The EMBO Journal vol.14 no.7 pp.1314–1321, 1995

A focal adhesion factor directly linking intracellularly motile *Listeria monocytogenes* and *Listeria ivanovii* to the actin-based cytoskeleton of mammalian cells

Trinad Chakraborty[1], Frank Ebel, Eugen Domann, Kirsten Niebuhr[2], Birgit Gerstel[2], Susanne Pistor[2], Constance J.Temm-Grove[3,4], Brigitte M.Jockusch[3], Matthias Reinhard[5], Ulrich Walter[5] and Jürgen Wehland[2]

Institut für Medizinische Mikrobiologie, Frankfurter Strasse 107, D-35392 Giessen, [2]Gesellschaft für Biotechnologische Forschung, Abteilung Zellbiologie/Immunbiologie, Mascheroder Weg 1, D-38124 Braunschweig, [3]Zoologisches Institut, Abteilung Zellbiologie, Spielmannstrasse 7, D-38106 Braunschweig and [5]Medizinische Universitätsklinik, Klinische Biochemie und Pathobiochemie, Josef-Schneider-Strasse 2, D-97080 Würzburg, Germany

[4]Present address: Cold Spring Harbor Laboratories, Demerec Laboratory, Cold Spring Harbor, NY 11724, USA

[1]Corresponding author

Communicated by K.Timmis

The surface-bound ActA polypeptide of the intracellular bacterial pathogen *Listeria monocytogenes* is the sole listerial factor needed for recruitment of host actin filaments by intracellularly motile bacteria. Here we report that following *Listeria* infection the host vasodilator-stimulated phosphoprotein (VASP), a microfilament- and focal adhesion-associated substrate of both the cAMP- and cGMP-dependent protein kinases, accumulates on the surface of intracytoplasmic bacteria prior to the detection of F-actin 'clouds'. VASP remains associated with the surface of highly motile bacteria, where it is polarly located, juxtaposed between one extremity of the bacterial surface and the front of the actin comet tail. Since actin filament polymerization occurs only at the very front of the tail, VASP exhibits properties of a host protein required to promote actin polymerization. Purified VASP binds directly to the ActA polypeptide *in vitro*. A ligand-overlay blot using purified radiolabelled VASP enabled us to identify the ActA homologue of the related intracellular motile pathogen, *Listeria ivanovii*, as a protein with a molecular mass of ~150 kDa. VASP also associates with actin filaments recruited by another intracellularly motile bacterial pathogen, *Shigella flexneri*. Hence, by the simple expedient of expressing surface-bound attractor molecules, bacterial pathogens effectively harness cytoskeletal components to achieve intracellular movement.

Key words: ActA/*Listeria ivanovii*/*Listeria monocytogenes*/*Shigella flexneri*/VASP

Introduction

The mode of locomotion of intracellular parasitic bacteria has recently gained much interest as a model system to study microfilament assembly and function. In the case of *Listeria monocytogenes*, a ubiquitously occurring Gram-positive bacterium which is responsible for an uncommon but severe form of infection in humans and animals, the main modes of entry usually follow transplacental (Gray and Killinger, 1966) or transintestinal routes (Gellin and Broome, 1989). However, during acute infection many tissues are infected, demonstrating the ability of these bacteria to invade different eukaryotic cells (reviewed in Kocks and Cossart, 1994). Following phagocytosis by the host cell, the bacterium is able to avoid digestion in the phagolysosome by breaching the surrounding membrane and entering the cytoplasm (reviewed in Portnoy *et al.*, 1992).

Once inside the host cell cytoplasm, *L.monocytogenes* induces polymerization of actin around itself which is subsequently organized into a 'comet tail', located at one pole of the bacterial surface (reviewed in Tilney and Tilney, 1993). The characterization of mutants unable to accumulate actin filaments led to the identification of the 90 kDa surface-bound listerial ActA polypeptide (Domann *et al.*, 1992; Kocks *et al.*, 1992). Immunolocalization of ActA in infected tissue culture cells demonstrated that ActA is located on the bacterial surface at the site of actin filament assembly but is not detected in the comet tails of motile bacteria (Kocks *et al.*, 1993; Niebuhr *et al.*, 1993). By directly expressing the ActA from a eukaryotic expression vector it was demonstrated that ActA is the sole listerial factor required to initiate recruitment of host actin filaments around intracellular bacteria (Pistor *et al.*, 1994).

The ability to grow in the host cytoplasm as well as the actin-based movement and cell-to-cell spreading has also been observed for the closely related pathogen *Listeria ivanovii* (Karunasagar *et al.*, 1993) and is shared by two other groups of pathogenic bacteria, *Shigella flexneri* (Ogawa *et al.*, 1966; Makino *et al.*, 1986; Bernadini *et al.*, 1989) and *Rickettsia conorii* and *Rickettsia rickettsi* (Heinzen *et al.*, 1993; Teysseire *et al.*, 1992). While the putative ActA homologue from *L.ivanovii* has yet to be detected, for *S.flexneri* a gene encoding an outer membrane protein variously denoted VirG or IcsA (Bernadini *et al.*, 1989; Lett *et al.*, 1989) is required for actin accumulation by these bacteria. In *Rickettsia* the genes involved in actin assembly have thus far not been identified.

Several studies have shown that actin polymerization is required for movement of *Listeria* (Tilney and Portnoy, 1989; Dabiri *et al.*, 1990). Polymerization takes place at the interface of the bacterium and the elongating tail, resulting in movement of the bacteria away from the older stationary portion of the tail (Sanger *et al.*, 1992; Theriot *et al.*, 1992). By examining the protein composition of the microfilamentous structures recruited by *L.monocytogenes*, numerous host actin-binding proteins, including α-actinin

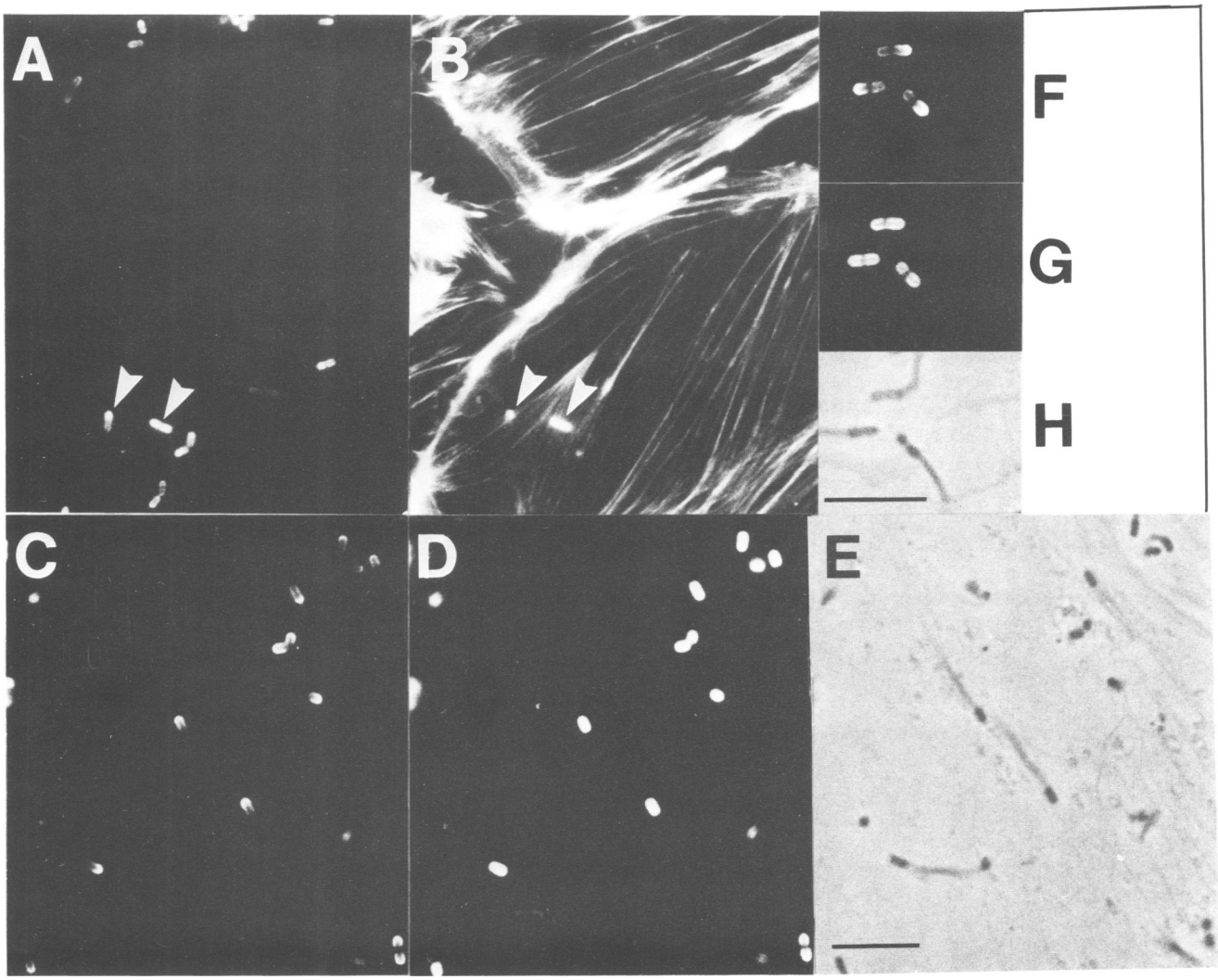

Fig. 1. Association of VASP with *L.monocytogenes* in infected PtK_2 cells. Double-fluorescence images (**A** and **B**; **C** and **D** and **F** and **G**) were obtained with monoclonal ActA antibodies (D and G) or polyclonal VASP antibodies (A, C and F) and FITC–phalloidin (B) followed by secondary rhodamine/fluorescein-labelled antibodies; (**E** and **H**) are corresponding phase-contrast images. One and a half hours after starting the infection VASP is evenly distributed on the bacterial surface even before actin 'clouds' accumulate around these bacteria (A and B). The presence of two bacteria showing F-actin staining is indicated with arrows. At later stages (4 h after starting the infection) VASP (C), unlike ActA (D) is polarly located on the surface of motile bacteria (E). A detailed view depicting ActA uniformly distributed on the surface of dividing bacteria but absent from the septal surfaces, is shown in G, while only faint staining with VASP is seen at the second pole opposite the actin comet tail (F). Bars represent 6 μm.

(Dabiri *et al.*, 1990), filamin (Dold *et al.*, 1994), fimbrin (Kocks and Cossart, 1993) and ezrin/radixin (Temm-Grove *et al.*, 1994), have been localized within the actin comet tails created by intracellularly motile *Listeria*. These proteins are associated even with the most distal actin filaments in the tails suggesting that none of them interacts directly with the bacterial surface. An exception is profilin (Theriot *et al.*, 1994), which appears to be concentrated at the back-half of intracellularly motile *Listeria* where it is 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 (Theriot *et al.*, 1994), indicating that an additional host factor is required to mediate the ActA–profilin interaction.

The vasodilator-stimulated phosphoprotein (VASP) is a novel cytoskeletal protein which is associated with microfilaments, and is particularly concentrated at sites of focal contacts as well as in the peripheral regions of protruding lamellae in locomoting or spreading cells (Reinhard *et al.*, 1992). VASP was originally characterized as a protein which is phosphorylated in response to both cAMP- and cGMP-elevating vasodilators and platelet inhibitors mediated by cAMP- and cGMP-dependent protein kinases (Waldmann *et al.*, 1987; Halbrügge and Walter, 1989; Halbrügge *et al.*, 1990; Nolte *et al.*, 1991; Eigenthaler *et al.*, 1992; Butt *et al.*, 1994). Phosphorylation of VASP in human platelets closely correlates with the inhibition of both platelet aggregation and phospholipase C activation (Waldman *et al.*, 1987; Halbrügge *et al.*, 1990; Geiger *et al.*, 1992). Intrigued by its role in signal transduction and its association with highly dynamic structures that promote cellular motility we examined its interaction with intracellularly motile *Listeria*. Our results demonstrate that VASP interacts directly with the ActA polypeptides of *L.monocytogenes* and *L.ivanovii* and hence

is the first cytoskeletal protein to be discovered that binds to intracellular pathogens. VASP also associates with the actin filaments of the unrelated, intracellularly motile Gram-negative bacterial pathogen *S.flexneri,* demonstrating its importance in generating and controlling microfilament structures and dynamics in cellular motility. We have identified VASP as one (and possibly the only) factor involved in the coupling of *Listeria* and *Shigella* to the host cytoskeleton.

Results

VASP has properties of a host factor promoting actin filament polymerization

In order to investigate a role for VASP in microfilament assembly, we first examined its distribution on intracellular *Listeria* using immunofluorescence microscopy. Soon after infection, VASP was found uniformly coating the bacterial surface and was seen even before F-actin 'clouds' were detectable around the bacteria (Figure 1A and B). Later, VASP localizes polarly on the surface of motile bacteria where it is juxtaposed between the bacterial surface and the front of the actin comet tail (Figure 1C and E). Hence, VASP is uniquely placed to promote actin polymerization during bacterial propulsion within the host cell cytoplasm.

Association of intracellular motile L.monocytogenes with VASP requires ActA

Since the ActA polypeptide is indispensable for the intracellular motility of *L.monocytogenes*, we examined the relative distribution of VASP and ActA on cytoplasmically motile bacteria. ActA was found to coat uniformly the surface of individual bacteria, including those of dividing cells (Figure 1D), whereas it was conspicuously absent from the septal surface between two dividing cells (Figure 1G). In contrast, VASP distribution on intracellularly motile bacteria was always highly polarized and showed a bi-lobed distribution on the opposite poles of dividing cells (Figure 1F and H), whereas the surface of non-motile bacteria was uniformly labelled by VASP antibodies. The association of VASP with *Listeria* is dependent on the presence of the ActA polypeptide exposed on the bacterial surface. An isogenic *act*A mutant strain lacking all but 55 amino acids of the ActA polypeptide (see Materials and methods) infected and multiplied normally within host cells, but was associated with neither VASP nor actin (data not shown). Hence, ActA is essential for the interaction of VASP with the bacteria.

VASP binds to the ActA polypeptide of L.monocytogenes

A possible direct interaction between VASP and ActA was initially tested in solid phase binding assays with purified proteins. For this purpose, purified ActA was coated onto the plastic surface of removable microtiter wells. After blocking the non-specific sites, VASP was applied as a soluble ligand. Detection of bound VASP by specific polyclonal antibodies (Halbrügge *et al.*, 1990; Reinhard *et al.*, 1992) and [^{125}I]protein A revealed significant binding of VASP to immobilized ActA (Figure 2).

To further investigate VASP's interaction with ActA, a ligand-overlay blot was performed using purified radiolabelled VASP as the soluble ligand to probe different

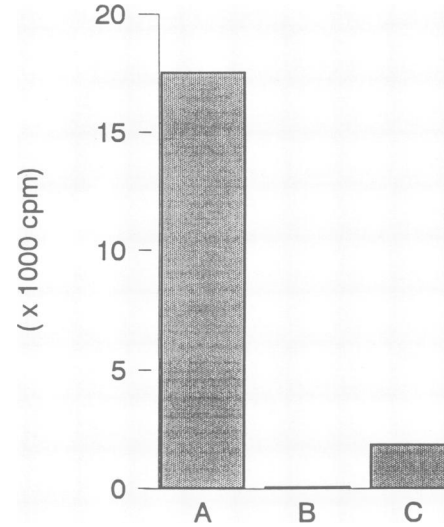

Fig. 2. Solid phase binding assay demonstrating the *in vitro* interaction of purified VASP and ActA proteins. Binding of VASP to microtiter-surface coated ActA from *L.monocytogenes* EGD was detected using VASP-specific antibodies and [^{125}I]protein A (column A). Wells without VASP-incubation (column B) and not coated with ActA (column C) served as controls. The results shown are the average of three independent experiments each performed in triplicate.

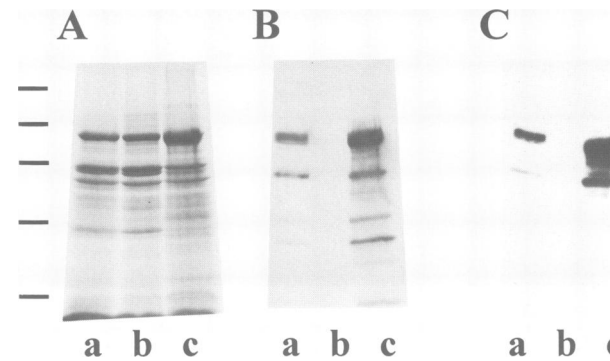

Fig. 3. Ligand-overlay and immunoblot assays demonstrating specific interaction between VASP and ActA. Cell wall extracts of *L.monocytogenes* EGD (lane a), *L.monocytogenes* EGD Δ*act*A2 (lane b), and *L.monocytogenes* EGD ΔactA2 harbouring plasmid pBPL1 (lane c) were separated by SDS–PAGE and analysed by Coomassie blue staining (**A**), the corresponding blots were processed with affinity-purified ActA antibodies (**B**) or with ^{32}P-labelled VASP in the ligand-overlay assay (**C**). The ActA antibodies reacted with several polypeptide species (B) all of which have previously been shown by N-terminal sequencing studies to be C-terminally truncated derivatives of the ActA polypeptide (Niebuhr *et al.*, 1993), and are clearly absent in the strain deleted for the *act*A gene (B, lane b). Molecular weight markers from top to bottom are: 205, 116, 80, 49 and 32 kDa.

listerial SDS extracts immobilized on polyvinylidene fluoride (PVDF) membranes. Extracts were derived from the wild type *L.monocytogenes* EGD strain, its isogenic *act*A deletion derivative (*L.monocytogenes* Δ*act* A2) and the same deletion strain complemented with a plasmid harbouring the wild type *act*A gene. The Coomassie-stained gel, the immunoblot using specific affinity-purified polyclonal ActA antibodies (Niebuhr *et al.*, 1993) and the autoradiogram corresponding to the overlay blot are shown in Figure 3. It can be concluded that VASP binds to immobilized ActA, since (i) both the immunoblot and the ligand overlay assays revealed no reaction with proteins

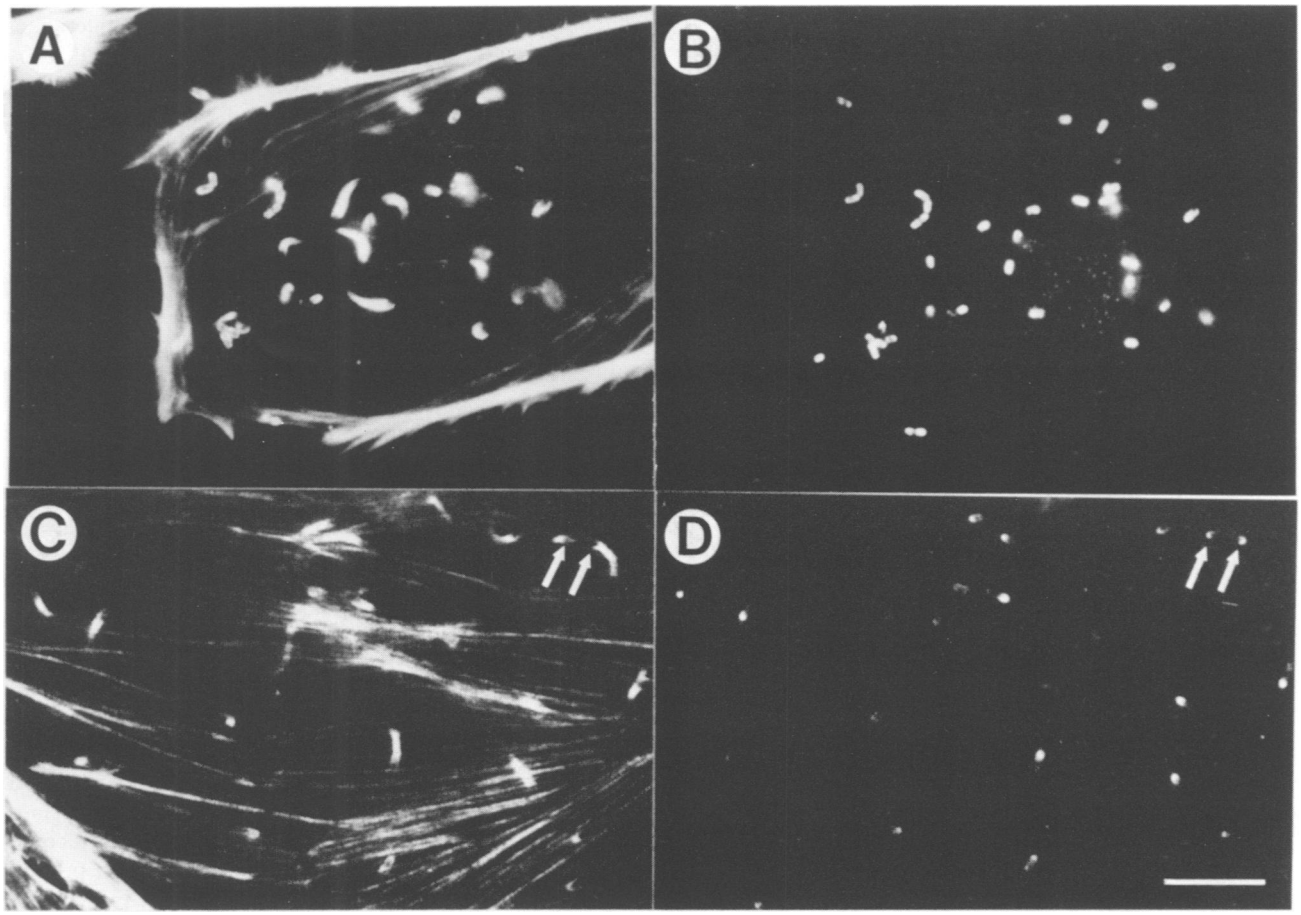

Fig. 4. Localization of VASP, ActA and actin in *L.ivanovii*-infected PtK_2 cells analysed by double fluorescence microscopy using FITC–phalloidin (**A** and **C**), polyclonal ActA (**B**) or VASP antibodies (**D**), and rhodamine-labelled secondary antibodies. The affinity-purified *L.monocytogenes* ActA antibodies reveal homogeneous labelling of the intracellularly bacteria (B). The presence of two bacteria exhibiting actin comet tails (C) and the corresponding VASP staining (D) are indicated by arrows. Bar represents 10 μm.

of the isogenic *act*A deletion mutant, (ii) ActA was detected by affinity-purified antibodies in the extracts of both the wild type strain and the complemented isogenic *act*A deletion mutant and (iii) radiolabelled VASP interacted directly with ActA in the SDS extracts of these two strains. Since the binding occurred in the absence of other host cytoplasmic factors, VASP appears to be a host factor that physically couples these intracellular bacteria to components of the host cytoskeleton.

The ActA homologue of L.ivanovii binds VASP

Although the actin dynamics and the morphology of the microfilament network induced by intracellular *L.ivanovii* are highly similar to those seen with *L.monocytogenes*, nothing is known of the ActA homologue encoded by this species (Karunasagar *et al.*, 1993; Gouin *et al.*, 1994). Cells infected with *L.ivanovii* were processed for double immunofluorescence microscopy using fluorescently labelled phalloidin for actin localization (Figure 4A and C) and either affinity-purified *L.monocytogenes* ActA (Figure 4B) or VASP antibodies (Figure 4D). As we had previously observed for intracellular *L.monocytogenes* (Niebuhr *et al.*, 1993) the ActA antibodies uniformly decorated the surface of intracytoplasmic *L.ivanovii* and not the actin comet tails of motile bacteria. VASP staining was found at the interface of the bacterium and the elongating actin tail as already seen for *L.monocytogenes*.

We surmised that a comparison of results obtained by immunoblotting with affinity-purified *L.monocytogenes* ActA antibodies with that obtained with a ligand-overlay blot using radiolabelled VASP should unambiguously allow the identification of ActA in cell wall extracts of these bacteria. Immunoblot analysis of SDS extracts revealed that the *L.ivanovii* ActA polypeptide was considerably larger than the 90 kDa ActA of *L.monocytogenes* and had an estimated molecular mass of ~150 kDa (Figure 5A, cf. lanes 1 and 3). No cross-reaction was seen with SDS extracts of the non-pathogenic *Listeria innocua* strain that is unable to either invade or survive in tissue culture cell lines (Figure 5A, lane 2). The size of the *L.ivanovii* ActA was confirmed by the ligand blot assay using radiolabelled VASP (Figure 5B, lane 2). Thus, VASP also binds to the ActA homologue of *L.ivanovii*.

VASP is associated with the actin-comet tails of S.flexneri

Another bacterium with a similar mode of intracellular movement is *S.flexneri*, a Gram-negative enteric bacterium that causes bacillary dysentery (reviewed in Hale, 1991). Using immunofluorescence microscopy of *S.flexneri*-

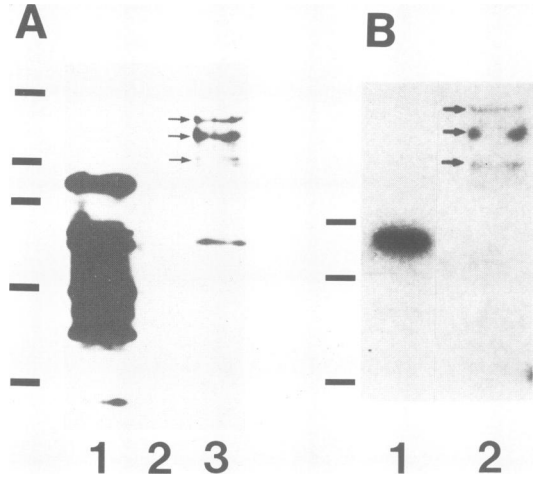

Fig. 5. Immunoblot and ligand-overlay assays using affinity-purified rabbit *L.monocytogenes* ActA-antibodies and radiolabelled VASP to probe SDS extracts of *L.ivanovii*. (**A**) Cell wall extracts of *L.monocytogenes* EGD (lane 1), *L. innocua* ATCC 33090 (lane 2) and *L.ivanovii* ATCC 19119 (lane 3) were separated on a 10% SDS-gel, immunoblotted and processed with affinity-purified rabbit *L.monocytogenes* ActA antibodies to reveal the size of the ActA homologue in *L.ivanovii*. (**B**) For the ligand-overlay blot performed with ^{32}P-labelled VASP the extracts were seperated on a 7.5% SDS-gel. Note that radiolabelled VASP interacts with identical bands as seen in the immunoblot assays. Molecular weight markers from top to bottom for A are: 205, 116, 80, 49 and 32 kDa and for B are: 116, 80 and 49 kDa.

infected human skin fibroblasts, we found that VASP was also associated with intracellularly motile *Shigella* (Figure 6B and E). In contrast to what was observed with *L.monocytogenes* and *L.ivanovii*, VASP was found evenly distributed throughout the F-actin comet tail (Figure 6A and D). In *S.flexneri* the IcsA polypeptide (Makino *et al.*, 1986; Bernadini *et al.*, 1989), a 120 kDa surface protein which is responsible for actin assembly at one pole of moving bacteria, is thought to be polarly secreted following proteolytic cleavage. Unlike the listerial ActA protein, the IcsA polypeptide is present throughout the actin comet of moving *Shigella* (Goldberg *et al.*, 1993). These data suggest that VASP might interact with this bacterial protein to generate the actin-based cytoskeleton of these pathogens. However, a ligand-overlay blot using radiolabelled VASP to probe cytoplasmic, membrane and water extracts of *S.flexneri* was negative (data not shown), suggesting that surface proteins of *S.flexneri*, unlike ActA of *L.monocytogenes*, do not directly interact with VASP.

Discussion

Numerous host actin-binding proteins, including α-actinin (Dabiri *et al.*, 1990), filamin (Dold *et al.*, 1994), fimbrin (Kocks and Cossart, 1993) or ezrin/radixin (Temm-Grove *et al.*, 1994) have been localized within the actin comet tails created by intracellularly motile *Listeria*, but no functional role for any one of them has yet been established. *Listeria* grown in broth culture do not associate efficiently with either purified actin (see Tilney and Tilney, 1993) or profilin (Theriot *et al.*, 1994), suggesting that the association of these proteins depends on the presence of a host cytoplasmic factor. Since the surface-bound ActA is the sole listerial product required for interaction with the actin cytoskeleton (Pistor *et al.*, 1994), the host factor sought should be recruited preferentially by this bacterial protein.

Immunolocalization of VASP on highly motile intracytoplasmic *Listeria* revealed its presence at the interface between the bacteria and the elongating actin tail where it is uniquely placed to promote actin polymerization during bacterial movement. Furthermore, VASP was found to bind *in vitro* directly to the ActA polypeptide of *L.monocytogenes*. This was also shown to be the case for *L.ivanovii,* and using the ligand-overlay blot developed in this study we identified the ActA homologue in this strain as a surface-bound polypeptide of ~150 kDa. Although we did not detect a direct interaction between VASP and the surface proteins of the Gram-negative intracellular pathogen *S.flexneri*, VASP was nevertheless a major component of the actin comet tails of intracellularly motile *Shigella*.

Our studies may have uncovered the initial interaction taking place between invading *Listeria* and the host cytoskeleton. VASP has many properties that would be predicted for a host factor inducing actin polymerization. First, VASP is associated with the bacterium prior to the formation of actin clouds. Second, VASP is polarly located on intracellularly motile *Listeria* and is restricted to the bacterial surface at the front of the actin comet tail. Third, VASP binds directly to purified ActA of these bacteria in the absence of any other host factor. Fourth, it has recently been demonstrated that VASP is the first known proline-rich ligand of profilin (Reinhard *et al.*, 1995). Fifth, VASP is a major substrate for two different intracellular signalling pathways, the cGMP/cG-PK and the cAMP/cA-PK (Waldmann *et al.*, 1987; Halbrügge and Walter, 1989; Halbrügge *et al.*, 1990; Nolte *et al.*, 1991; Eigenthaler *et al.*, 1992; Butt *et al.*, 1994).

In cultured cells VASP and profilin co-localize in highly dynamic membrane regions consistent with a direct interaction of these two proteins (Reinhard *et al.*, 1995). Profilins are ubiquitous G-actin sequestering proteins involved in the regulation of actin polymerization and, like VASP, linked to signal transduction (Theriot and Mitchison, 1993). Hence, the unique locations of profilin and VASP in rapidly moving structures together with their signal integration properties suggest that they may act in concert to confer signal perception to microfilament organization.

Several important issues remain to be addressed concerning the VASP–ActA interaction. The extreme polarity of VASP on the bacterial surface as compared with its ActA distribution suggests that only subpopulations of ActA and VASP molecules interact. Since both proteins exist in phosphorylated and non-phosphorylated forms (Brundage *et al.*, 1993; Butt *et al.*, 1994), efficient binding may depend on the phosphorylation status of either protein. Studies are currently in progress to examine the effect of phosphorylation on the binding properties of both proteins.

Although we have not directly assessed the particular region on the ActA polypeptide which is the substrate for VASP binding, indirect evidence suggests that the central proline-rich repeats of ActA may be required for this interaction. As recently reported, microinjection of *Listeria*-infected PtK2 cells with a peptide derived from one of

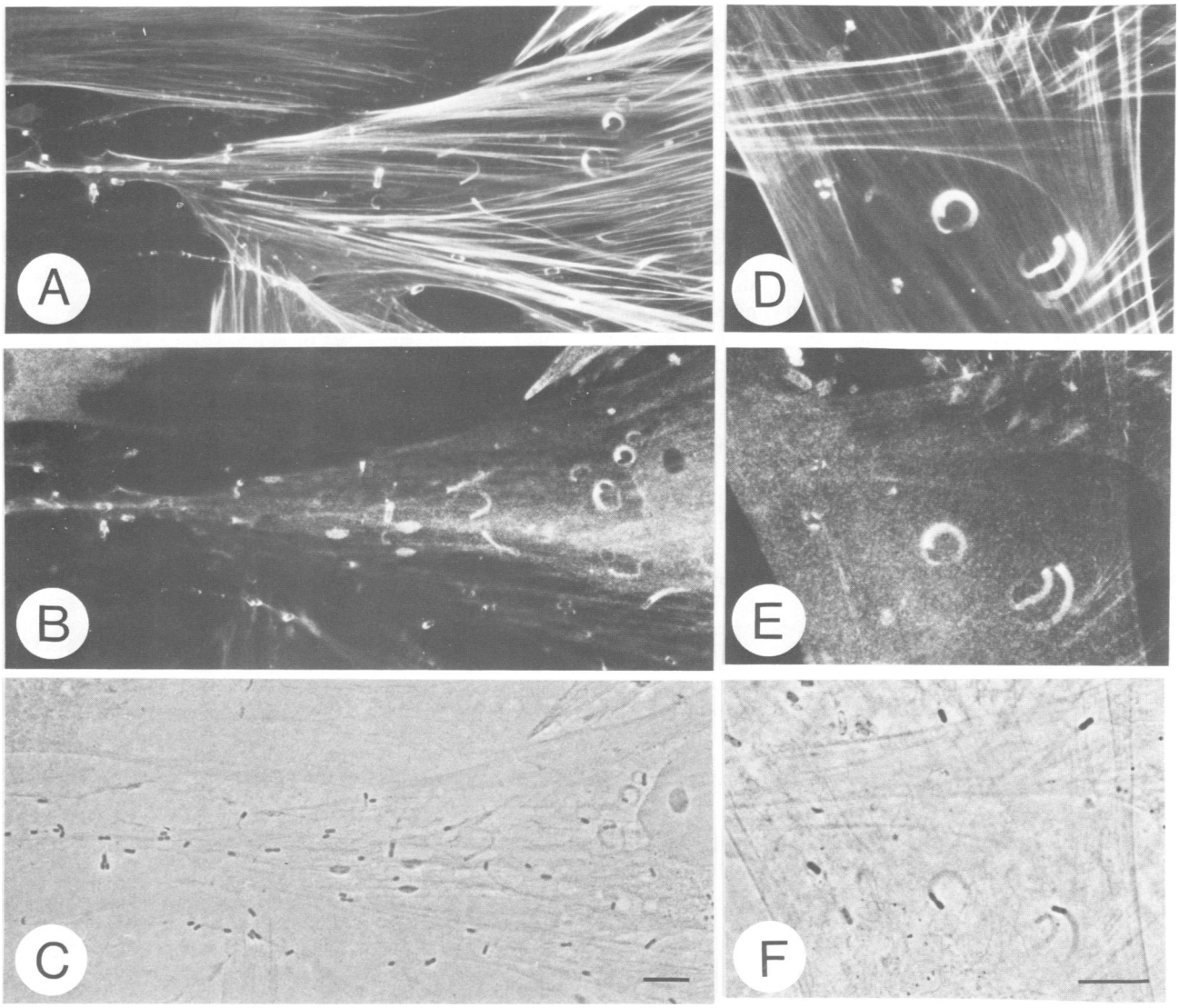

Fig. 6. Localization of actin and VASP in *S.flexneri*-infected human skin fibroblasts using FITC–phalloidin (**A** and **D**), polyclonal VASP antibodies and rhodamine-labelled secondary antibodies (**B** and **E**). The double fluorescence images show that VASP is present in the actin comet tails of intracellularly motile *Shigella*. Phase contrast micrographs of the respective cells are depicted in **C** and **F**. Bars represent 10 μm.

the central proline-rich repeats of ActA arrests both bacterial movement and dynamic activity in the cortical regions of the host cell (Southwick and Purich, 1994), a phenotype that might be predicted if the peptide directly interacts with VASP and/or profilin. These results in turn imply that host-cell analogues of ActA that are capable of directly interacting with VASP must exist.

Previous studies on the animal pathogen *L.ivanovii* indicated that, despite its ability to accumulate and reorganize host cell actin to effect intracellular and cell-to-cell spread, there was no evidence for the presence of an ActA homologue (Karunasagar *et al.*, 1993). More recently, nucleotide sequences corresponding to the proline-rich repeats of ActA have been detected in this species (Gouin *et al.*, 1994). However, the ActA polypeptide of this species has not been identified nor is it known whether a functional product is expressed. In this study we demonstrated that *L.ivanovii* encodes for an enlarged ActA homologue of ~150 kDa which is also capable of direct interaction with purified VASP. The interaction appeared to be much weaker suggesting low sequence similarities between the ActA proteins of *L.monocytogenes* and *L.ivanovii*.

Although VASP was present in the actin comet tails of intracellularly motile *S.flexneri*, it did not appear to bind directly to the surface of these bacteria. These data add to the increasing evidence that emphasizes detailed differences in generating the actin comet tails by these two bacteria. Although mutants defective in IcsA or ActA of *Shigella* or *Listeria*, respectively (Domann *et al.*, 1992; Kocks *et al.*, 1992; Bernadini *et al.*, 1989; Makino *et al.*, 1986) are phenotypically similar, these are distinct and unrelated genes, and their products are differently distributed on the respective bacterial surface and possess unique properties. The finding of a differential location of VASP contributes to this catalogue and suggests that the mode of attracting VASP differs between *Listeria* and *Shigella*. An attractive hypothesis is that *Shigella* recruits an additional host cell protein which in turn binds VASP. A prime candidate for this interaction is vinculin, a protein

associated with the actin-based cytoskeleton of intracellularly motile *Shigella* (Kadurugamuwa *et al.*, 1991; Sansonetti *et al.*, 1994), where it has a distribution similar to VASP. Vinculin, a major focal adhesion protein (Geiger *et al.*, 1980), contains a sequence motif with strong homology to the polyproline-rich repeats of the ActA polypeptide (Domann *et al.*, 1992).

The actin dynamics and the morphology of the filament network in the actin comet tails of intracellularly motile *Listeria* share similarities with the organization and behaviour of actin filaments at the leading edge of highly motile cells. It has been postulated (Theriot and Mitchison, 1992) that the driving force for movement in both cases is the random nucleation of short actin filaments at the leading edge, whereby the barbed ends are closely apposed to either the membrane or the bacterial surface, from which they are continuously released and flow away from the nucleation site. Our results now shed some light onto the nature of some of the key molecules in the postulated plasma membrane-associated cellular actin nucleation machinery. One is clearly an analogue of the ActA polypeptide and it may have the important property of being able to bind to VASP.

The listerial ActA polypeptide can be viewed as a prototype for attractor molecules ('attractins') expressed by an intracellular bacterium to effectively recruit host cytoskeletal components. Clearly, as is demonstrated by *Listeria* and *Shigella*, different steps in the pathway may be harnessed to achieve the same end result of actin-based motility. Our results imply that VASP is a key protein required for the initiation and maintenance of dynamic changes in the actin-based cytoskeleton and suggest that eukaryotic ActA analogues may be important components of this pathway.

Materials and methods

Bacterial strains, media and reagents

The *L.monocytogenes* serotype 1/2a EGD, the *L.innocua* ATCC 33090 and the *L.ivanovii* ATCC 19119 strains have been described previously (Domann *et al.*, 1992; Karunasagar *et al.*, 1993) as well as the *S.flexneri* serotype Y strain (SFL 1) (Kärnell *et al.*, 1992). All strains were grown routinely in brain–heart infusion (BHI) broth (Difco) at 37°C. The construction of the isogenic *L.monocytogenes* Δ*act*A2 mutant is detailed below as well as its complementation. The *L.monocytogenes* Δ*act*A2 mutant harbouring plasmid pBPL1 was grown in the presence of 100 μg/ml spectinomycin. All salts and ancillary agents were purchased from Sigma (Deisenhofen, Germany) unless explicitly mentioned.

Construction of an actA deletion mutant of L.monocytogenes

The *act*A2 deletion was generated using the polymerase chain reaction (PCR) employing specific primers to introduce an in-frame deletion that removes 584 amino acids (91.4%) of the wild type *act*A gene of *L.monocytogenes* EGD. The oligonucleotide pair A (5′-GCTGAAGC-TTGGGATCCAGTTGGGGTTAACTG-3′) and B (5′-GCAATTGGC-AGTAATGAAAACCACC-3′) were used to amplify a 288 bp DNA fragment at the 5′ region enclosing the first 19 N-terminal amino acid residues, and the oligonucleotide pair C (5′-GCGAAGGAAGAACC-AGGG-3′) and D (5′-TTGGCGTGCATAGGTTGAC-3′) served to amplify a 689 bp DNA fragment at the 3′ region including the last 36 C-terminal amino acids (Domann *et al.*, 1992). Following Klenow treatment to produce blunt ends, the PCR products were ligated at room temperature for 1 h. The ligation product harbouring the deletion was selectively amplified using the oligonucleotide pair A and C and subsequently cloned into the *Sma*I restriction endonuclease site of the thermosensitive shuttle vector pAULA (Chakraborty *et al.*, 1992) to create pAULA-ΔactA2. The construct was sequenced to verify the deleted region and introduced into *L.monocytogenes* by electroporation (Park and Stewart, 1990). Transformants were isolated by growth at 30°C on BHI agar containing 5 μg/ml erythromycin. Single colonies were isolated by streaking bacteria onto BHI plates plus erythromycin at 42°C following overnight growth. These colonies contain chromosomally integrated plasmids. To select for excision of the integrated plasmid, a single colony was propagated in 20 ml BHI and grown to stationary phase. Cultures were diluted 1:1000 into fresh media and grown to stationary phase; this step was repeated up to five times. Erythromycin-sensitive colonies were identified by streaking colonies from BHI agar onto BHI agar containing erythromycin. *L.monocytogenes* Δ*act*A2 was identified by Southern analysis of chromosomal DNA (data not shown) as well as by immunoblotting with affinity-purified ActA antibodies to verify absence of the gene product (see Figure 3).

The *L.monocytogenes act*A gene, bases 6037–8234, was amplified from EGD chromosomal DNA by PCR and ligated into pAT28 (Trieu-Cuot *et al.*, 1990) digested with *Sal*I and *Bam*HI to generate pBPL1. The restriction sites *Xho*I and *Bam*HI of the amplified product were created as non-complementary ends of the amplification primers. Primers used were 5′-TGAAGTCGAGAAGCAGTTGGGGT-3′ and 5′-TTTTG-AATTTCGGATCCTTCACCTCACTTT-3′. The construct was sequenced and introduced into *L.monocytogenes* Δ*act*A2 by electroporation.

Polyacrylamide gel electrophoresis, ligand-overlay and immunoblot assays

Cell wall extracts of *Listeria* were isolated and processed for SDS–PAGE and immunoblotting as described (Niebuhr *et al.*, 1993). For the ligand overlay blot assay samples were separated by SDS–PAGE and blotted onto Immobilon P membranes (Millipore, Eschborn, Germany). After saturation of non-specific binding sites with blocking medium (Reinhard *et al.*, 1992) containing 0.5 mM DTT, and a 2 h incubation with radiolabelled VASP (0.1 μg/ml in blocking medium/DTT) at room temperature, the nylon sheets were washed with PBS containing 0.5 mM DTT, 0.3% (v/v) Triton X-100, 0.05% (v/v) Tween 20, dried, and exposed to an autoradiographic film. ^{32}P-labelled VASP was prepared as follows: human platelet VASP, purified essentially as described (Reinhard *et al.*, 1992) with an additional hydroxylapatite step (Bio-gel HT; Bio-Rad, Richmond, CA) was phosphorylated by cGMP-dependent protein kinase (Halbrügge and Walter, 1989) for 1 h at 30°C in the presence of 2.5 μM [γ-^{32}P]ATP (5.9 TBq/mmol) followed by 30 min with 200 μM [γ-^{32}P]ATP (64 GBq/mmol). After phosphorylation one volume of double concentrated blocking medium/DTT was added and free ATP was removed by centrifugation through a MicroSpin S-200 HR column (Pharmacia) equilibrated with blocking medium/DTT.

Solid phase binding assays

Microtiter wells (Removawell, Dynatech Laboratories) were coated with purified ActA (Niebuhr *et al.*, 1993) at 30 μg/ml in PBS for 1.5 h at room temperature and left overnight at 4°C. The following day, the supernatant was removed, the wells were washed thrice with PBS and treated with a blocking solution of 3% BSA in PBS for 1 h. After three washes with PBS, VASP was added at a final concentration of 1.5 μg/ml in a reaction volume of 400 μl and incubated for a further 2 h at room temperature. The reaction wells were rinsed thrice with PBS, and binding of VASP was detected using the VASP antiserum M4 (1:1000 in PBS/BSA) for 1 h followed by [^{125}I]protein A (3.7 kBq/ml in PBS/BSA; ICN Radiochemicals, Irvine, CA) incubation for a further hour. Individual wells were removed, and the radioactivity bound was measured in a γ-counter.

Infection of cultured cells and fluorescence microscopy

All cell lines were cultivated in the absence of antibiotics. PtK_2 potooro kidney epithelial cells (ATCC CCL 56) were cultured in minimum essential medium (MEM; Gibco) supplemented with 10% FCS, glutamine and non-essential amino acids: human skin fibroblasts (Reinhard *et al.*, 1992) were cultivated in Dulbecco's MEM (Gibco) supplemented with 10% FCS and glutamine. Bacteria were grown as described above and added directly to the tissue culture medium at a dilution of 1:100. Cells were grown on small glass coverslips in 6-well plates. In the case of *S.flexneri*, the plates were centrifuged at 145 *g* for 10 min after adding the bacteria. After 1–2 h, the plates were extensively washed with fresh medium supplemented with gentamycin (25 μg/ml) to kill extracellular bacteria. At predetermined times the cells were washed once with pre-warmed PBS, and cells on coverslips were processed for immunofluorescence microscopy as described previously (Niebuhr *et al.*, 1993). Cells were fixed in 3.7% formaldehyde in PBS and permeabilized with 0.2% (v/v) Triton X-100 in PBS. Coverslips were incubated with fluorescein-

labelled phalloidin mixed with the primary poly- or monoclonal antibodies followed by rhodamine-labelled secondary antibodies (Dianova, Hamburg, Germany) or with a mixture of the primary polyclonal VASP and monoclonal ActA antibodies followed by rhodamine-labelled anti-mouse and fluorescein-labelled anti-rabbit antibodies and further processed as described previously (Niebuhr *et al.*, 1993). The monoclonal ActA antibody N81 and the affinity-purified rabbit antibodies against ActA (Niebuhr *et al.*, 1993), the VASP-M4 antiserum and the affinity-purified VASP-M4 antibodies (Reinhard *et al.*, 1992) have been described previously. Samples were examined with a Zeiss Axiophot microscope equiped with epifluorescence, and photographs were taken with Kodak T-MAX 400 films.

Acknowledgements

We thank Kenneth N.Timmis for his generous support, Elisha Orr (Department of Genetics, University of Leicester, UK) for fruitful discussions and Tibor Pál (Institute of Microbiology, University of Pécs, Hungary) for providing the *S.flexneri* strain. The work reported herein was supported by grants from the Deutsche Forschungsgemeinschaft (SFB 249/A13 for T.C., F.E. and E.D; Jo 55/11 for C.J. T-G and B.M.J. and SFB 176/A21 for M.R. and U.W.).

References

Bernadini,M.L., Mounier,J., D'Hauteville,H., Coquis-Rondon,M. and Sansonetti,P.J. (1989) *Proc. Natl Acad. Sci. USA*, **86**, 3867–3871.

Brundage,R.A., Smith,G.A., Camilli,A., Theriot,J. and Portnoy,D.A. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 11890-11894.

Butt,E., Abel,K., Krieger,M., Palm,D., Hoppe,V., Hoppe,J. and Walter,U. (1994) *J. Biol. Chem.*, **269**, 14509–14517.

Chakraborty,T., Leimeister-Wächter,M., Domann,E., Hartl,M., Goebel,W., Nichterlein,T. and Notermans,S. (1992) *J. Bacteriol.*, **174**, 568–578.

Dabiri,G.A., Sanger,J.M., Portnoy,D.A. and Southwick,F.S. (1990) *Proc. Natl Acad. Sci. USA*, **87**, 6068–6072.

Dold,F.G., Sanger,J.M. and Sanger,J.W. (1994) *Cell Motil. Cytoskelet.*, **28**, 97–107.

Domann,E., Wehland,J., Rohde,M., Pistor,S., Hartl,M., Goebel,W., Leimeister-Wächter,M., Wuenscher,M. and Chakraborty,T. (1992) *EMBO J.*, **11**, 1981-1990.

Eigenthaler,M., Nolte,C., Halbrügge,M. and Walter,U. (1992) *Eur. J. Biochem.*, **205**, 471–481.

Geiger,B., Tokuyasu,K.T., Dutton,A.H. and Singer,S.J. (1980) *Proc. Natl Acad. Sci. USA*, **77**, 4127–4131.

Geiger,J., Nolte,C., Butt,E., Sage,S.O. and Walter,U. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 1031–1035.

Gellin,B.C. and Broome,C.V. (1989) *Am. Med. Assoc.*, **261**, 1313–1320.

Goldberg,M., Barzu,O., Parsot,C. and Sansonetti,P.J. (1993) *J. Bacteriol.*, **175**, 2189–2196.

Gouin,E., Mengaud,J. and Cossart,P. (1994) *Infect. Immun.*, **62**, 3550–3553.

Gray,M.L. and Killinger,A.H. (1966) *Bacteriol. Rev.*, **30**, 309–382.

Halbrügge,M. and Walter,U. (1989) *Eur. J. Biochem.*, **185**, 41–50.

Halbrügge,M., Friedrich,C., Eigenthaler,M., Schanzenbächer,P. and Walter,U. (1990) *J. Biol. Chem.*, **265**, 3088–3093.

Hale,T.L. (1991) *Microbiol. Rev.*, **55**, 206–224.

Heinzen,R.A., Hayes,S.F., Peacock,M.G. and Hackstadt,T. (1993) *Infect. Immun.*, **61**, 1926–1935.

Kadurugamuwa,J., Rohde,M., Wehland,J. and Timmis,K.N. (1991) *Infect. Immun.*, **59**, 3463–3471.

Karunasagar,I., Krohne,G. and Goebel,W. (1993) *Infect. Immun.*, **61**, 1926–1935.

Kärnell,A., Sweiha,H. and Lindberg,A.A. (1992) *Vaccine*, **10**, 167–172.

Kocks,C. and Cossart,P. (1993) *Infect. Agents Dis.*, **2**, 207–209.

Kocks,C. and Cossart,P. (1994) *Mol. Microbiol.*, **13**, 395–402.

Kocks,C., Gouin,E., Tabouret,M., Berche,P., Ohayon,M. and Cossart,P. (1992) *Cell*, **68**, 521–531.

Kocks,C., Heilio,R., Gounon,R., Ohayon,H. and Cossart,P. (1993) *J. Cell Sci.*, **105**, 699–710.

Lett,M., Sasakawa,C., Okada,N., Sakai,T., Makino,S., Yamada,M., Komatsu,K. and Yoshikawa,M. (1989) *J. Bacteriol.*, **171**, 353–359.

Makino,S., Sasakawa,C., Kamata,K., Kurata,T. and Yoshikawa,M. (1986) *Cell*, **46**, 551–555.

Niebuhr,K., Chakraborty,T., Rohde,M., Gazlig,T., Jansen,B., Köllner,P. and Wehland,J. (1993) *Infect. Immun.*, **61**, 2802–2973.

Nolte,C., Eigenthaler,M., Schanzenbächer,P. and Walter,U.J. (1991) *Biol. Chem.*, **266**, 14808–14812.

Ogawa,H., Nakamura,A. and Nakaya,R. (1968) *Jap. J. Med. Sci. Biol.*, **21**, 259–273.

Park,S.F. and Stewart,G.S.A.B. (1990) *Gene*, **94**, 129–132.

Pistor,S., Chakraborty,T., Niebuhr,K., Domann,E. and Wehland,J. (1994) *EMBO J.*, **13**, 758–763.

Portnoy,D.A., Chakraborty,T., Goebel,W. and Cossart,P. (1992) *Infect. Immun.*, **60**, 1263–1268.

Reinhard,M., Halbrügge,M., Scheer,U., Wiegand,C., Jockusch,B.M. and Walter,U. (1992) *EMBO J.*, **11**, 2063–2070.

Reinhard,M., Giehl,K., Abel,K., Haffner,C., Jarchaii,T., Hoppe,V., Jocküsch,B.M. and Walter,U. (1995) *EMBO J.*, **14**, in press.

Sanger,J.M., Sanger,J.W. and Southwick,F.S. (1992) *Infect. Immun.*, **60**, 3609–3619.

Sansonetti,P.J., Mounier,J., Prévost,J.C. and Mége,R.M. (1994) *Cell*, **76**, 829–839.

Southwick,F.S. and Purich,D.L. (1994) *Proc. Natl Acad. Sci* USA, **91**, 5168–5172.

Temm-Grove,C.J., Jockusch,B.M., Rohde,M., Niebuhr,K., Chakraborty,T. and Wehland,J. (1994) *J. Cell Sci.*, **107**, in press.

Teysseire,N., Chiche-Portiche,C. and Raoult,D. (1992) *Res. Microbiol.*, **143**, 821–829.

Theriot,J. and Mitchison,T.J. (1992) *Trends Cell Biol.*, **2**, 219–222.

Theriot,J., Mitchison,T.J., Tilney,L.G. and Portnoy,D.A. (1992) *Nature*, **357**, 257–260.

Theriot,J.A. and Mitchison,T.J. (1993) *Cell*, **75**, 835–838.

Theriot,J.A., Rosenblatt,J., Portnoy,D.A., Goldschmidt-Clermont,P.J. and Mitchison,T.J. (1994) *Cell*, **76**, 505–517.

Tilney,L.G. and Portnoy,D.A.J. (1989) *J. Cell Biol.*, **109**, 1597–1608.

Tilney,L.G. and Tilney,M.S. (1993) *Trends Microbiol.*, **1**, 25–31.

Trieu-Cuot,P., Carlier,C., Poyart-Salmeron,C. and Courvalin,P. (1990) *Nucleic Acids Res.*, **18**, 4296.

Waldman,R., Nieberding,M. and Walter,U. (1987) *Eur. J. Biochem.*, **167**, 441–448.

Received on October 13, 1994; revised on December 19, 1994