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A novel proline-rich motif present in ActA of *Listeria monocytogenes* and cytoskeletal proteins is the ligand for the EVH1 domain, a protein module present in the Ena/VASP family

Kirsten Niebuhr, Frank Ebel[1], Ronald Frank, Matthias Reinhard[2], Eugen Domann[1], Uwe D.Carl, Ulrich Walter[2], Frank B.Gertler[3], Jürgen Wehland and Trinad Chakraborty[1,4]

Abteilung Zellbiologie und Immunologie/AG Molekulare Erkennung, Mascheroder Weg 1, 38124 Braunschweig, Germany, [1]Justus-Liebig-Universität Gießen, Institut für Medizinische Mikrobiologie, Frankfurter Straße 107, 35392 Gießen, Germany, [2]Medizinische Universitätsklinik, Institut für Klinische Biochemie und Pathobiochemie, Josef-Schneider Straße 2, 97080 Würzburg, Germany and [3]Department of Biology, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 02138-4307, USA

[4]Corresponding author

K.Niebuhr and F.Ebel contributed equally to this paper

The ActA protein of the intracellular pathogen *Listeria monocytogenes* induces a dramatic reorganization of the actin-based cytoskeleton. Two profilin binding proteins, VASP and Mena, are the only cellular proteins known so far to bind directly to ActA. This interaction is mediated by a conserved module, the EVH1 domain. We identify E/DFPPPPXD/E, a motif repeated 4-fold within the primary sequence of ActA, as the core of the consensus ligand for EVH1 domains. This motif is also present and functional in at least two cellular proteins, zyxin and vinculin, which are in this respect major eukaryotic analogs of ActA. The functional importance of the novel protein–protein interaction was examined in the *Listeria* system. Removal of EVH1 binding sites on ActA reduces bacterial motility and strongly attenuates *Listeria* virulence. Taken together we demonstrate that ActA–EVH1 binding is a paradigm for a novel class of eukaryotic protein–protein interactions involving a proline-rich ligand that is clearly different from those described for SH3 and WW/WWP domains. This class of interactions appears to be of general importance for processes dependent on rapid actin remodeling.

Keywords: Ena/VASP family/EVH1 domain/proline-rich motif/vinculin/zyxin

Introduction

The organization of actin filaments at the leading edge of highly motile cells shares strong similarities with the actin filament network in the comet tails of the motile intracellular bacterial pathogen *Listeria monocytogenes* (recently reviewed in Theriot, 1995). *Listeria monocytogenes* is a widespread, rapidly growing, Gram-positive bacterium that causes food-borne infections in animals and humans with severe implications, especially for pregnant women, newborns and immunocompromised individuals (Gellin and Broome, 1989). The virulence and cell to cell spreading of *L.monocytogenes* is dependent on its ability to exploit the actin-based cytoskeleton of host cells to support its motility (Tilney and Portnoy, 1989; Kuhn *et al.*, 1990; Domann *et al.*, 1992; Kocks *et al.*, 1992). A single bacterial surface protein, ActA, is both necessary and sufficient for the recruitment of host actin filaments (Pistor *et al.*, 1994, 1995; Friederich *et al.*, 1995; Kocks *et al.*, 1995; Smith *et al.*, 1995). A study with serially truncated ActA derivatives revealed that there are two separable domains required for interaction with the actin cytoskeleton: an N-terminal 23 amino acid region that is essential for actin filament nucleation and the proline-rich repeat region required for efficient actin filament recruitment (Pistor *et al.*, 1995). Hence, ActA associates with multiple host cytoskeletal proteins to generate intracellular bacterial motility. Since ActA is able to initiate a cascade of events leading to formation of a specific F-actin structure and subsequent actin-based movement, it is likely that it mimics host proteins that regulate and control actin assembly.

Currently only two cellular proteins have been shown to interact directly with ActA: the vasodilator-stimulated phosphoprotein (VASP) (Chakraborty *et al.*, 1995) and Mena, the mammalian homolog of *Drosophila* Enabled (Ena) (Gertler *et al.*, 1995, 1996). Both proteins belong to the newly discovered Ena/VASP family, whose members share a similar organization including three blocks of similarity: the N-terminal 113 amino acids termed Ena-VASP homology domain 1 (EVH1), the proline-rich central domain and the C-terminal 130 amino acids (EVH2) (Gertler *et al.*, 1996). So far the Ena/VASP family comprises four proteins: Ena, which was identified in genetic screens for dominant mutations that alleviate phenotypes associated with mutations in the *Drosophila* homolog of Abelson tyrosine kinase (Abl) (Gertler *et al.*, 1990), VASP, which was originally characterized as a major substrate for cAMP- and cGMP-dependent protein kinases in human platelets (Halbrügge and Walter, 1989) and two murine proteins, Mena and Evl (Ena-VASP-like), that were identified by their similarity to *Drosophila* Enabled (Gertler *et al.*, 1996).

Apart from their direct interaction with ActA on the surface of intracellular *Listeria*, VASP and Mena have several properties in common. They are strongly enriched in focal contact, along stress fibers and in regions of dynamic actin rearrangement, such as lamellipodia (Reinhard *et al.*, 1992; Gertler *et al.*, 1996) and both are natural ligands for the actin monomer binding protein profilin (Reinhard *et al.*, 1995a; Gertler *et al.*, 1996). These findings led to the model that ActA attracts profilactin to the interface of the bacterium and its actin tail by recruiting VASP and Mena, thereby enhancing local actin polymerization and enabling rapid actin-based movement (reviewed by Pollard, 1995).

In this report we describe the binding requirements and specificities of Mena and VASP. We show that binding is mediated by the common EVH1 domain and, using an overlay assay to probe arrays of immobilized peptides, we define the EVH1 binding motif. Analysis of peptides derived from the ActA sequence led to the identification of a minimal consensus binding motif for both VASP and Mena and indicated that the binding of EVH1 domains to their ligands represents a novel class of protein–protein interactions. We have also identified the EVH1 binding motif in eukaryotic proteins involved in microfilament assembly and regulation. Finally, we have examined the biological consequences of specifically eliminating the EVH1 binding sites from the ActA protein of *L.monocytogenes*.

Results

Characterization of the EVH1 binding site within the ActA protein of L.monocytogenes

A set of immobilized overlapping synthetic peptides covering the entire sequence of ActA was used to identify the specific binding site for VASP. Peptides that bound VASP have the sequence FPPP/IPTD/E in common, demonstrating that each proline-rich repeat of ActA acts individually as a VASP ligand (Figure 1a). Removal of the phenylalanine residue preceding the proline residues in these peptides completely abrogated VASP binding (cf. spots 51 and 52 in Figure 1a). To verify and extend these results, we examined the ability of overlapping soluble peptides covering the sequence of the second ActA repeat to compete with radiolabeled VASP for binding to ActA in a blot overlay assay. Equal amounts of *L.monocytogenes* SDS surface extracts containing the ActA protein were probed with radiolabeled VASP either in the presence or absence of competing peptides. Peptides carrying the sequence FPPPPTDEEL effectively eliminated the ability of VASP to bind full-length ActA (lanes P1 and P2 in Figure 1b).

To determine the VASP recognition core sequence in ActA, we synthesized truncated versions of the starting peptide sequence, $_{1}$SFEFPPPPTD$_{10}$ (Figure 2a). Considerable binding was still detected when peptides were sequentially C-terminally truncated down to the sequence SFEFPPPP. Successive deletions from the N-terminus were tolerated up to position E3, whereas removal of the F4 residue completely abrogated VASP binding. Identical results were obtained with *in vitro* translated Mena (data not shown). These results demonstrate that the core of the VASP/Mena binding motif corresponds to the amino acid sequence FPPPP.

Sequence requirements for VASP and Mena binding were characterized further using ^{35}S-labeled *in vitro* translated proteins and a series of immobilized synthetic peptides. Examination of peptides corresponding to the four natural repeats of ActA showed that they were bound with different efficiencies (Figure 2b). When the FPPIP core sequence of the fourth repeat was embedded in the context of the second repeat, a 2-fold increase in VASP binding to this chimeric peptide was observed, illustrating the importance of the residues flanking the core sequence. Complete loss of VASP and Mena binding was observed when the phenylalanine was substituted by an alanine and when the peptide sequence of repeat 2 was reversed, suggesting polarity in the recognition sequence. Examination of a peptide covering one of the eight identical proline-rich repeats of the recently identified ActA homolog from *Listeria ivanovii* (Kreft *et al.*, 1995; Gouin *et al.*, 1995; Gerstel *et al.*, 1996) revealed a weaker reactivity as compared with the *L.monocytogenes* repeats (see Figure 2b).

To determine whether the EVH1 domain, which is conserved between Ena, VASP, Mena and Evl, interacts specifically with the ActA proline-rich motifs, we examined the ability of radiolabeled GST–EVH1 fusion proteins of Mena (amino acids 6–170) and Evl (amino acids 1–153) to bind to purified ActA following preincubation in the presence of increasing amounts of the peptide SFEFPPPPTDEELRL. As shown in Figure 1c, this peptide competitively inhibited binding of the Mena and Evl EVH1 domains to ActA, demonstrating that these domains share not only extended sequence identity, but also exhibit similar ligand specificity.

Replacement analysis of the EVH1 binding sequence

To analyze the importance of individual residues in the EVH1 binding site in greater detail we examined variations of the peptide sequence SFEFPPPPTD obtained by single amino acid replacements (Figure 3). Exchanges of single proline residues revealed that in general P6 and P7 were more sensitive to substitutions than P5 and P8. When the threonine (T9) was replaced by any of the 19 amino acids in a peptide scan, all substitutions were well tolerated at this position.

Substitution of F4 by all possible amino acids revealed that the aromatic amino acid preceding the polyproline stretch was an important determinant in mediating strong binding between VASP and Mena and the respective peptide. Only three other amino acids were tolerated at this position. Surprisingly, a significant increase in binding was observed when F4 was substituted by tryptophan, whereas substitution by tyrosine was tolerated but less effective. Peptides containing a leucine at this position were poor targets for VASP binding, whereas Mena accepted them equally well as a ligand.

The importance of the aromatic residue was confirmed by competition assays. We investigated the ability of peptides comprising either the wild-type motif FPPPPT or its variations APPPPT and WPPPPT embedded in the context of the second ActA repeat sequence (SFEFPPPPT-DEELRL) to compete with full-length ActA in a solid phase binding assay using VASP as ligand (Figure 4C). The affinity of the peptide harboring the WPPPPT motif was clearly higher than that of the wild-type motif, as half maximal inhibition of VASP binding was observed with 2 μM WPPPPT peptide, versus 15.5 μM FPPPPT peptide, while the APPPPT peptide showed a weak effect only at very high peptide concentrations (250 and 500 μM). To investigate the significance of these results in the context of the living cell, we microinjected HeLa cells with the FPPPPT and APPPPT peptides. As described previously, microinjection of the wild-type peptide depleted VASP and Mena from its normal location, the focal adhesions (Pistor *et al.*, 1995; Gertler *et al.*, 1996; compare Figure 4A and A′), whereas microinjection of

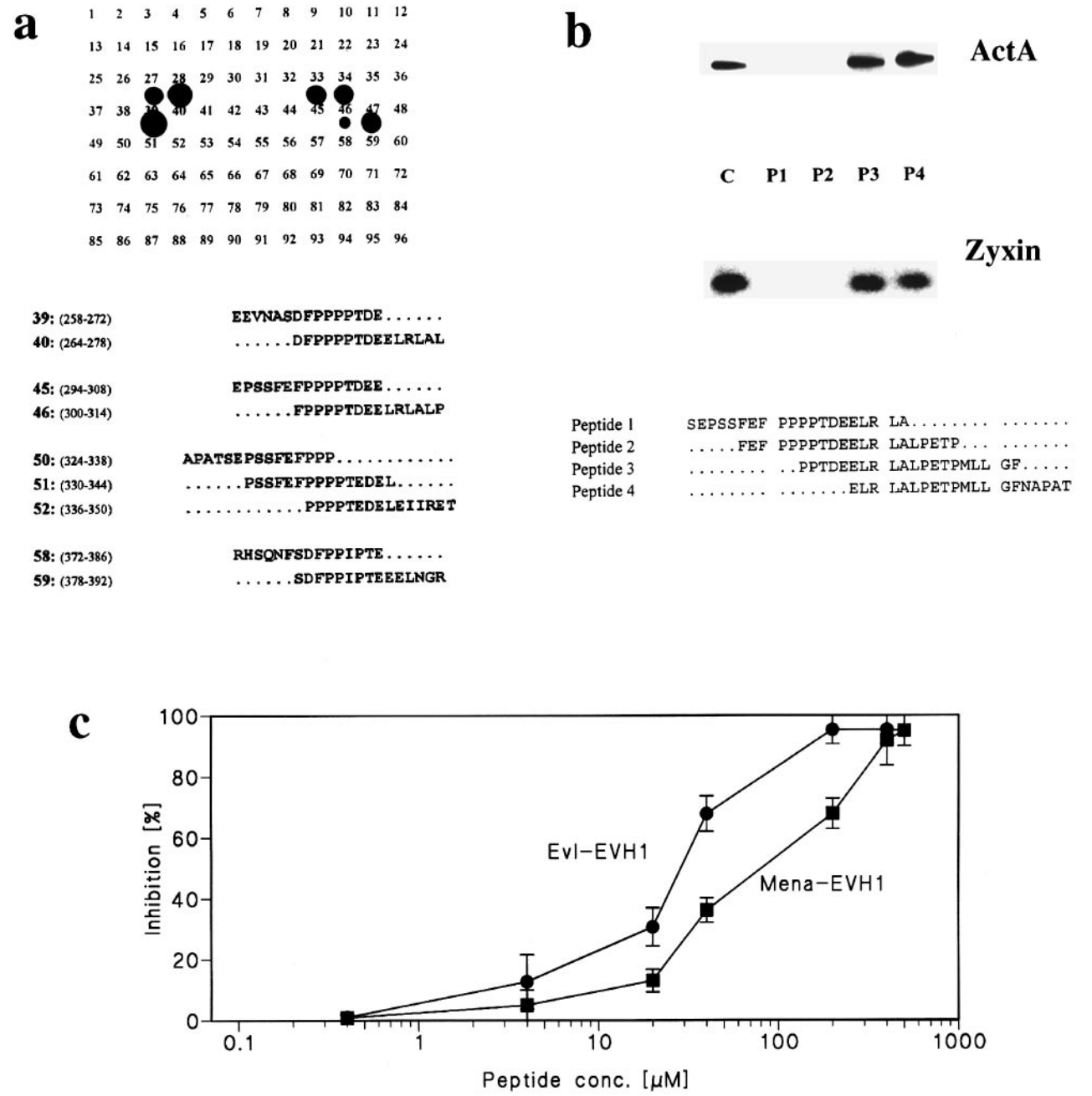


Fig. 1. VASP and other proteins containing the EVH1 domain bind to the proline-rich repeat motif of ActA. (**a**) Delineation of the VASP binding site in the ActA sequence. Overlapping peptides corresponding to the amino acid sequence of ActA of *L.monocytogenes* were synthesized on a derivatized cellulose membrane. Each peptide comprised 15 amino acids with sequences overlapping for nine amino acids starting with amino acids 30–44 of ActA (i.e. without signal sequence) at position 1 of the scan and ending with amino acids 600–614 (i.e. without membrane anchor) at position 96. The peptide sheet was incubated with ^{32}P-labeled VASP and exposed to autoradiography. The peptide sequences of relevant spots are given below. (**b**) Competition of VASP binding to ActA and to zyxin by soluble overlapping ActA-derived peptides. ^{32}P-labeled VASP (0.1 μg/ml) was incubated with the peptides P1, P2, P3 and P4 (750 μM) for 2 h at 30°C before performing an overlay on cell wall extracts of *L.monocytogenes* and whole lysates of porcine platelets. Non-preincubated VASP served as a control (lane C). (**c**) The EVH1 domains of Evl and Mena bind to the proline-rich motif within ActA. ^{32}P-labeled GST fusion proteins comprising the EVH1 domains of Evl (closed circles, amino acids 1–153) and Mena (closed boxes, amino acids 6–170) were preincubated with a range of concentrations of the peptide SFEFPPPPTDEELRL and binding to ActA was tested in a solid phase binding assay.

the APPPPT peptide had no effect on the localization of VASP (Figure 4B and B′) and Mena (data not shown).

Identification of host cell proteins harboring the EVH1 binding motif

Since ActA induces the formation of an actin-based cytoskeleton, it might mimic host cell proteins involved in the initiation and maintenance of dynamic changes in the actin-based cytoskeleton, processes in which VASP and Mena are also implicated. We used affinity-purified polyclonal anti-ActA antibodies to search for such host cell analogs. Human skin fibroblasts or HeLa cells labeled with anti-ActA antibodies and examined by immunofluoresence microscopy showed intense staining of the

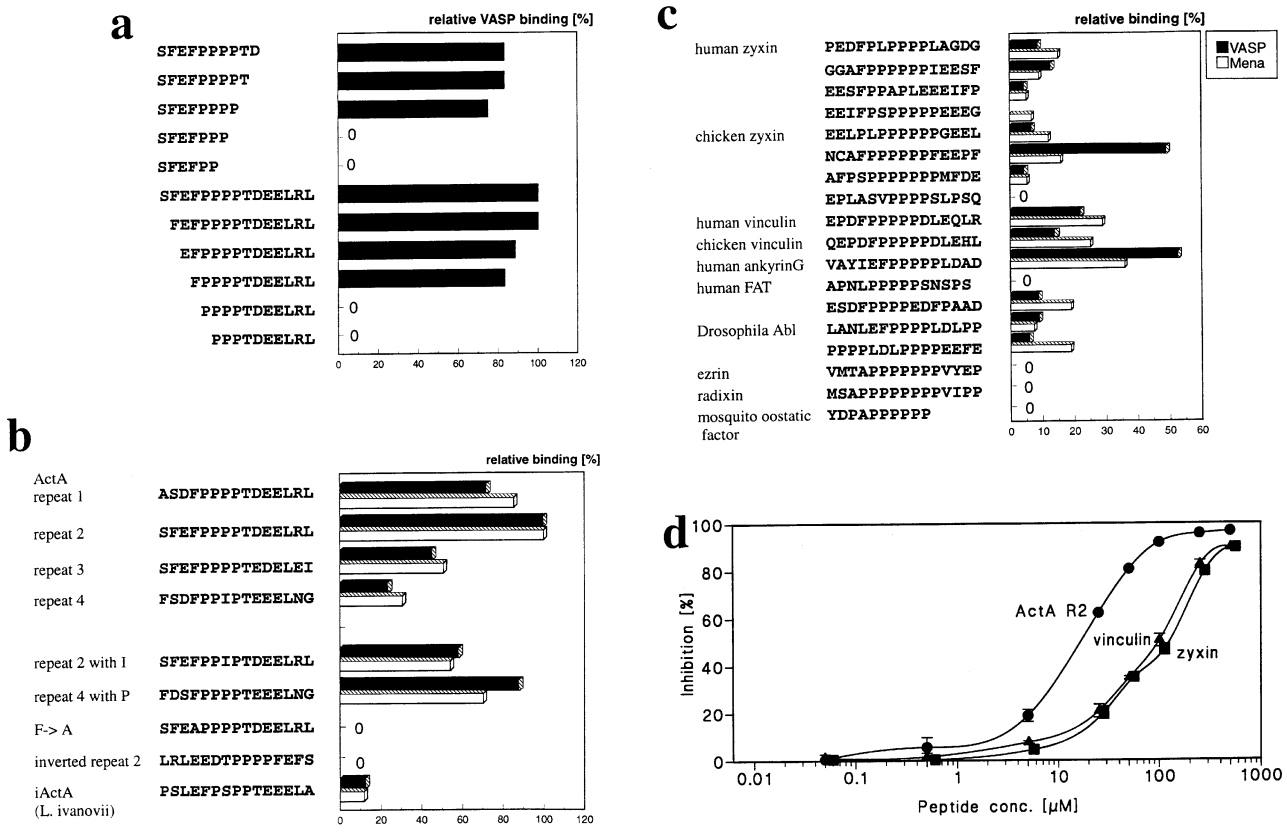


Fig. 2. Characterization of the EVH1 binding motif and its identification in eukaryotic proteins. (**a**) Definition of the core binding sequence using ^{32}P-labeled VASP and immobilized peptides representing truncated forms of the second repeat of ActA. (**b**) Comparison of the relative binding affinities of ^{35}S-labeled VASP (solid bars) and Mena (open bars) to peptides representing the four ActA repeats, several variations thereof and the iActA repeat motif of *L.ivanovii*. (**c**) Binding of radiolabeled VASP and Mena to immobilized peptides representing proline-rich sequences derived from cytoskeletal proteins. The signal obtained with the second ActA repeat in (a) and (b) was taken as 100% relative binding. (**d**) Comparision of the ligand affinity of soluble peptides derived from the EVH1 binding motifs of ActA, vinculin and zyxin. Different concentrations of peptides derived from ActA (SFEFPPPPTDEELRL, closed circles), human vinculin (EPDFPPPPPDLEQLR, closed triangles) and human zyxin (GGAFPPPPPPIEESF, closed boxes) were used for competition of binding of ^{32}P-labeled VASP (0.1 μg/ml) to immobilized ActA in a solid phase binding assay.

focal adhesion complexes (Figure 5A). A similar staining pattern was observed with affinity-purified polyclonal antibodies raised against a peptide comprising the EVH1 binding sequence (SFEFPPPPTDEEL) of ActA (Figure 5C), which co-localized with VASP (Figure 5D), and Mena (not shown). Immunoblot analysis performed with these antibodies on total lysates of human epithelial cells showed a distinct cross-reaction with a protein of 83 kDa (Figure 5a, lane 1). Longer exposure of these blots revealed additional distinct reactions, predominantly with proteins of 42 and 116 kDa, while very faint signals were also seen with polypeptides of ~35, 55 and 75 kDa (Figure 5a, lane 2). Preincubation of these antibodies with the corresponding peptide (see above) completely abrogated these reactions in immunofluorescence and Western blots (data not shown). Comparative immunoblots performed in parallel using antibodies raised against zyxin and vinculin, two proteins that contain sequences related to the EVH1 binding motif, showed that the 116 kDa protein co-migrated with vinculin and the 83 kDa protein with zyxin (Figure 5b). The specific cross-reaction of the ActA antibodies was confirmed by testing it on purified zyxin (Figure 5c) and vinculin (data not shown). The third predominant band corresponding to a 42 kDa protein did not show any reaction with the antibodies specific for zyxin and vinculin and is the subject of further investigation.

To analyze the similarity between the VASP–ActA and the VASP–zyxin interactions, we repeated the competition experiment shown in Figure 1b, upper panel, using whole lysates of porcine platelets. It has previously been shown by Reinhard *et al.* (1995b) that in blot overlay assays on cell lysates VASP specifically interacts with a 83 kDa protein, which was identified as mammalian zyxin. The results of the peptide competition were identical to those obtained with the ActA protein; only ActA-derived peptides containing the sequence FPPPPTDEEL inhibited VASP binding to zyxin (see Figure 1b).

A novel, proline-rich protein binding motif in eukaryotic proteins

We used the core motif F/W/Y/LPPPP to screen several databases for its presence in other cytoskeletal proteins. Using this information, immobilized peptides comprising the putative EVH1 binding sites were synthesized and screened for their ability to interact with ^{35}S-labeled *in vitro* translated VASP and Mena (Figure 2c). As expected, VASP and Mena binding to peptide motifs derived from vinculin and zyxin (Price *et al.*, 1989; Sadler *et al.*, 1992; Macalma *et al.*, 1996) was prominent, but these peptides were bound with lower efficiency compared with the ActA-derived sequence. To draw quantitative

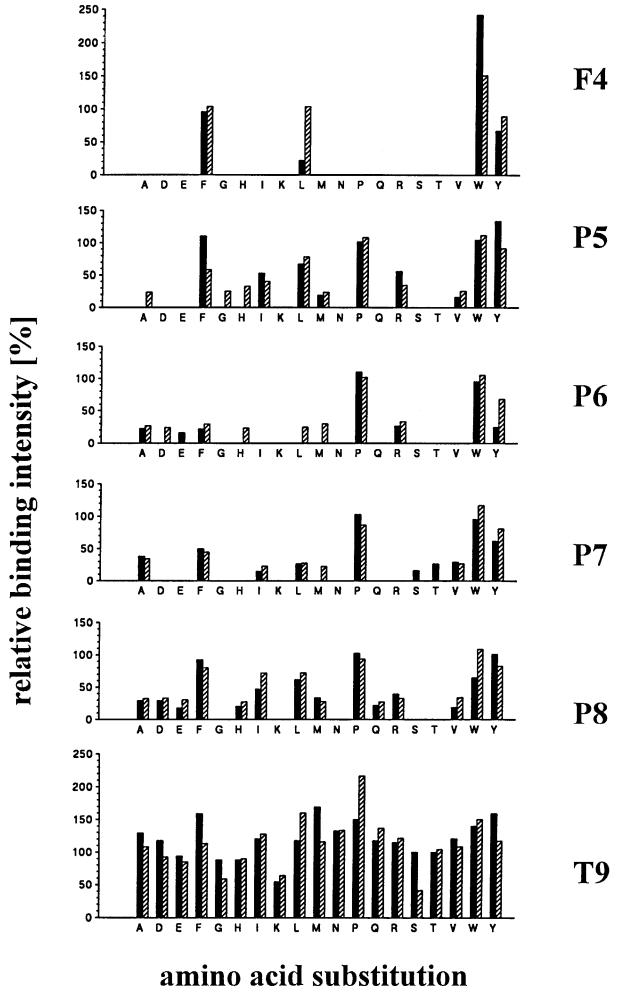


Fig. 3. Characterization of the ligand sequence requirements for EVH1 binding. (**A**) The individual amino acids of the VASP binding core sequence (FPPPPT) in the peptide SFEFPPPPTD were systematically exchanged for all amino acids by synthesizing the corresponding set of immobilized peptides. Cysteine derivatives require an *S*-acetamidomethyl protection group and were not included in the synthesis as they do not represent a naturally occurring amino acid. Relative binding values were determined by scanning the peptide sheets with a phosphorimager and taking the signal obtained with the original motif as 100%. Solid bars represent VASP binding and hatched bars show Mena binding.

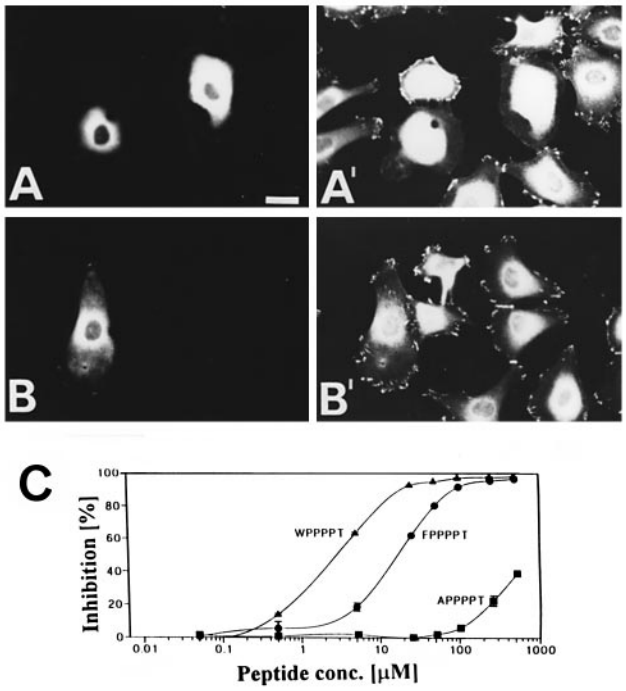


Fig. 4. Importance of the aromatic amino acid preceding the prolines for EVH1 binding. Comparison of the abilities of microinjected peptides FPPPPT and APPPPT in the context of the ActA repeat motif SFEFPPPPTDEELRL to recruit VASP from the focal adhesions of HeLa cells. The peptides were coupled to ovalbumin and coinjected with BSA–rhodamine as a fluorescent tag (**A** and **B**). (**A′** and **B′**) Immunostaining for VASP after microinjection of the peptide corresponding to the ActA repeat (A′) and the peptide with a substitution of the phenylalanine by alanine (B′). The bar in (A) is also valid for panels (A′), (B) and (B′) and represents 17 μm. (**C**) Analysis of the ligand affinity of peptides harboring a replacement of the phenylalanine residue (FPPPPT, closed circles) by tryptophan (WPPPPT, closed triangles) and alanine (APPPPT, closed boxes). ^{32}P-labeled VASP was preincubated with different concentrations of the peptides and its binding to ActA analyzed in a solid phase binding assay.

conclusions about the ligand affinity, we examined VASP binding to ActA in the presence of increasing concentrations of competing peptides derived from ActA, human zyxin and human vinculin (Figure 2d). Indeed, the affinity of VASP for the ActA repeat motif was higher than for the zyxin and vinculin peptides: a concentration of 15.5 μM ActA peptide was sufficient for 50% inhibition of VASP binding, as compared with 80 and 110 μM of the vinculin and zyxin peptides respectively.

In peptide overlays strong signals were also obtained when similar sequences found in human ankyrin$_G$ (Kordeli *et al.*, 1995) and in the human homolog of *Drosophila* FAT (hFAT; Dunne *et al.*, 1995) were probed with VASP and Mena (Figure 2c). Interestingly, two peptides derived from adjacent sequences in the C-terminus of *Drosophila* Abl (Henkemeyer *et al.*, 1988) were ligands for Mena and VASP.

In contrast, no detectable reaction was obtained with peptide sequences derived from ezrin (Gould *et al.*, 1989) and radixin (Wilgenbus *et al.*, 1993) (Figure 2c), several proline-rich peptides present in the yeast proteins verprolin (Donelly *et al.*, 1993) and ABP1 (Drubin *et al.*, 1990), the GPPPPP-rich peptides within VASP itself (Haffner *et al.*, 1995) or other peptides comprising similar motifs, such as the mosquito oostatic factor (YDPAPPPPPP; Borovsky *et al.*, 1990), demonstrating the specificity of this interaction. When the ^{32}P-labeled GST–EVH1 fusion proteins of Mena and Evl were used to probe these peptides, they showed basically the same binding properties as full-length VASP and Mena, but exhibited some differences in the relative binding intensities (data not shown).

Proline-rich sequences have also been identified as ligands for SH3 and WWP/WW domains (Ren *et al.*, 1993; Chen and Sudol, 1995). To test whether EVH1 domain specificity could indeed overlap, we synthesized 20 peptides which have been identified as SH3 ligands by phage display and 45 peptides comprising natural sequences known to interact with SH3 domains and respective consensus motifs (Sparks *et al.*, 1996), as well as five peptides which have been reported to bind to WWP/WW domains (Chen and Sudol, 1995; Chan *et al.*,

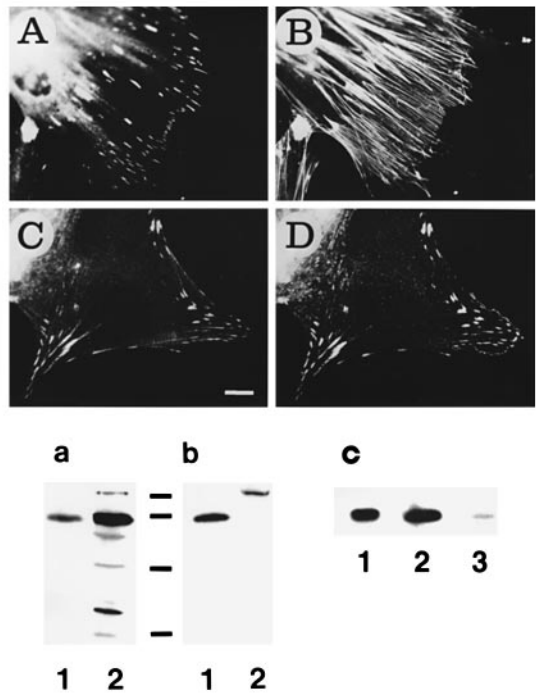


Fig. 5. Cross-reactivity of polyclonal antibodies raised against the ActA protein and a peptide comprising the EVH1 binding motif of ActA with eukaryotic proteins. (Above) Human skin fibroblasts were stained simultaneously with affinity-purified polyclonal antibodies raised against the ActA protein (**A**) and phalloidin (**B**) and with affinity-purified antibodies raised against a peptide comprising the EVH1 binding motif (SFEFPPPPTDEELRL) within the ActA protein (**C**) and monoclonal VASP-specific antibodies (**D**) respectively. Bar represents 5 μm. (Below) Immunoblots with whole lysates of HeLa cells were probed with affinity-purified antibodies raised against a peptide comprising the EVH1 binding motif. The blots were developed by chemiluminescence with a short (**a**, lane 1) and a longer (lane 2) exposure time. In parallel, identical blots were probed with antibodies against zyxin (**b**, lane 1) and vinculin (lane 2). (**c**) Cross-reaction of the ActA-specific antibodies with zyxin conventionally purified (Reinhard *et al.*, 1995b) (lane 1) or immunoprecipitated with specific polyclonal antibodies (lane 2) from human platelet lysates and, as a control, an immunoprecipitation with preimmune serum (lane 3). Bars represent molecular mass standards as follows: 112, 84, 53 and 35 kDa (top to bottom).

1996). When these peptides were probed in a VASP overlay assay, none of these ligands showed significant interaction with VASP (Figure 6).

Chromosomal deletion of the EVH1 binding sites from ActA of L.monocytogenes

To assess the biological significance of the interaction of proteins harboring an EVH1 domain with ActA, we deleted the proline-rich repeat region (amino acids 265–389) from the chromosomal copy of the *act*A gene of *L.monocytogenes*. Bacterial surface extracts and total lysates of HeLa cells infected with the wild-type and the mutant were analyzed in Western blots using ActA-specific antibodies, which showed that the truncated ActA protein was stable and expressed at levels comparable to that of the wild-type (data not shown). Following infection of HeLa cells, the mutant ActAΔR1-R4 polypeptide, like wild-type ActA (Niebuhr *et al.*, 1993; Chakraborty *et al.*, 1995), was uniformly distributed on the intracellularly localized bacteria, as detected by immunofluoresence (Figure 7B). In contrast to the wild-type *Listeria*, no VASP (compare Figure 7E and H) and Mena staining could be detected on the surface of the mutant bacteria. However, they were still capable of accumulating actin, albeit at greatly reduced levels. At later time points following infection short actin stubs, unlike the actin comet tails seen with wild-type bacteria, were observed at one end of the intracellularly motile bacteria (compare Figure 7A and G with D). These bacteria produced short surface protrusions and were still capable of spreading from cell to cell when examined in a plaque assay, with an ~40% reduction in diameter when compared with the wild-type strain (Figure 7J). Video time lapse microscopy demonstrated that these mutant bacteria were still capable of directed movement, but they were dramatically reduced with respect to their speed within the host cell. The wild-type moved at an average speed of 29.4 μm/min (maximum speed measured 55 μm/min), whereas the mutant only reached an average speed of 5.6 μm/min and a maximum speed of 11.3 μm/min.

To investigate the impact of the ActA–VASP/Mena interaction on the virulence potential of *L.monocytogenes*, mouse infection studies were performed. Bacteria lacking the binding site for proteins from the VASP/Mena family were strongly attenuated for virulence as compared with the wild-type strain. At day 5 all mice infected with EGD succumbed to infection, whereas mice infected with the *actA*ΔR1-R4 mutant survived with lowered bacterial numbers in both liver and spleen (Figure 7K).

Discussion

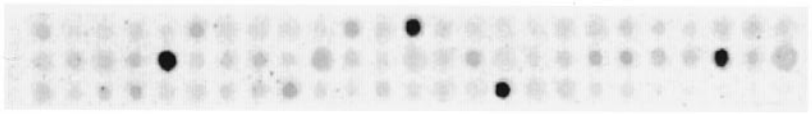
The EVH1 domain engages a novel proline-rich motif in protein–protein interactions

The actin-based motility of intracellular *L.monocytogenes* is a model system to investigate the dynamics of the microfilament system. Recently the finding that VASP and Mena are ligands of the bacterial surface protein ActA and of profilin (Chakraborty *et al.*, 1995; Reinhard *et al.*, 1995a; Gertler *et al.*, 1996) led to the hypothesis that both proteins enable intracellular *Listeria* efficiently to exploit the profilactin pool in the infected cell. In this study we have established that VASP and Mena bind to the peptide motif E/DFPPPPTD/E on ActA via their conserved EVH1 domains.

Currently two well-defined protein modules, the SH3 and the WWP/WW binding domains, are known to mediate binding to proline-rich ligands (Ren *et al.*, 1993; Chen and Sudol, 1995; Chan *et al.*, 1996). Evidence that binding of the EVH1 domain to its ligand represents a novel class of such protein–protein interactions came from the inability of VASP to bind to representatives of SH3 and WWP/WW ligands. Interestingly, all EVH1 domains identified so far are highly homologous and embedded in proteins of similar structure (Gertler *et al.*, 1996), whereas SH3 and WWP/WW domains show low sequence similarities and are separate modules, often present in otherwise unrelated polypeptides.

Three components within the EVH1 binding motif could be discerned: the essential N-terminal aromatic residue, the polyproline core sequence and the flanking acidic residues. The aromatic residue preceding the polyproline sequences is the most characteristic feature of the EVH1 binding motif and clearly distinguishes it from those of

a



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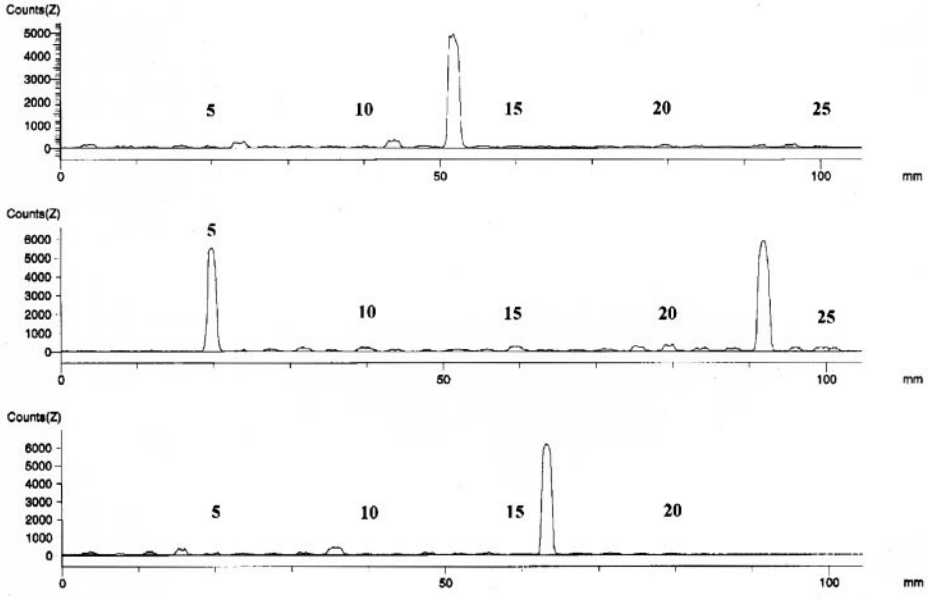

c

1	Abl	PPWWAPPPIPNSPQVL	Src	GPPPQVPSRPN	Grb2 N	WLPRPIPKVPVS
		PTYPPPPPPDTAKGA		RAPPGVPSRSG		LQAPELPTKTR
	Cortactin	LTPQSKPPLPPKPSAV		PLPPPPPPRGG		AVSPLLPRKER
		LGEFSKPPIPQKPTWM		APPPPPVPRGR		KTAPTPPKRSS
5	Grb2 N	KWDSLLPALPPAFTVE	**ActA**	**SFEFPPPPTDEELRL**		ASLPPVPPRLD
		YWDMPLPRLPGEEPSL		AVPPPVPPPPS		GGAPPVPSRPG
		YYQRPLPPLPLSHFES		QPAPALPPKPP		GPPPQVPSRPN
		YFSRALPGLPERQEAH		GGPPPGPGRRG		RAPPGVPSRSG
		SLWDPLPPIPQSKTSV		TKSPPQPPRPD		PVPPPVPPRRR
10		DPYDALPETPSMKASQ	Yes	PVKQPPPLAPQS		DSPPAIPPRQP
	p53bp2	SSAPQRPPLPVRKSRP	Abl	RAPTMPPPLPPV		ESPPLLPPREP
		VVPLGRPEIPLRKSLP		YPPAYPPPPVPV		IAGPPVPPRQS
	ActA	**SFEFPPPPTDEELRL**		PGPGYGPPPVPP		NLPEPAPPRPS
	Plcγ	MPPPVPPRPPGTLQVA	Plcγ	APPVPSRPGASP		PPGPAGPIRQA
15		VPPLVAPRPPSTLNSL		PPQVPSRPNRNR	WBP-1	HHPGTPPPPYTVGPG
	Crk N	GQPAGDPDPPPLPAKF		LPPVPPRLDLLP	**ActA**	**SFEFPPPPTDEELRL**
		ATSEGLPILPSKVGSY	Crk N	QAPELPTKTR	WBP-2	MYVQPPPPPYPGPME
	Src	PPQMPLPEIPQQ		VSPLLPRKER		MPTSQPPPPYYPPED
		PPDNGPPPLPTS		EKPALPRKRA	WWP/SH3	APPTPPPLPP
20		TAPKPMPPRPPL		PPPALPPKKR		APPAPPPLPP
		SRARNLPLPPPP		TPPALPEKKR		
		TVTRGVPPPPTV		KPPPLPEKKN		
		RPPRPLPVAPGS	**ActA**	**SFEFPPPPTDEELRL**		
		VRKQMLPPPPCP		PPPALPPKQR		
25		GGAPPVPSRPG	Grb2 N	PQRRPLPCTPGD		

Fig. 6. VASP binding to SH3 and WW/WWP ligands. Peptides corresponding to published SH3 and WW/WWP ligands and, as a control, the ActA-derived EVH1 binding motif were synthesized on cellulose paper, incubated with ^{35}S-labeled VASP and analyzed with a phosphorimager (**a**). The intensity of the signals is shown (**b**). The amino acid sequences of the single peptides are given (**c**); the ActA-derived control sequences are in bold. Positions 1–17 in lane 1 represent SH3 ligands identified previously in phage display libraries (two representatives of each consensus motif; Sparks *et al.*, 1996). No reaction was obtained with Src ligands LASRPLPLLPNSAPGQ and ISQRALPPLPLMSDPA and Yes ligands ITMRPLPALPGHGQIH and RSGRPLPPIPGVGHNV (not shown). Positions 18 in lanes 1 to 13 in lane 3 comprise peptide sequences derived from SH3 binding proteins (Sparks *et al.*, 1996). Putative WW domain ligands are represented in lane 3 (positions 14, 16 and 17; Chen and Sudol, 1995) and in addition a motif found to be a ligand for WW/WWP as well as SH3 domains (positions 18 and 19; Chan *et al.*, 1996).

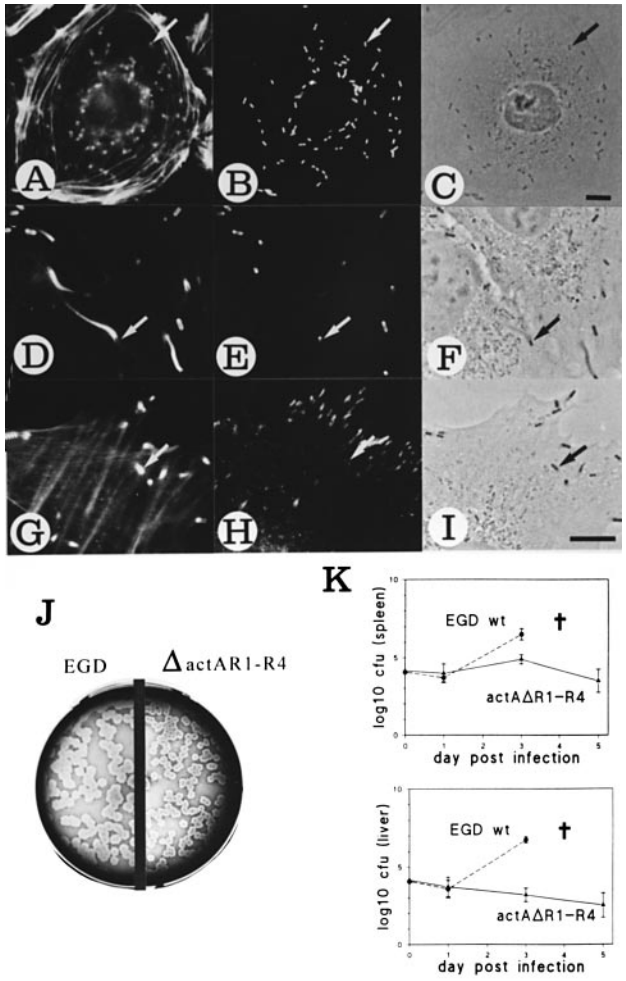

Fig. 7. Characterization of a *L.monocytogenes* mutant lacking the EVH1 binding sequence within the ActA polypeptide. HeLa cells were infected with wild-type *L.monocytogenes* EGD (D–F) and with the mutant EGDΔR1-R4 (A–C and G–I) for 4 h. After fixation the cells were stained for immunofluorescence with ActA-specific polyclonal antibodies (**B**), a VASP-specific monoclonal antibody (**E** and **H**) and with FITC–phalloidin (**A**, **D** and **G**) to visualize bacterially induced actin polymerization. The corresponding phase contrast micrographs are shown in (**C**), (**F**) and (**I**). Note that due to the strong enrichment of VASP at the bacterial surface in (E) the exposure time was much shorter than that of (H), resulting in the loss of signals derived from VASP in its normal localizations, e.g. focal adhesion complexes. Bars represent 10 μm. (**J**) A comparison of the plaque size formed by the wild-type (left) and the mutant (right). The results of the mouse infection studies are shown in (**K**).

WWP/WW and most SH3 domains characterized so far. Replacement analysis revealed that the phenylalanine is more than a mere non-polar elongation of the polyproline core. Indeed, it confers both specificity and polarity to the binding motif. This was confirmed *in vivo* by microinjection experiments: whereas a peptide harboring the EVH1 binding motif (FPPPP) efficiently depleted VASP and Mena from their normal locations within the cell (Pistor *et al.*, 1995; Gertler *et al.*, 1996), an APPPP-containing peptide failed to do so. A Phe→Trp replacement significantly increased the efficiency of binding of VASP and Mena to such peptides. However, database searches did not identify proteins harboring such high-affinity EVH1 binding motifs, possibly reflecting the necessity for weaker protein–protein interactions that permit a regulated equilibrium of association and dissociation of the partner molecules.

Interestingly, peptides harboring a Phe→Leu substitution were strongly bound by Mena, whereas the binding of VASP and the EVH1 domain of Evl was dramatically reduced. These findings indicate that VASP, Evl and Mena have overlapping, but slightly different binding specificities for their ligands, suggesting interactions with common and distinct partner molecules within the eukaryotic cell. Similar overlaps and differences in the binding specificities have also been observed with different SH3 and WWP/WW domains (Rickles *et al.*, 1994; Chan *et al.*, 1996; Sparks *et al.*, 1996), thus enabling a finely tuned system of protein–protein interactions within the cell.

Efficient intracellular motility and virulence of L.monocytogenes require interaction with EVH1-containing molecules

Since ActA appears to be a highly optimized ligand for VASP and Mena, we expected that the biological consequences of this interaction could be best discerned by abrogating EVH1-mediated binding and monitoring its effect on intracellular bacterial motility. Southwick and Purich (1994) observed that microinjection of a peptide covering the proline-rich sequence of ActA resulted in arrest of *Listeria* movement within infected host cells. We found that removal of the EVH1 binding sites on ActA reduced the bacterial speed within infected PtK_2 cells to <20% of that of the wild-type and compromised cell-to-cell spread in a plaque assay. Hence, while the direct interaction of *Listeria* with EVH1 domain-containing proteins is not an absolute prerequisite for movement, the results suggest that VASP, Mena and their relatives act as efficient accelerators of actin filament assembly, probably by delivering profilactin to sites of high actin filament dynamics. A similar experimental approach has also been taken by other groups. Lasa *et al.* (1995) complemented an *actA* deletion mutant with a plasmid harboring an *actA* derivative lacking the proline-rich region and measured a significantly lower speed in *Xenopus laevis* oocyte extracts. In a recent study Smith *et al.* (1996) compared a variety of *Listeria* mutants and found a relationship between the number of repeats and the rate of intracellular movement. Also, replacement of the FPPPP motif by FGGGG reduced the bacterial speed to 30% of the wild-type and was indistinguishable from an ActA mutant lacking all EVH1 binding sites.

Though the process of intracellular movement itself was independent of the direct interaction of *Listeria* with EVH1 domain-containing proteins, the biological significance of the bacterial acceleration mediated by VASP, Mena and related proteins became obvious when the *actA*ΔR1-R4 mutant was tested in mouse infection studies. The mutant was strongly compromised with respect to its virulence potential, indicating that the efficiency in movement and spread within the infected host is critical in determining the outcome of the infection.

Apart from *L.monocytogenes* and *L.ivanovii*, we have previously demonstrated that another unrelated intracellular pathogen, *Shigella flexneri*, also recruits VASP to its actin comet tails (Chakraborty *et al.*, 1995). Recently it has been shown that the IcsA nucleator protein of *S.flexneri* binds directly to the head portion of vinculin (Suzuki

et al., 1996). The finding that EVH1-containing proteins bind to a proline-rich motif present in vinculin now provides a molecular basis for the association of VASP with *S.flexneri*-induced actin tails. Thus EVH1 domain interactions may have been specifically usurped by intracellular pathogens because of their important role in the generation and maintenance of dynamic actin structures. We postulate that other pathogens that interact with host cell microfilament structures, such as *Rickettsia*, enteropathogenic *Escherichia coli* or vaccinia virus, also recruit EVH1 domain-containing proteins for the formation of the actin-based structures that they induce (Knutton *et al.*, 1989; Theysseire *et al.*, 1992; Cudmore *et al.*, 1995).

Evidence for molecular mimicry by a pathogenic bacterium: zyxin and vinculin harbor similar EVH1 binding sequences

Antibodies specific for the EVH1 binding site intensely stained the focal adhesion complexes of human epithelial cells, suggesting that host cell ActA analogs, which represent the 'natural' ligands for VASP and Mena, are enriched at this location. The major cross-reacting protein was identified as zyxin, a focal adhesion constituent which was previously identified as a ligand for VASP and Mena *in vitro* (Reinhard *et al.*, 1995b; Gertler *et al.*, 1996). The similarity of ActA and zyxin with respect to VASP binding was further corroborated by the finding that peptides derived from the ActA and zyxin EVH1 binding sequences could inhibit VASP binding to the respective other protein. Cross-reactivity was also observed with vinculin and very recent findings showed that purified VASP and Mena indeed interact directly with vinculin *in vitro* (Brindle *et al.*, 1996; Gertler *et al.*, 1996; Reinhard *et al.*, 1996). However, vinculin and zyxin differed with respect to their interaction with EVH1-containing proteins: zyxin was bound avidly in blot overlays and solution binding assays, whereas vinculin was less efficiently bound (Reinhard *et al.*, 1995b, 1996; Gertler *et al.*, 1996). Like ActA, zyxin harbors four EVH1 binding motifs, which may increase its ability to interact with the multimeric VASP and Mena complexes. In the vinculin molecule only one copy of this proline-rich sequence is found in the hinge region (Weller *et al.*, 1990), which can potentially be masked by intramolecular head-to-tail binding (Johnson and Craig, 1994, 1995; Kroemker *et al.*, 1994; Menkel *et al.*, 1994). Thus, the interaction of vinculin with EVH1-containing proteins may be conformationally dependent and could in turn influence its ability to interact with other cytoskeletal proteins. If this were the case, zyxin would be an example of a constitutive EVH1 ligand, whereas vinculin represents a conformationally regulated ligand.

The sequence similarities shared between zyxin, vinculin and ActA are restricted to the proline-rich motifs. ActA also harbors a second functional region in the N-terminus that is essential for actin filament nucleation (Friederich *et al.*, 1995; Lasa *et al.*, 1995; Pistor *et al.*, 1995). Significantly, this element harbors the motif KKRRK which is also conserved in iActA of *L.ivanovii* (Gerstel *et al.*, 1996), which otherwise shares only 30% overall sequence homology. The absence of similar sequences in zyxin or vinculin suggests that in ActA the combination of both domains in one molecule generates the basis for an efficient actin nucleation complex. In the eukaryotic cell these domains appear to be located on different molecules, which have to be spatially combined to allow a fine tuned regulation of this process. It will be a challenge to identify these cellular actin nucleators and to elucidate the mechanisms by which they cooperate with members of the Ena/VASP family.

Sequence databases were searched for proteins that harbor polyproline motifs similar to those found in ActA, zyxin and vinculin and a peptide corresponding to a sequence found in human ankyrin$_G$ was bound avidly by VASP and Mena. Among the ankyrins, the EVH1 binding sequence is unique to ankyrin$_G$, which is primarily found in cells of neuronal origin (Kordeli *et al.*, 1995), a cell type where novel developmentally regulated forms of Mena have also been detected (Gertler *et al.*, 1996). Another protein harboring an EVH1 recognition sequence is human FAT, a new member of the human cadherin superfamily (Dunne *et al.*, 1995). Its cytoplasmic sequence contains two proline-rich motifs, one of which is a ligand of VASP and Mena. Further studies with cells from different tissues will reveal whether ankyrin$_G$, FAT and other yet unknown proteins are natural ligands of EVH1-containing proteins.

The role of the EVH1 domain in proteins of the Ena/VASP family

A conspicuous site of VASP and Mena localization in the cell is focal adhesion complexes. However, both proteins can be redistributed within the cell if the EVH1 binding sequence is provided ectopically, as for example on intracellular *Listeria* or ActA targeted to mitochondria and even on microinjected soluble peptides. In all these experiments the focal adhesion complexes appeared intact, as judged by staining for phosphotyrosine and for vinculin and zyxin (Gertler *et al.*, 1996; unpublished results), indicating that VASP and Mena are dynamic and peripheral constituents of these structures. These findings imply that the highly specific interaction between EVH1 domains and their ligand motifs primarily serves to mediate the proper localization of proteins from the Ena/VASP family and to link them to other molecules with which they cooperate.

Apart from the focal adhesions, VASP and Mena are found in highly dynamic structures of cells. Given the structural similarities between the actin filament organization at the leading edge of lamellipodia and in the tails of *Listeria*, it is tempting to speculate that VASP and Mena are also recruited to these areas of rapid actin remodeling by the EVH1-mediated interaction to act as accelerators of the actin polymerization process. VASP is a substrate for cAMP- and cGMP-dependent kinases (Halbrügge and Walter, 1989) and Mena has been shown to be serine/threonine- and tyrosine-phosphorylated and to bind to the SH3 domains of the Abl and Src kinases (Gertler *et al.*, 1996). Perhaps EVH1-mediated interactions are regulated by the state of phosphorylation, thereby linking signal transduction pathways to processes requiring rapid actin polymerization.

In summary, our attempts to elucidate host cell components required for the generation of an actin-based cytoskeleton by a bacterial nucleator protein have led to the discovery of a novel proline-rich motif and the demonstration of its interaction with the EVH1 domain

of the Ena/VASP protein family. We propose that the ActA protein engages in molecular mimicry, having incorporated into its primary sequence a motif that is present in a number of cytoskeletal proteins. The major host cell analogs of ActA with respect to VASP binding known so far are zyxin and vinculin, two microfilament proteins with multiple sites for binding other cytoskeletal proteins. Given that VASP and Mena share no sequence homology with SH3 and WWP/WW domains, we propose that ActA–VASP/Mena binding represents a new class of eukaryotic protein–protein interaction involving a novel proline-rich ligand and the EVH1 domain. Elucidation of the mechanisms controlling these interactions will significantly contribute to our understanding of the regulation of the dynamic actin-based cytoskeleton.

Materials and methods

Peptide synthesis

Spot synthesis was performed according to Frank (1992) with an Abimed ASP 222 Automated SPOT Robot. Free, soluble peptides were synthesized with an AMS 222 Multiple Peptide Synthesizer using TentaGel S resin (Rapp Polymere, Germany) and purified by HPLC.

Radioactive protein labeling

Porcine platelet VASP was purified and phosphorylated as described by Reinhard *et al.* (1995b). ^{35}S-Labeled full-length VASP and Mena (80 kDa form) were produced *in vitro* using the TNT™ coupled transcription/translation system (Promega). The GST fusion protein comprising the EVH1 domain of Mena (amino acids 6–170) has been described previously (Gertler *et al.*, 1996). A fragment encoding amino acids 1–153 of Evl was generated by PCR and the EVH1 domains were cloned into the pGEX-2TK vector (Pharmacia) harboring a phosphorylation site. The fusion proteins were phosphorylated following the instructions of the vendor.

Protein binding assays

The overlay and solid phase binding assays used to detect binding of VASP and Mena to proteins or peptides were performed as previously described (Chakraborty *et al.*, 1995; Reinhard *et al.*, 1995b). Dried membranes were exposed to autoradiographic films overnight at –70°C or quantified using a phosphorimager (Molecular Dynamics). Western blots were performed using horseradish peroxidase-coupled secondary antibodies and a chemoluminescence detection kit (Amersham). For the peptide competition experiments aliquots of ^{32}P-labeled VASP (0.1 μg/ml in blocking medium) or the fusion proteins (5 μg/ml) were preincubated with synthetic peptides for 2 h at 30°C before testing on Removawells (Dynatech) coated with 0.25 μg purified ActA (Niebuhr *et al.*, 1993) or in blot overlays.

Cell culture and immunofluorescence microscopy

PtK_2 (ATCC CCL 56), HeLa cells (ATCC CCL 2.2), L929 fibroblasts (ATCC CCL 1) and human skin fibroblasts (Reinhard *et al.*, 1992) were grown in MEM or DMEM (Gibco) supplemented with 10% fetal calf serum, glutamine and non-essential amino acids in the absence of antibiotics. Microinjection and immunofluorescence experiments were performed as described previously (Pistor *et al.*, 1995; Gertler *et al.*, 1996). The VASP-specific monoclonal antibody used in this study was raised against purified human VASP (K.Reinhard, A.Lingnau, K.Niebuhr, J.Wehland and U.Walter, unpublished results), the vinculin-specific antibody was purchased from Sigma and the polyclonal ActA- and zyxin-specific antibodies have been described previously (Niebuhr *et al.*, 1993; Reinhard *et al.*, 1995b). The peptide–antibodies against the ActA repeat motif (SFEFPPPPTDEELRL) coupled to ovalbumin were raised according to a protocol described previously (Domann *et al.*, 1992) and affinity purified with the respective peptide coupled to Sepharose.

Infection of cultured cells with L.monocytogenes, and video microscopy

Listeria monocytogenes serotype 1/2a EGD and the *actA*ΔR1-R4 mutant (see below) were grown in brain/heart infusion (BHI) broth (Difco) at 37°C with aeration. The infection experiments were performed as described previously (Niebuhr *et al.*, 1993). The coverslips were either observed with a temperature controlled Zeiss inverted microscope equipped with a video camera (electronic SN76; Grundig), contrast enhancement (ACE Micro Systems, Zeiss) and a time lapse video recorder (HS-S5600 E; Mitsubishi) or processed for immunofluorescence. The plaque assay using L929 fibroblasts was performed according to standard protocols.

Construction of the chromosomal deletion mutant actAΔR1-R4

A PCR employing specific primers was used to introduce an in-frame deletion mutant in the *actA* gene, lacking the four proline-rich repeats. The oligonucleotide pair A (5′-CGGAACAAATTAGTGAAAATGAA-GGCCG-3′) and B (5′-GTCCGAAGCATTTACCTCTTACTTTTC-3′) was used to amplify a 873 bp DNA fragment [A, positions 120–147 and B, positions 993–966 of the published sequence (Domann *et al.*, 1992; Kocks *et al.*, 1992)] in the 5′-region of *actA* encoding the first 264 N-terminal amino acid residues. The oligonucleotide pair C (5′-AACGGG-AGAGGCGGTAGACCAACATC-3′) and D (5′-TTGGCG- TGCATA-GGTTGAC-3′) served to amplify a 1331 bp DNA fragment in the 3′-region encoding the last 250 C-terminal amino acids of ActA (C, positions 1369–1394 and D, positions 2699–2681). The two PCR products were used in a ligation reaction. The ligation product harboring the deletion was selectively amplified with oligonucleotides A and D. The resulting PCR product was cloned into the TA cloning vector pCRII (Invitrogen), generating plasmid pCR-*act*AΔR1-R4. The plasmid pCR-*act*AΔR1-R4 was digested with the restriction endonucleases *Hin*dIII and *Xba*I and the resulting DNA fragment, harboring the deletion in the *actA* gene, was cloned into the suicide vector pAUL-A. The resulting plasmid pAUL-*act*ΔAR1-R4 was transformed into *L.monocytogenes* via protoplast transformation and mutants were isolated as described previously (Chakraborty *et al.*, 1995).

Mouse infection studies

NMRI mice were infected i.p. with 10^4 colony forming units (c.f.u.) of *L.monocytogenes* EGD wild-type and the isogenic mutant *act*AΔR1-R4 respectively. Five mice per control group were killed at days 1, 3 and 5 post-infection and c.f.u. in spleen and liver were determined by homogenizing the organs and plating appropriate dilutions of the samples on BHI plates.

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