypeptide derivatives.

ActA polypeptides and their ability to recruit actin was assessed using specific antibodies against ActA (Niebuhr *et al.*, 1993) and fluorescently-labelled phalloidin, respectively, in transiently transfected cells.

The ActA polypeptide is targeted to the mitochondria in eukaryotic cells

Immunofluorescence localization following transfection revealed that the ActA polypeptide (pSPL18 and pSPL21; Figures 1b, 2a, 3a and c, and 4a) was presented on vesicle- and tubule-like structures within the cytoplasm of transfected cells. The appearance of tubular or vesicular structures correlated with the level of expression of ActA, with vesicular structures dominating at high expression levels as judged by staining intensity (compare Figure 3a and c). The tubule-like structures are reminiscent of mitochondrial distribution in eukaryotic cells and double immunofluorescence microscopy with antibodies against the β-subunit of the mitochondrial F_1 ATP synthetase (Jenkins *et al.*, 1990) confirmed this to be the case (Figure 2a and b). Hence, the full-length ActA polypeptide is targeted to mitochondria when expressed in mammalian cells.

To identify the mitochondrial targeting signal of ActA, PtK_2 cells were transfected with *act*A gene derivatives encoding ActA proteins lacking either the N-terminal bacterial signal peptide (pSPL21; Figure 1b) or the signal peptide plus the C-terminally located bacterial membrane anchor (pSPL26; Figure 1b). Deletion of the bacterial signal peptide (pSPL21; Figure 1b) had no effect on mitochondrial localization (Figures 2a, 3a and c, and 4a), while the truncated ActA polypeptide lacking its C-terminal membrane anchor (pSPL26; Figure 1b) was diffusely distributed throughout the cytoplasm and nucleus (Figure 3e). Thus, the C-terminal membrane anchor of ActA effectively directs this polypeptide to mitochondria.

Actin accumulation on the surface of ActA-coated mitochondria requires the presence of the internal proline-rich repeats of ActA

We next examined the distribution of actin in PtK_2 cells transfected with various derivatives of the *act*A gene. In those cells expressing high levels of the full-length ActA on mitochondria, phalloidin-stained actin filaments colocalized with the ActA polypeptide and accumulated on the surface of these organelles (Figure 3b). This was particularly evident with the mitochondria that were vesicular rather than tubular in structure (compare Figure 3a and b with c and d). Actin filament accumulation on the mitochondrial surface appeared to lead to the formation of disperse vesicular structures reminiscent of the effects of microtubule depolymerizing drugs on mitochondria (Johnson *et al.*, 1980). The localization of tubulin always revealed an intact microtubular network in transfected cells (data not shown). Deletion of the signal peptide did not abrogate actin accumulation (pSPL21; Figure 3b). However, in cells where the truncated ActA polypeptide lacked the membrane anchor sequence and was diffusely distributed throughout the cytoplasm and nucleus, low but discernible redistribution of actin was detected as increased diffuse cytoplasmic labelling (data not shown). The fluorescence images of actin accumulation around mitochondria in transfected cells resemble those seen around intracellular *Listeria* (Mounier *et al.*, 1990; Sanger *et al.*, 1992), suggesting the presence of short actin filaments on the mitochondrial surface.

The ability of the ActA polypeptide when localized to mitochondria to induce actin accumulation around these organelles allowed us to examine regions within the ActA polypeptide that are required for this interaction. Because of the similarity of the internal proline-rich repeat in ActA to those in the actin-associated protein vinculin (Weller *et al.*, 1990), this region of the ActA polypeptide has recently been suggested to be involved in the process of bacterial actin recruitment (Domann *et al.*, 1992). When PtK_2 cells transfected with an *act*A derivative encoding a polypeptide

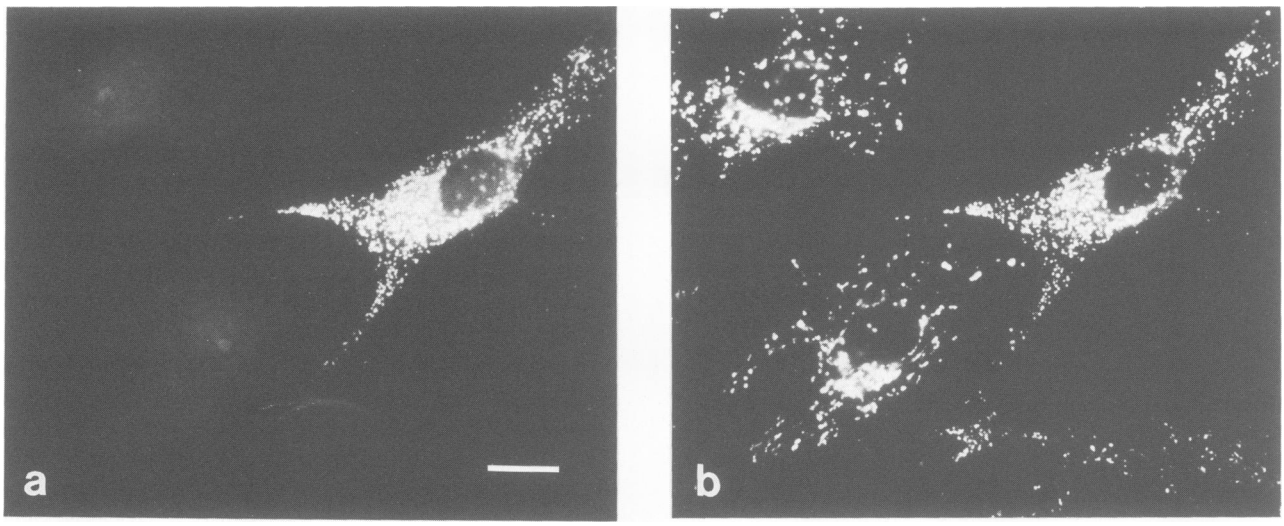


Fig. 2. ActA is targeted to mitochondria when expressed in eukaryotic cells. PtK_2 cells were transiently transfected with the pSPL21 plasmid and processed for double immunofluorescence microscopy using the N20 ActA mAb (**a**) and polyclonal antibodies against the ATP synthetase (**b**), followed by fluorescein-labelled goat anti-mouse and rhodamine-labelled goat anti-rabbit antibodies. Non-transfected cells reveal a tubular pattern typical for mitochondria (b), whereas disperse vesicular structures that are also labelled by ActA antibodies dominate in the transfected cell (compare a with b). Bar represents 15 μm.

lacking the internal proline repeat sequences (pSPL32; Figure 1b) were examined by immunofluorescence microscopy, no actin was detected around mitochondria, even though these were intensively labelled by ActA antibodies (Figure 3g and h). Surprisingly, an ActA derivative, lacking both the proline repeats and its membrane anchor (pSPL22; Figure 1b), accumulated predominantly in the nucleus (Figure 3i and j).

Colocalization of α-actinin with actin on the surface of mitochondria in cells expressing the ActA polypeptide

Since it has been shown previously that the actin filaments surrounding invading *Listeria* are stabilized and cross-linked by other microfilament-associated proteins (Dabiri *et al.*, 1990; Sanger *et al.*, 1992), we examined whether this was also the case for the structures described here. Examination of the distribution of α-actinin in PtK_2 cells transfected with the pSPL21 *actA* construct indicated that, apart from its normal cytoskeletal distribution, α-actinin colocalizes with ActA on mitochondria (Figure 4a and b). Hence, we conclude that recruitment of cytoskeletal elements by ActA, when targeted to mitochondria, resembles that naturally displayed on the surface of cytoplasmically located *Listeria*.

Discussion

By expressing the ActA polypeptide of *L.monocytogenes* in eukaryotic cells, we have demonstrated its capacity to act as a nucleator seeding the polymerization of actin filaments around the subcellular structure on which it is expressed, be it a mitochondrion or the intracytoplasmically located invading bacterium. Remarkably, the accumulating actin filaments have properties much like those observed around intracellular *Listeria,* as though the same eukaryotic control mechanisms operate in both situations to limit unrestricted filament formation. Expression of the ActA polypeptide alone mimics the initial phase of actin accumulation seen with infecting *Listeria* (Tilney and Portnoy, 1989). Formation of the polarized actin 'tail' appears to be dictated by temporal events governing bacterial growth and division, with the likely participation of further bacterial and host cell factors (Theriot *et al.*, 1992; Tilney *et al.*, 1992; Niebuhr *et al.*, 1993).

A striking observation in transfected cells was the appearance of mitochondria having a vesicular morphology which correlated with the level of expression of ActA. Since mitochondria exhibit a microtubule-dependent movement within the cytoplasm (for references see Schliwa, 1984), a physical interaction between mitochondria and microtubules most probably occurs via microtubule-associated motor proteins (see Walker and Sheetz, 1993). Upon depolymerization of microtubules, mitochondria not only cease their cytoplasmic movement but also lose their extended filamentous form in many cell types (Johnson *et al.*, 1980). Hence, the accumulation of a dense cross-linked microfilamentous network on ActA-coated mitochondria could easily abolish the physical interaction with microtubules, leading to their vesicular shape in transfected cells.

The region involved in inducing actin accumulation was delineated to the internal proline-rich repeats of the ActA polypeptide. Proline-rich sequences have recently been shown to be recognition elements for SH3 domain-containing proteins (Pawson and Schlessinger, 1993, and references therein). This domain appears to be involved, via protein–protein interactions, in targeting proteins to their site of action, as well as in the regulation of protein movement within the cell. The ActA polypeptide could overcome host cell-controlled actin nucleation by interacting with SH3 domain-containing cytoskeletal proteins to generate new sites of nucleation and actin filament formation. Perhaps the ActA polypeptide has a higher affinity for such a SH3 domain-containing protein than its endogenous counterpart, which would explain the potency of *Listeria* in promoting actin accumulation on its surface in infected cells. Co-immunoprecipitation with ActA may in future lead to the identification of such a putative protein and open up the possibility of mutational analysis to pinpoint amino acids required for this interaction.

Our results also uncovered the presence of two functional

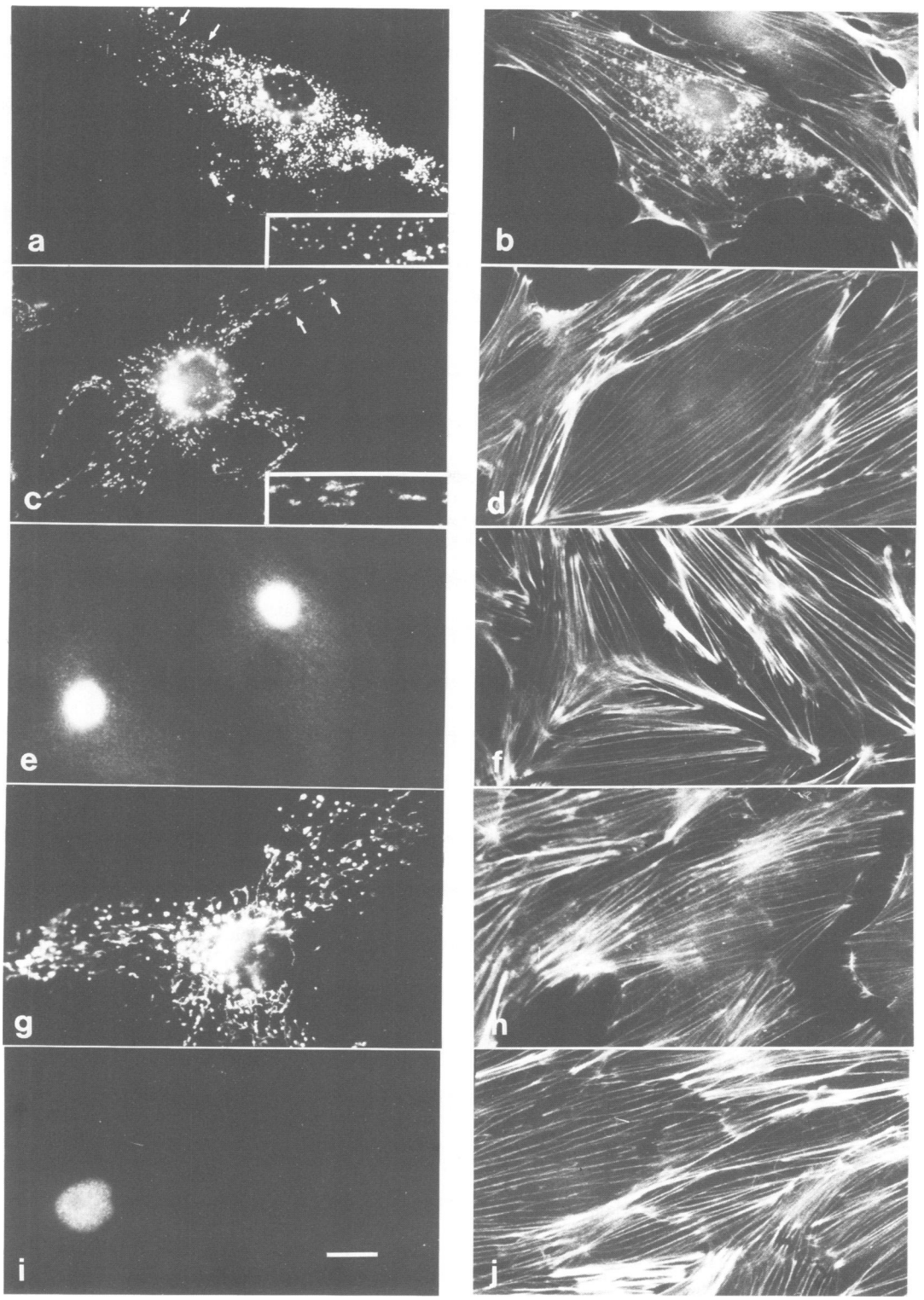


Fig. 3. Immunofluorescence microscopy of PtK_2 cells transiently transfected with various derivatives of the *act*A gene of *L.monocytogenes*. Cells were transfected with the following plasmids: pSPL21 (**a**–**d**), pSPL26 (**e** and **f**), pSPL32 (**g** and **h**) and pSPL22 (**i** and **j**). 48 h after starting the transfection, cells were processed for double fluorescence microscopy using the affinity-purified polyclonal ActA antibodies (a, c, e, g and i) and fluorescein-labelled phalloidin (b, d, f, h and j), followed by rhodamine-labelled goat anti-rabbit antibodies. Strong reactivity of ActA antibodies with tubular and vesicular structures is only observed in transfected PtK_2 cells expressing ActA polypeptides containing the bacterial membrane anchor [pSPL18 (data not shown), pSPL21 in a and c, and pSPL32 in g], whereas the deletion of the bacterial signal peptide has no influence (pSPL21 in a and c). Arrowheads in a and c indicate those cellular regions that are enlarged in the respective inserts to visualize the vesicular and tubular structures respectively. In the absence of the membrane anchor (pSPL26 in e and f, pSPL22 in i and j), ActA polypeptides are diffusely distributed throughout the cytoplasm and the nucleus (e and i). Actin colocalizes with ActA on mitochondria in transfected cells expressing high levels of the respective ActA derivative (a and b). Note that deletion of the internal proline-rich repeat domain abolishes actin recruitment to mitochondria (g and h). The occasional weak mitochondrial staining in the fluorescein channel (h) is due to some bleed through from the rhodamine channel (g) because of the high expression level and the intensive labelling of the truncated ActA derivative in this cell. Bar represents 15 μm (a–j) and 5 μm in the enlarged inserts in a and c, respectively.

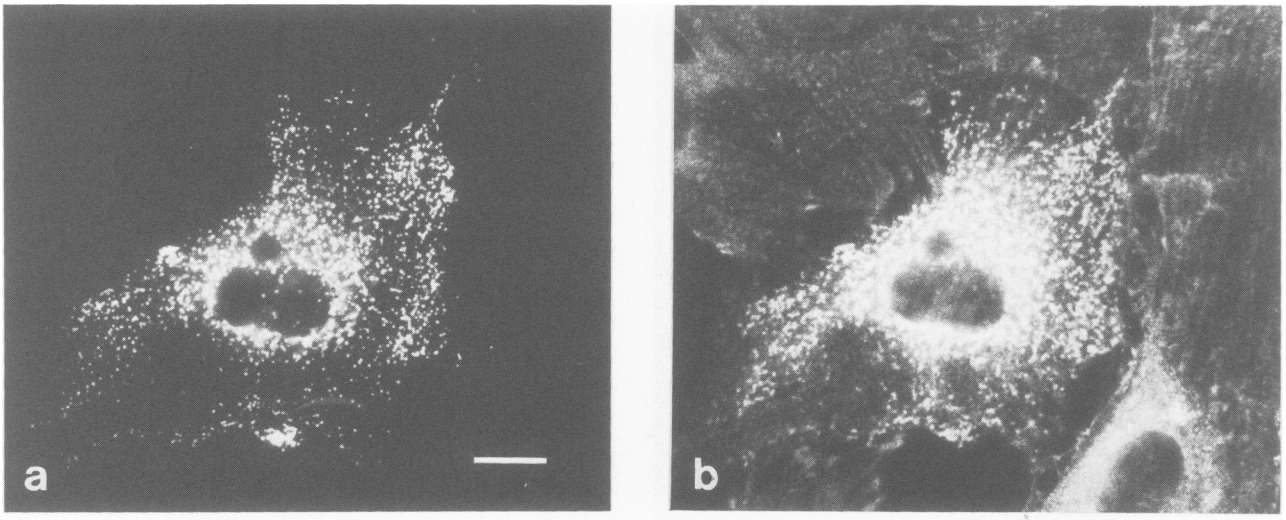


Fig. 4. Immunofluorescence localization of α-actinin and ActA in PtK_2 cells transiently transfected with the pSPL21 plasmid. Cells were processed for double immunofluorescence microscopy using an α-actinin mAb (**b**) and the affinity-purified polyclonal rabbit ActA antibodies (**a**), followed by fluorescein-labelled goat anti-rabbit antibodies and rhodamine-labelled goat anti-mouse antibodies, respectively. Note that, apart from its normal microfilamentous distribution, α-actinin also colocalizes with ActA on mitochondria in the transfected cell (b). Bar represents 9 μm.

regions within the ActA molecule that direct transport of variously truncated versions of this polypeptide to either mitochondria (Figure 3a, c and g) or the nucleus (Figure 3e and i). The mitochondrial targeting sequence of ActA, which displayed certain motifs of characteristic mitochondrial import targeting signals, i.e. positively-charged and hydrophobic residues (Hartl *et al.*, 1989), was located to the C-terminal 26 amino acid residues (Figure 1a). The location of a mitochondrial target signal at the C-terminal end of the ActA polypeptide is unique because there is no clear example of a C-terminal location for such a signal on mitochondrial proteins. At this point it is of interest to note that the ActA polypeptide is also unusual because, unlike many surface proteins of Gram-positive bacteria, it lacks characteristic membrane anchor sequences. The mechanism by which the ActA polypeptide is directed to the mitochondria and its exact localization on this subcellular structure is currently being investigated. To date, there is no other example of a bacterial surface protein being directed to the mitochondria of the host cell.

Nuclear transport was most striking in the absence of the membrane anchor and the proline-rich repeats, and may be associated with stretches of lysines clustered within the N-terminal domain of ActA. Although a well defined consensus sequence or structure is not apparent, nuclear localization sequences frequently consist of a contiguous stretch of basic amino acids often combined with helix-breaking residues such as proline or glycine (Dingwall and Laskey, 1991). Indeed, the ActA sequence PKVFKKIKDAGK, extending from amino acid residues 213–224, has strong similarity to the well characterized nuclear localization sequence of the simian virus 40 large T-antigen (Kalderon *et al.*, 1984).

We have previously found that the ActA polypeptide of growing *L.monocytogenes* is specifically degraded by C-terminal end processing events to generate sequentially truncated polypeptides lacking some of the discrete regions described above (Niebuhr *et al.*, 1993). The significance of these findings for the pathophysiology of *Listeria* infection is not clear at the moment.

In conclusion, we have demonstrated that the ActA polypeptide alone is sufficient to induce reorganization of the actin cytoskeleton on the surface of the subcellular structure upon which it is located. Like *Listeria*, other intracellularly mobile pathogens, including *Shigella* (Bernardini *et al.*, 1989) and *Rickettsia* spp. (Heinzen *et al.*, 1993), appear to have evolved a strategy employing surface-bound nucleator components to interdict and overcome host cell controls on extraneous actin accumulation. Yet, despite their functional similarity, there exists no sequence homology between the ActA polypeptide and the IcsA protein of *Shigella flexneri* (Lett *et al.*, 1989). This suggests to us that there is either functional redundance with respect to the proteins capable of acting as nucleators of the host cell actin or that, in the complex phenomenon of actin cytoskeleton reorganization, these proteins exert their influence at different steps which are not discernible in the assays used so far to assess their function. The novel experimental model of actin filament nucleation and microfilament recruitment on mitochondria described here will permit biochemical analysis to catalogue the events and players involved in generating this reorganization of the actin cytoskeleton. Finally, the mitochondrial targeting of ActA can now, in principle, be used to direct any potential nucleator protein to this organelle and provides a facile system for assessing function and identifying interacting proteins.

Materials and methods

Reagents

All chemical reagents, including fluorescein-labelled phalloidin, were purchased from Sigma (Deisenhofen, Germany) unless indicated otherwise. Restriction endonucleases and ligase were purchased from Gibco BRL (Eggenstein, Germany), Biolabs (Schwalbach, Germany), Perkin Elmer (Ueberlingen, Germany), Applied Biosystems (Weiterstadt, Germany) and Boehringer Mannheim (Germany) following the manufacturer's instructions.

Cloning of actA derivatives

The ActA polypeptides were transiently overexpressed using the eukaryotic expression vector pMPSVHE (Artelt *et al.*, 1988). To clone *act*A sequences under control of the constitutively expressing promoter of MPSV, the *act*A sequences were amplified from genomic DNA of *L.monocytogenes* EGD strain by PCR (Keohavong *et al.*, 1988) using oligonucleotide primers. The

5′ primers were designed so as to be optimal for eukaryotic expression (Kozak, 1991). To clone the *act*A sequences into the pMPSVHE vector, the restriction sites *Hin*dIII and *Bam*HI (bold) were introduced into the 5′- and 3′-ends of the *act*A sequences respectively by using specific PCR primers. Nucleotides deviating from the published *act*A sequence (Domann *et al.*, 1992) are indicated in italic letters. The different *act*A derivatives are schematically shown in Figure 1b. To construct pSPL18 the following primer pairs 5′-end/3′-end were used: 5′-AGGAGG***AAGCTT***AA*A*TGG-GATTAAACAG and 5′-TTTGAATTTC***GGATCC***TTCACCTCACTTT for pSPL21; 5′-GACATA***AAGCTT***GCA*AT*GACAGATAGCG and 5′-TTTGAATTTC***GGATCC***TTCACCTCACTTT for pSPL26; 5′-GACATA***AAGCTT***GCA*AT*GACAGATAGCG and 5′-CATTGCAAG-*TT*ATA***GGATCC***TATGGTTCCC for pSPL22: the 5′- and 3′-ends were derived with the same primers that had been used for constructing pSPL26. For the deletion of amino acid positions 264–426, a fusion between two cloned PCR products was constructed. To obtain the 3′ position of ActA terminating at amino acid position 264, the following primer was used: 5′-TGGCGGG*GGA*TCCGAAGCATTTAC. The resulting fragment was cloned and, following digestion with *Bam*HI, was fused to the *Mbo*I–*Bam*HI fragment encoding the C-terminal end (minus the membrane anchor) from pSPL26. In the case of pSPL32, the *Mbo*I–*Bam*HI fragment from pSPL18 encoding the C-terminal end (including the membrane anchor) was used. 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 373A automated DNA Sequencer.

Antibodies

The N20 ActA mAb has been described previously, as well as the affinity-purified polyclonal rabbit antibodies against ActA (Niebuhr *et al.*, 1993). The affinity-purified polyclonal rabbit antibodies against the β-subunit of the F_1 ATP synthetase from yeast were a generous gift from Dr E.Orr (Department of Genetics, University of Leicester, UK). The α-actinin mAb (A5044) was purchased 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 supplemented with glutamine, non-essential amino acids and 10% fetal calf serum at 37°C in 8% CO_2. Subconfluent grown cultures were transfected with 2 μg specific plasmid and 2 μg genomic DNA isolated from PtK_2 cells with 0.5 ml precipitate for 5 ml volume of medium using the $Ca_3(PO_4)_2$ DNA coprecipitation method (Wigler *et al.*, 1977). 7 h after adding the precipitate, cells were washed once with fresh complete MEM medium. 48 h after starting the transfection, cells were washed once with PBS and processed for immunofluorescence microscopy.

For labelling of mitochondria with the anti-ATP synthetase antibodies and for the localization of α-actinin, cells were fixed and permeabilized with cold (−20°C) methanol. For actin labelling with fluorescent phalloidin PtK_2, 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 and/or fluorescein–phalloidin, coverslips were further processed for double immunofluorescence microscopy using fluorescein- or rhodamine-labelled goat anti-rabbit antibodies and/or 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 Ilford 400 Delta films or Kodak Tri-X Pan films respectively.

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Note added in proof

In our recent studies on the expression of the ActA polypeptide in transfected or *Listeria*-infected cells we found that the intracellular expressed ActA shifts to higher molecular weight forms. This has been shown to be the result of protein phosphorylation.