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# Aromatic and basic residues within the EVH1 domain of VASP specify its interaction with proline-rich ligands

Uwe D. Carl\*, Marc Pollmann\*, Elisha Orr†, Frank B. Gertler‡, Trinad Chakraborty§ and Juergen Wehland\*

Short contiguous peptides harboring proline-rich motifs are frequently involved in protein–protein interactions, such as associations with Src homology 3 (SH3) and WW domains. Although patches of aromatic residues present in either domain interact with polyprolines, their overall structures are distinct, suggesting that additional protein families exist that use stacked aromatic amino acids (AA domains) to bind polyproline motifs [1–3]. A polyproline motif (E/DFPPPTD/E in the single-letter amino-acid code), present in the ActA protein of the intracellular bacterial pathogen *Listeria monocytogenes*, serves as a ligand for the Ena/VASP protein family – the vasodilator-stimulated phosphoprotein (VASP), the murine protein Mena, *Drosophila* Enabled (Ena) and the Ena/VASP-like protein Evi [4–7]. These share a similar overall structure characterized by the two highly conserved Ena/VASP homology domains (EVH1 and EVH2) [5]. Here, using three independent assays, we have delineated the minimal EVH1 domain. Mutations of aromatic and basic residues within two conserved hydrophilic regions of the EVH1 domain abolished binding to ActA. Binding of an EVH1 mutant with reversed charges could partially be rescued by introducing complementary mutations within the ligand. Like SH3 domains, aromatic residues within the EVH1 domain interacted with polyprolines, whereas the ligand specificity of either domain was determined by reciprocally charged residues. The EVH1 domain is therefore a new addition to the AA domain superfamily, which includes SH3 and WW domains.

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## Results and discussion

Using the yeast two-hybrid system, sequentially truncated carboxy-terminal and amino-terminal derivatives of VASP, Mena and Evi fused to the transcriptional activation domain of GAL4 (AD) were assessed for their ability to bind to different ActA derivatives fused to the GAL4 DNA-binding domain (BD). All fusion proteins that were truncated at the carboxyl terminus were able to bind ActA except those that were shorter than the predicted EVH1 domain (Table 1). Two VASP–AD fusion proteins that were truncated at the amino terminus, VASP<sub>56–380</sub> and VASP<sub>106–380</sub>, failed to bind to ActA, confirming the requirement of the amino-terminal domain of VASP (Table 1).

To corroborate these results, we transfected PtK<sub>2</sub> cells with plasmids expressing VASP, Mena or Evi derivatives fused

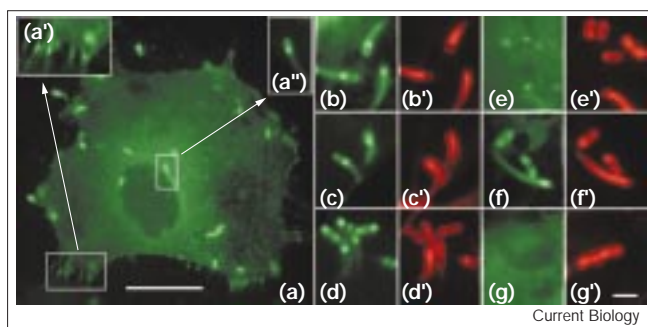
Table 1

Interactions of truncated derivatives of Mena, Evi and VASP with each other and ActA.

	ActA <sub>7–422</sub> –BD	ActA <sub>7–263</sub> –BD	VASP–BD	VASP <sub>227–380</sub> –BD	Mena <sub>355–541</sub> –BD	Evi <sub>210–393</sub> –BD
VASP–AD	+	–	+	+	+	+
VASP <sub>1–304</sub> –AD	+	–	–	–	n.d.	n.d.
VASP <sub>1–192</sub> –AD	+	–	–	–	n.d.	n.d.
VASP <sub>1–149</sub> –AD	+	–	–	–	n.d.	n.d.
VASP <sub>1–116</sub> –AD	+	–	–	–	–	–
VASP <sub>1–98</sub> –AD	–	–	–	–	n.d.	n.d.
VASP <sub>1–79</sub> –AD	–	–	–	–	n.d.	n.d.
VASP <sub>56–380</sub> –AD	–	–	+	+	+	+
VASP <sub>106–380</sub> –AD	–	–	+	+	+	+
Mena–AD	+	–	+	+	+	+
Mena <sub>1–196</sub> –AD	+	–	–	–	n.d.	n.d.
Mena <sub>1–154</sub> –AD	+	–	–	–	n.d.	n.d.
Mena <sub>1–114</sub> –AD	+	–	–	–	n.d.	n.d.
Mena <sub>1–96</sub> –AD	–	–	–	–	n.d.	n.d.
Evi–AD	+	–	+	+	+	+
Evi <sub>1–148</sub> –AD	+	–	–	–	n.d.	n.d.
Evi <sub>1–117</sub> –AD	+	–	–	–	n.d.	n.d.
Evi <sub>1–96</sub> –AD	–	–	–	–	n.d.	n.d.

Data were determined using the yeast two-hybrid system. See Supplementary material for diagrams of the truncated proteins and the yeast two-hybrid data. The numbers indicate amino-acid positions. ActA<sub>7–263</sub>–BD, which lacks the polyproline motifs, served as a control. +, an interaction; –, no interaction; n.d., not determined.

Figure 1

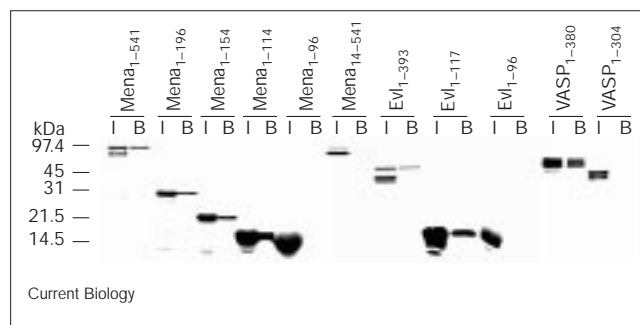


Fluorescence micrographs of PtK<sub>2</sub> cells transiently transfected with constructs expressing various VASP, Mena and Evl derivatives fused to GFP. Cells were transfected with the following constructs: (a) full-length GFP-VASP; (b,b') GFP-VASP<sub>1-116</sub>; (c,c') GFP-Mena<sub>1-114</sub>; (d,d') GFP-Evl<sub>1-117</sub>; (e,e') GFP-VASP<sub>1-98</sub>; (f,f') GFP-VASP<sub>14-380</sub>; and (g,g') GFP-VASP<sub>14-304</sub>, and subsequently infected with *L. monocytogenes* EGD. (a–g) Distribution of the transiently expressed GFP fusion proteins; (b'–g') distribution of actin, shown by phalloidin staining of the cells in (b–g). (a) In infected cells, full-length GFP-VASP<sub>1-380</sub> was concentrated in focal contacts (enlarged in inset a') and on the surface of intracellular *L. monocytogenes*. On motile *Listeria*, VASP was concentrated at the interface between the bacterial pole and the actin tail (enlarged in inset a''). (b–d,b'–d') GFP fusion proteins bearing only the EVH1 domain of either VASP, Mena or Evl were still recruited to the bacterial surface. (e,e') No further binding was detectable when the carboxy-terminally truncated derivative GFP-VASP<sub>1-98</sub> was used, whereas (f,f') the amino-terminally truncated protein GFP-VASP<sub>14-380</sub> was able to decorate *Listeria*, probably as a result of EVH2-mediated multimerization between the transiently expressed VASP protein and endogenous Ena/VASP proteins. (g,g') No binding could be observed when GFP-VASP<sub>14-304</sub>, which is truncated at both termini, was used. Note that the brightness has been enhanced in (a',e,g) and reduced in (a'') to improve visualization of focal contacts, bacteria or the polarized distribution of GFP-VASP. Bars represent 10 μm in (a) and 2 μm in (b–g).

to the green fluorescent protein (GFP). As shown for GFP-VASP (Figure 1a), all fusion proteins were localized in the focal adhesions of transfected cells, as long as the respective GFP fusion protein carried the complete EVH1 domain (data not shown). To determine the extent that these fusion proteins could be recruited by intracellular *L. monocytogenes*, we infected the transfected cells with *L. monocytogenes* wild-type strain EGD. Intracellular bacteria accumulated full-length GFP-VASP<sub>1-380</sub> and the carboxy-terminally truncated GFP-VASP<sub>1-116</sub>, GFP-Mena<sub>1-114</sub> and GFP-Evl<sub>1-117</sub> fusion proteins on their surface (Figure 1a–d). In contrast, GFP-VASP<sub>1-98</sub>, which did not have an intact EVH1 domain, was not detectable on intracellular bacteria (Figure 1e).

To determine *in vitro* binding to ActA, the VASP, Mena and Evl derivatives shown in Table 1 were produced in a protein translation system using [<sup>35</sup>S]methionine and subsequently analyzed in an affinity-binding assay with immobilized ActA. Full-length Mena, and its derivatives carrying the EVH1 domain, bound ActA strongly whereas

Figure 2



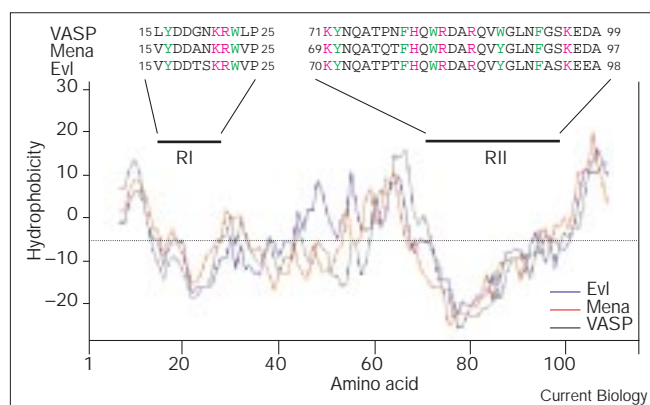
Affinity-binding assay using *in vitro* translated <sup>35</sup>S-labeled derivatives of Mena, Evl, VASP with ActA immobilized on Affigel. Bound protein was released by boiling in sample buffer and analyzed by SDS-PAGE and autoradiography. I, protein input applied to the affinity matrix; B, protein retained by the Affigel (for example, bound to ActA).

virtually no labeled protein was retained by the ActA affinity matrix when Mena<sub>1-96</sub> was used (Figure 2). Similar results were obtained with corresponding Evl derivatives. In contrast, only full-length VASP was retained by the ActA affinity matrix, suggesting that, unlike Mena and Evl, the activity of the VASP EVH1 domain depends on the integrity of the EVH2 domain (see below). The derivative Mena<sub>14-541</sub> showed no detectable binding to immobilized ActA (Figure 2), suggesting that the domain required for the interaction with ActA extends into the very amino-terminal region of Ena/VASP proteins. Together, these results allowed us to delineate the EVH1 domains of VASP, Mena and Evl to within their amino-terminal 116, 114 and 117 amino acids, respectively.

The differences in VASP binding observed in the two-hybrid system and the *in vitro* binding assay could be the result of a requirement for the EVH2 domain. We therefore constructed plasmids that express full-length VASP (VASP–BD) or its EVH2 domain (VASP<sub>227-380</sub>–BD) fused to BD. Both fusion proteins interacted with full-length VASP–AD and with those truncated VASP–AD proteins that contained an intact EVH2 domain but not with VASP<sub>1-304</sub>–AD. Further experiments with analogous Mena and Evl constructs indicated that the EVH2 domain is responsible for homomultimerization and heteromultimerization of the VASP, Mena and Evl proteins (see Table 1).

When PtK<sub>2</sub> cells were transfected with a plasmid expressing GFP-VASP<sub>14-380</sub> and subsequently infected with *L. monocytogenes* EGD, fluorescence was detected on bacteria (Figure 1f). This suggested that in the absence of a functional EVH1 domain, the EVH2 domain is capable of binding to endogenous VASP and Mena. Substantiating this hypothesis, GFP-VASP<sub>14-304</sub> was diffusely distributed throughout the cytoplasm and no accumulation was

Figure 3



Hydropathy profile of the predicted amino-acid sequences of the EVH1 domains of VASP, Mena and Evl. The bars RI and RII indicate hydrophilic regions, which are highly conserved in all three proteins as shown in the alignment of amino-acid sequences. Green, conserved aromatic residues; pink, conserved basic amino acids.

detectable either on intracellular bacteria or in focal contacts (Figure 1g).

To identify residues within the minimal EVH1 domain that are crucial for interacting with proline-rich ligands, we generated a hydropobicity plot and found two distinct potentially surface-exposed hydrophilic regions, which we termed RI and RII (Figure 3). To select amino-acid residues for site-directed mutagenesis, we relied on structural analysis performed previously with SH3 domains and their proline-rich ligands [8]. SH3 domains are composed of numerous hydrophobic and aromatic amino acids (for example, each has a hydrophobic pocket embedded in a hydrophilic area containing acidic residues), whereas their ligands carry basic amino acids adjacent to prolines [8]. Several highly conserved aromatic

residues are also present in the RI and RII regions of the different EVH1 domains. The core EVH1-binding motif FPPPP — previously also termed the ABM1 homology sequence [9] — is, however, flanked by acidic residues in the natural ligands ActA (SFEFPPPTDEEL), vinculin (EPDFPPPPDLEQ) and zyxin (EEIFSPPPPPPEEEG) [6,10,11]. We therefore anticipated that, unlike SH3 domains, EVH1 domains should harbor basic residues in close vicinity to the aromatic amino acids. Indeed, inspection of the RI and RII regions revealed several conserved basic residues that might form salt bridges with acidic residues within the EVH1 ligand (Figure 3). Using the yeast two-hybrid system, we found that substitutions within RI or RII completely abolished binding to ActA, whereas mutations located outside RI and RII had no effect (Table 2). These results were further corroborated by the *in vitro* affinity-binding assay (data not shown) and by expressing, in *Listeria*-infected cells, a GFP-VASP fusion protein in which the Phe79 and His80 residues of VASP were replaced by Leu and Asp, respectively (GFP-VASP<sub>F79L/H80D</sub>; see Supplementary material published with this paper on the internet).

We next considered whether the specific charges of the residues flanking the ActA polyproline motif were essential for binding to VASP. A set of five 15-mer peptides corresponding to the ActA polyproline motif (S<sub>1</sub>F<sub>2</sub>E<sub>3</sub>F<sub>4</sub>P<sub>5</sub>P<sub>6</sub>P<sub>7</sub>P<sub>8</sub>T<sub>9</sub>D<sub>10</sub>E<sub>11</sub>E<sub>12</sub>L<sub>13</sub>R<sub>14</sub>L<sub>15</sub>) was synthesized on a cellulose membrane in such a way as to sequentially replace the acidic residues Glu3, Asp10, Glu11 and Glu12 by arginines, and incubated with radiolabeled VASP. As expected, strong binding was observed to the peptide corresponding to the wild-type ActA motif (Figure 4a, column i). Binding to the remaining four peptides was reduced by 66% to 86% (Figure 4a, columns ii–v), revealing the pivotal role of the acidic patches flanking the polyproline core motif. We next examined the influence of a corresponding change in the

Figure 4

Binding of (a) wild-type VASP and (b) VASP<sub>K21E/R22E</sub> to immobilized synthetic 15-mer peptides corresponding to the core EVH1-binding motif within ActA. Each acidic amino acid within the sequence SFEFPPPTDEELRL was replaced by a basic arginine in succession (i–v) as indicated by blue residues. Peptides synthesized on a membrane were incubated with *in vitro* transcribed/translated (a) [<sup>35</sup>S]VASP or (b) [<sup>35</sup>S]VASP<sub>K21E/R22E</sub>, and subjected to autoradiography. The insets (a', b') show the spot intensities; the contrast in b' was increased relative to a' to improve visualization of the respective binding differences. Experiments were performed in triplicate; Standard deviations are indicated by error bars.

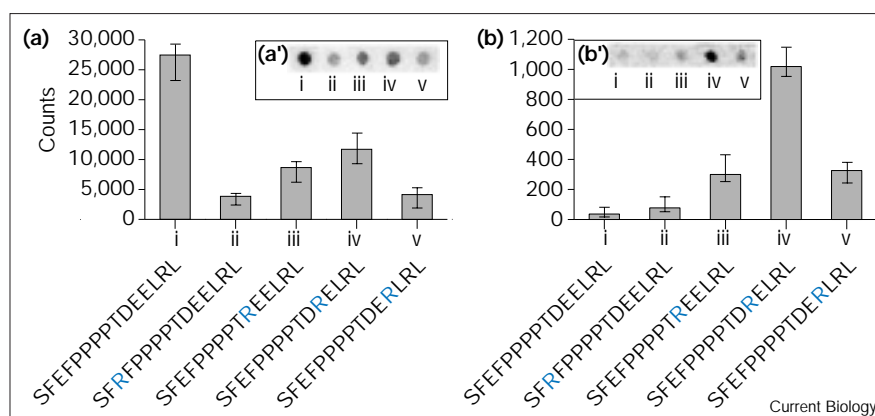


Table 2

Interactions of mutated VASP derivatives with themselves and with ActA derivatives.

	Mutated domain	ActA <sub>7-422</sub> -BD	ActA <sub>7-263</sub> -BD	VASP-BD	VASP <sub>227-380</sub> -BD
VASP-AD		+	-	+	+
VASP-N <sub>R10A/M14C</sub> -AD		+	-	-	-
VASP-N <sub>K21A/R22A</sub> -AD	RI	-	-	-	-
VASP-N <sub>W23T</sub> -AD	RI	-	-	-	-
VASP-N <sub>F33T</sub> -AD		+	-	-	-
VASP-N <sub>Y39T/N41S</sub> -AD		+	-	-	-
VASP-N <sub>F79L/H80D</sub> -AD	RII	-	-	-	-
VASP <sub>F79L/H80D</sub> -AD	RII	-	-	+	+

VASP-N contains residues 1–116 of VASP; it was chosen because it displayed very strong binding to ActA in the yeast two-hybrid system. The VASP-N fusion proteins carry single or double amino-acid substitutions and have been designated accordingly; the domain containing the mutation(s) is indicated in the first column. Only substitutions within the RI and RII regions completely abolished binding. The full-length mutant protein VASP<sub>F79L/H80D</sub>-AD interacted with full-length VASP-BD and with VASP<sub>227-380</sub>-BD through their EVH2 domains, whereas binding to ActA-BD was not detectable.

charged residues within the EVH1 domain on its binding to the same set of peptides. The yeast two-hybrid analysis presented above (see Table 2) demonstrated that the substitution of the basic residues Lys21 and Arg22 of the EVH1 domain by neutral alanines abolished binding. We speculated that this was due to the loss of positively charged residues that would normally interact with the negatively charged residues of the ligand. If this were the case, introduction of negatively charged residues at these positions would be expected to partially reverse the binding properties of such a variant. A VASP mutant in which the residues Lys21 and Arg22 were replaced by glutamate (VASP<sub>K21E/R22E</sub>) was therefore generated, and indeed was no longer able to bind to the polyproline motif SFEP PPPPTDEELRL of ActA, possibly as a result of repulsion effects of like charges. This mutant now exhibited a significant interaction with the peptide SFEP-PPPPTDREELRL, indicating that simple reversal of charges in key amino acids in either the binding domain or the ligand affects substrate specificity (Figure 4b).

Our data point to many common features between EVH1 and SH3 domains that are fundamental for binding to their respective proline-rich ligands. Like SH3 domains, EVH1 domains bind short, contiguous and stereotypical peptide sequences. The main difference, and the most probable reason why EVH1 domains fail to bind all SH3 ligands tested so far [6], is the reverse distribution of charged residues in ligands and binding domains that are necessary for forming salt bridges to stabilize the

hydrophobic interactions, as suggested previously [9]. Whereas acidic residues of SH3 domains interact with basic residues of their ligands, EVH1 domains use positively charged residues to establish electrostatic interactions with negatively charged residues present in its ligands ActA, vinculin and zyxin (for a model, see Supplementary material). Although we have produced artificial EVH1 variants that are 'reverse-charged', we suspect that such natural variant modules, that engage ligands with specificities similar to those we have defined above, may exist within the cell.

#### Supplementary material

Additional figures showing yeast two-hybrid data, fluorescence micrographs of PtK<sub>2</sub> cells expressing GFP-VASP mutants, a model for the binding of the ActA polyproline motif to the EVH1 domain, and additional methodological details are published with this paper on the internet.

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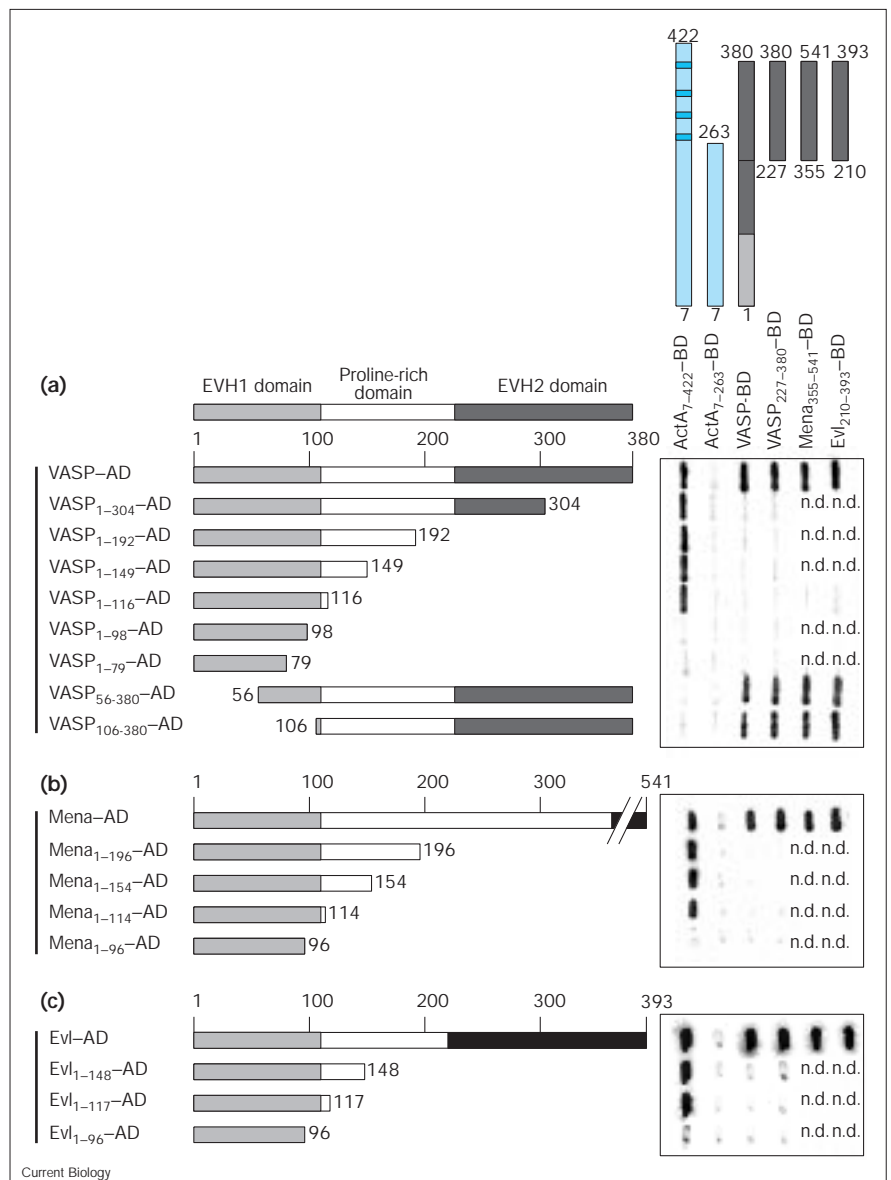
## Aromatic and basic residues within the EVH1 domain of VASP specify its interaction with proline-rich ligands

Uwe D. Carl, Marc Pollmann, Elisha Orr, Frank B. Gertler, Trinad Chakraborty and Juergen Wehland

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Figure S1

Interaction of various derivatives of (a) VASP, (b) Mena and (c) Evi with each other and with ActA (blue) as determined by the yeast two-hybrid system. Results of X-gal staining are shown on the right – blue colonies (which appear black in this figure) indicate an interaction; white colonies indicate no interaction. The numbers indicate amino-acid positions.



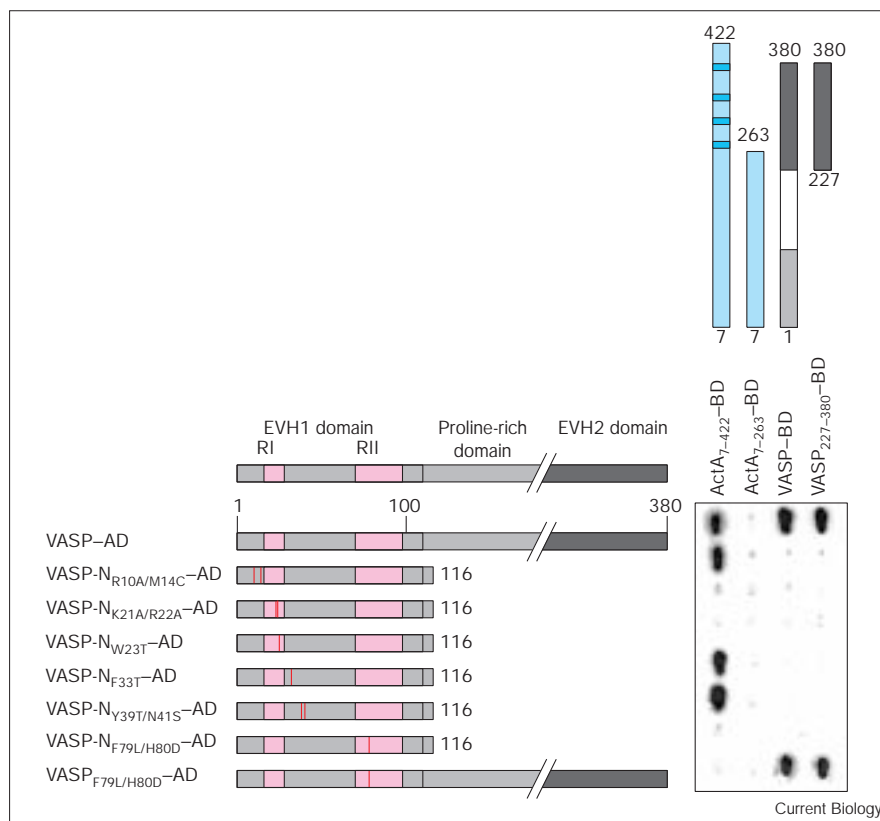
### Supplementary materials and methods

#### Plasmid construction

The various DNA fragments encoding VASP, Mena, Evi or ActA were generated by PCR using, as templates, a VASP EST clone (GenBank accession number AA491171, IMAGp998L102036, RZPD, Berlin), Mena and Evi cDNA clones [S1], or genomic DNA from *Listeria*

*monocytogenes*, respectively. Amplification primers were designed so that the resulting truncated protein began at amino-acid residues 1, 56, 106 or 227 for VASP; 1, 66, 95 or 355 for Mena; 1 or 210 for Evi; 7 for ActA; and ended at amino-acid residues 79, 98, 116, 149, 192, 304 or 380 for VASP; 96, 114, 154, 196 or 541 for Mena; 96, 117, 148 or 393 for Evi; 263, 332 or 422 for ActA. The numbers represent positions of amino acids according to the published sequences of

Figure S2



Yeast two-hybrid binding assays using the indicated constructs which express full-length VASP or VASP-N (residues 1–116) fused to AD. VASP-N was chosen because it displayed very strong binding to ActA in the yeast two-hybrid system as determined by  $\beta$ -galactosidase activity. The VASP-N fusion proteins carry single or double amino-acid substitutions and have been designated accordingly; red lines in the schematic diagram of the proteins indicate the positions of the substitutions. Only substitutions within the RI and RII regions (pink) completely abolished binding. The full-length mutant protein VASP<sub>F79L/H80D</sub>-AD interacted with full-length VASP-BD and with VASP<sub>227-380</sub>-BD through their EVH2 domains, whereas binding to ActA-BD was not detectable.

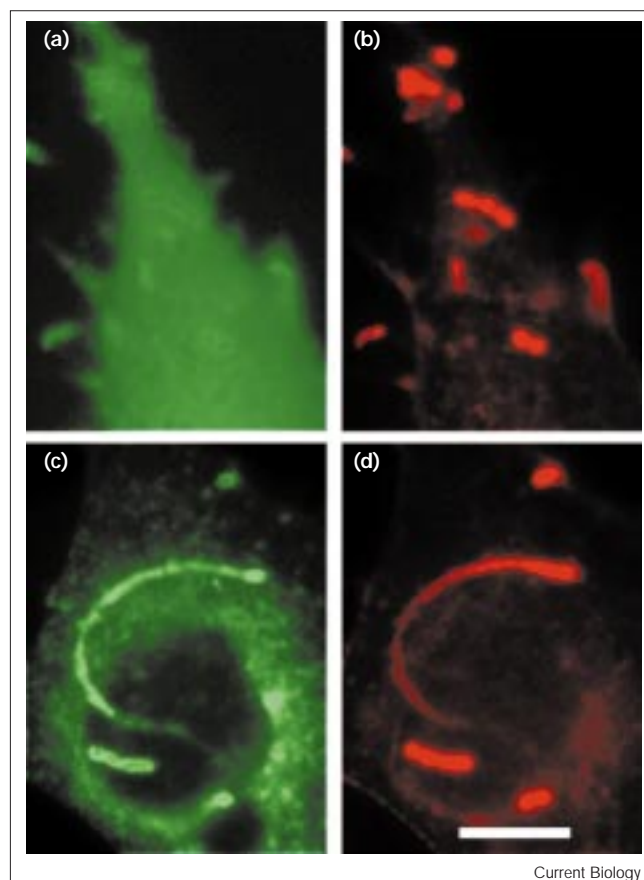
VASP [S2]; Mena and Evi [S1]; and ActA [S3]. The plasmid vectors pAS1 and pACTII, which have been described [S4], were cut at the unique *Nco*I and *Bam*HI sites located at the 3' end of sequences encoding the GAL4 DNA-binding domain and the GAL4 activation domain, respectively. The VASP, Mena or Evi PCR fragments were digested with *Nco*I and *Bam*HI and inserted into the pACTII or pAS1 vector. ActA PCR fragments were digested with *Nco*I and *Bam*HI or *Bgl*II. For digestion of the fragment encoding ActA<sub>7-422</sub>, the natural internal *Sau*3AI site corresponding to the amino-acid residue 422 was used. All ActA fragments were cloned into the pAS1 vector. The pMT7HE plasmid [S5] was modified to generate a *Nco*I site in the multilinker region corresponding to those of pACTII and was used for ligation of the different *Nco*I–*Bam*HI fragments of VASP, Mena or Evi. The pEGFP-C2 and pEGFP-N1 plasmids (Clontech) were digested at the unique *Hind*III and *Bam*HI sites located at the 3' and 5' end of the EGFP gene, respectively. The VASP, Mena or Evi constructs in pMT7HE were double digested with *Hind*III and *Bam*HI, inserts purified from agarose gels and used for ligation into the pEGFP-C2 and pEGFP-N1 plasmids. The mutant VASP plasmids were constructed either by fusing fragments encoding mutated VASP<sub>80-380</sub> to the 3' end of VASP<sub>1-79</sub> gene in the former cloned plasmid, or by using the PCR site-directed mutagenesis method as described [S6]. Therefore, PCR was used to generate two DNA fragments having overlapping ends in which site-directed mutations due to nucleotide changes within the primers were introduced. These fragments were combined in a subsequent 'fusion' PCR reaction, leading to a fusion product in which the mutated nucleotides had been incorporated. For the first PCR reaction, a primer located upstream of the VASP gene was used in combination with the following 3' primers: 5'-AAAGCA~~CCACAGTGGC~~-CGCGCTGGAAC-3'; 5'-GCAGGGAGCCATGCCGCGTTGCC-3'; 5'-GCAGGGAGCGTTTCGCTTGTGCC-3'; 5'-GCGGCTGGGCTGGCCTGGGACCCGT-3'; 5'-GCTGTGGGTGATCTGGACGCGGCT-

GAAG-3'; and 5'-AGGGAGCCATTCCTCGTTGCCATCATA-3' to generate amino-terminal VASP fragments encoding proteins mutated at amino-acid residues 10 and 13; 21 and 22; 23; 33; 39 and 41; and 21 and 22, respectively. The carboxy-terminal VASP fragments mutated in the same nucleotides were generated using a primer located downstream of the VASP gene in combination with the following 5' primers: 5'-TGTTCCAGCGCGGCCACTGTGTGCTTT-3'; 5'-TGGCAACGCGGCATGGCTCCCTG-3'; 5'-TGGCAACAAGCGCAACGCTCCCTG; 5'-GTCCCCAGGCCACCAGCCGCG-3'; 5'-CTTCAGCCGCGTCCAGATCACCCACAGCCCCACG-3'; 5'-TATGATGATGGCAACGAGGAATGGCTCCCT-3'. Nucleotides deviating from the published VASP sequence are indicated in italics. The combined PCR fragment was generated with the same primers used for constructing plasmids VASP<sub>1-116</sub> and VASP<sub>1-380</sub>. All inserts and fusion areas of the constructs were verified by sequencing using Taq Dye Deoxy Terminator cycle sequencing (Applied Biosystems) and analyzed on an Applied Biosystems 373A automated DNA Sequencer.

#### Yeast two-hybrid system

The protein interactions were analyzed by the yeast two-hybrid system as described [S4]. All pAS1 plasmids expressing the GAL4 DNA-binding domain (amino acids 1–147) fused to the ActA fragments were transformed into the yeast strain Y187 (*MAT $\alpha$* , *ura3-52*, *his3*, *ade2-101*, *trp1-901*, *leu2-3*, 112, *met*<sup>-</sup>, *gal4 $\Delta$* , *gal80 $\Delta$* , *URA3::GAL1-lacZ*, Clontech) by electroporation and were plated out onto synthetic complete plates lacking tryptophan (Sc-W). The pACTII plasmid derivatives expressing the GAL4 activation domain (starting at amino acid 768) were transformed into yeast strain Y190 (*MAT $\alpha$* , *ura3-52*, *his3-200*, *ade2-101*, *trp1-901*, *leu2-3*, 112, *gal4 $\Delta$* , *gal80 $\Delta$* , *URA3::GAL1-lacZ*, *LYS2::GAL1-HIS3*, *cyf12*, Clontech) and plated out on synthetic complete plates lacking leucine (Sc-L). To examine *in vivo* interaction between the proteins, the appropriate

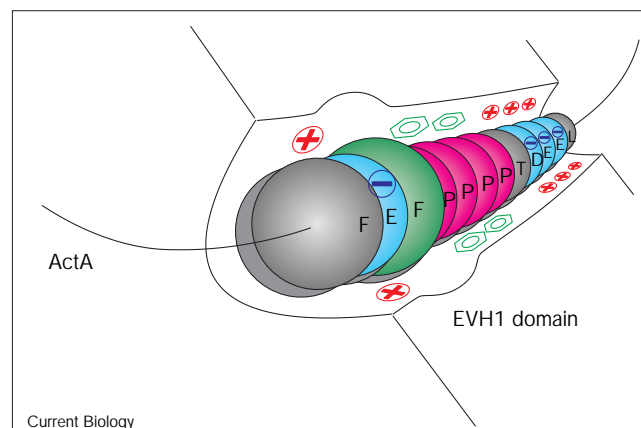
Figure S3



Fluorescence micrographs of PtK<sub>2</sub> cells transiently expressing (a,b) GFP–VASP<sub>1–116</sub>(F79L/H80D) or (c,d) GFP–VASP<sub>1–380</sub>(F79L/H80D) and infected with *L. monocytogenes* EGD. (a,c) Distribution of GFP fusion proteins; (b,d) phalloidin staining of the cells shown in (a,c), respectively. (a,b) In contrast to GFP–VASP<sub>1–116</sub> (see Figure 1b in the paper), the mutated version GFP–VASP<sub>1–116</sub>(F79L/H80D) was no longer able to bind to intracellular *Listeria*. Thus, all three assay systems indicated that the substitution of the two amino-acid residues Phe79 and His80 abrogated the interaction between VASP and ActA *in vitro* as well as *in vivo*. Nevertheless, when a full-length fusion protein, GFP–VASP<sub>1–380</sub>(F79L/H80D), which carries the same two substitutions, was expressed, fluorescence could be detected, as was also observed with the amino-terminally truncated GFP–VASP<sub>14–380</sub> (see Figure 1f in the paper). Both fusion proteins harbor ‘disabled’ EVH1 domains but still carry a functional EVH2 domain, which is obviously responsible for the targeting. In addition, actin comet tails showed more intense labeling with GFP–VASP<sub>1–380</sub>(F79L/H80D), which might be explained by the recent finding that the EVH2 domain of VASP interacts with F-actin *in vitro* [S12]. The bar in (d) represents 5 μm.

transformants were either mated, or the pACTII constructs were transformed into yeast already containing pAS1 constructs. For the mating, fresh overnight colonies of Y190 and Y187 were picked from Sc-L and Sc-W plates, respectively, mixed together with a toothpick on a YPD plate and incubated at 30°C. After 6 h, an inoculum of the mating mixture was streaked on synthetic complete plates lacking tryptophan and leucine. For measuring the β-galactosidase activity, multiple resulting diploid yeast colonies were picked and X-gal tests were performed as described [S7].

Figure S4



Model for the binding of the ActA polyproline motif to the EVH1 domain of the Ena/VASP protein family. The hydrophobic interaction involving the prolines of ActA and distinct aromatic residues of the EVH1 domain are thought to be responsible for the initial protein–protein contact. The phenylalanine residue directly preceding the polyproline cluster is essential for the binding and may serve as a key residue [S13]. The negatively charged residues flanking the polyproline core can form salt bridges with distinct positively charged amino acids (such as Lys21 and/or Arg22) of the EVH1 domain.

#### Peptide synthesis

Spot synthesis was performed according to Frank [S8] with an Abimed ASP 222 Automated SPOT Robot.

#### In vitro interactions

ActA was purified from supernatants of *L. monocytogenes* as recently described [S9] and coupled to an Affigel 15 matrix following recommendations by the manufacturer (Biorad); <sup>35</sup>S-labeled proteins were prepared by *in vitro* transcription/translation of VASP, Mena or Evl DNA fragments cloned in the pMT7HE vector using the TNT-coupled rabbit reticulocyte lysate system (Promega). These proteins were diluted 1:100 with incubation buffer (1× PBS, 0.05% Triton X-100, 0.1% BSA, 2 mM EDTA, 1 mM PMSF), incubated with the Affigel-15-immobilized ActA protein at 4°C with gentle shaking overnight and washed five times with 15-fold volume of TBS-T (0.2 M Tris-Cl pH 7.6, 137 mM NaCl, 0.1% Tween-20) at room temperature. Bound protein was released by boiling in gel sample buffer and separated by SDS–PAGE in comparison with the input protein. The gel was dried and analyzed with a phosphorimager (Molecular Dynamics). For peptide spot analysis, radiolabeled proteins were diluted 1:50 with TBS-T and incubated with the peptide membrane for 2 h with gentle shaking at room temperature. The membrane was washed five times with TBS-T and analyzed with a phosphorimager.

#### Transfection of GFP fusion constructs

PtK<sub>2</sub> cells (ATCC CCL56) were grown and transfected as described [S10]; 24 h after transfection, cells were washed once with MEM medium and infected with *L. monocytogenes*.

#### Infection of cultured cells with *L. monocytogenes*

*L. monocytogenes* serotype 1/2a EDG were grown in brain–heart infusion broth (Difco) at 37°C with aeration. For infections, bacteria from an overnight culture were added to PtK<sub>2</sub> cells at a dilution of 1:1000 and centrifuged for 2 min with 500g. After 1–2 h, plates were washed and incubated with fresh medium containing 25 μg/ml gentamycin. Three hours later, coverslips were washed with PBS, cells were fixed with 3.7% paraformaldehyde in PBS and permeabilized with 0.2% (v/v) Triton X-100 in PBS. For localization of actin, cells were incubated with



rhodamine-labeled phalloidin. Samples were examined with a Zeiss Axiovert 135 TV microscope equipped with epifluorescence and a cooled CCD camera driven by IPLab Spectrum software (Princeton Instruments). Images were processed using Adobe Photoshop 3.0.

#### Hydropathy profile

The plots were obtained by standard computer-assisted analysis using the algorithm and hydropathy values of Kyte and Doolittle [S11].

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