The EVH2 Domain of the Vasodilator-stimulated Phosphoprotein Mediates Tetramerization, F-actin Binding, and Actin Bundle Formation*

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Vasodilator-stimulated phosphoprotein (VASP) is a member of the Ena/VASP family of proteins that are implicated in regulation of the actin cytoskeleton. All family members share a tripartite structural organization, comprising an N-terminal Ena/VASP homology (EVH) 1 domain, a more divergent proline-rich central part, and a common C-terminal EVH2 region of about 160–190 amino acids. Using chemical cross-linking, sucrose gradient sedimentation, and gel filtration analyses of different truncated VASP constructs, we demonstrate that the VASP EVH2 region is both necessary and sufficient for tetramerization. Moreover, co-sedimentation and fluorescent phalloidin staining showed that the EVH2 region binds and bundles F-actin in vitro and localizes to stress fibers in transfected cells. Analysis of the functional contribution of highly conserved blocks within this region indicated that residues 259–276 of human VASP are essential for the interaction with F-actin, whereas residues 343–380 are required for tetramerization, probably via coiled-coil formation. Interactions with F-actin are enhanced by VASP tetramerization. The results demonstrate that the C-terminal EVH2 segment is not only conserved in sequence but also forms a distinct functional entity. The data suggest that the EVH2 segment represents a novel oligomerization and F-actin binding domain.

The mammalian vasodilator-stimulated phosphoprotein (VASP)1 (1) and Drosophila Enabled (Ena) (2) are the founding members of the Ena/VASP family of proteins. While Ena is a substrate of the Abelson tyrosine kinase (Abl) and is also genetically linked to the Abl signaling pathway (2), VASP is a substrate of both cGMP- and cAMP-dependent protein kinases (Ref. 3; for a review see Ref. 4). Three common cGMP-dependent protein kinase/cAMP-dependent protein kinase phosphorylation sites have been biochemically identified in human VASP (Ser-157, Ser-239, and Thr-278) (5, 6), two of which are also conserved in Mena (mammalian Enabled) and one in Evl (Ena-VASP-like), two other family members (7). VASP phosphorylation in response to cyclic nucleotide regulating vasodilators (i.e. cAMP-elevating prostaglandins and cGMP-elevating NO donors) closely correlates with platelet inhibition and in particular with the inhibition of fibrinogen binding to the human platelet integrin αIIbβ3 (3, 8). In agreement with these earlier studies, recent analysis of platelets from VASP-deficient mice support the concept that VASP is an important component in modulating agonist-induced integrin αIIbβ3 and P-selectin activation (9, 10). Similar to Ena (11) and its mammalian homologue Mena (7), VASP is an actin filament-associated protein that is predominantly localized at stress fibers, cell-matrix and cell-cell adherens junctions, and highly dynamic membrane areas (12, 13). There is a large body of evidence that both VASP and Mena are involved in the facilitation of spatially confined actin filament formation (for a review see Refs. 14 and 15).

All Ena/VASP family members share a tripartite structural organization consisting of highly homologous N-terminal and C-terminal parts (Ena-VASP homology domains 1 and 2 (EVH1 and EVH2)) that are separated by a central proline-rich core (1, 2, 7, 13). Hydrodynamic analyses suggested that VASP purified from human platelets is a homotetramer with an elongated structure (1). In addition, there are preliminary data indicating that both Ena and Mena may form hetero-oligomers with VASP (7, 11).

The EVH1 domain comprises about 111–133 amino acids with a high proportion of aromatic and aliphatic residues. Biochemical data indicate that the EVH1 domain binds to proline-rich (E/D)(P)PPPP(P/E) motifs (FP4 motifs) (16) present in the VASP and Mena binding proteins vinculin (7, 17–19), zyxin (7, 20), and the Listeria spp. surface protein ActA (7, 21). ActA shares some structural and functional features with zyxin (16, 20, 22, 23) and is essential for actin polymerization-based intracellular motility of Listeria (for a review see Ref. 14). Microinjection of peptides that are known to interfere with VASP and Mena binding to their FP4-containing ligands (16) displaces VASP and Mena from focal adhesions and causes retraction of membrane protrusions and cessation of listerial motility (7, 16, 24, 25). Furthermore, deletion of the VASP binding FP4 motifs in ActA impairs ActA-dependent F-actin accumulation, reduces the speed of listerial motility, and attenuates listerial virulence (16, 25–27). Analysis of a Drosophila recessive lethal ena allele revealed an A97V point mutation within the EVH1 domain that impairs Ena binding to zyxin in vitro (11). Mutant Ena fails to co-localize with zyxin at focal adhesions and actin stress fibers in transfected cells (11). These data are also consistent with an Ena EVH1 domain function in zyxin binding.

The proline-rich central part of Ena/VASP proteins is the most variable protein segment with no extensive sequence con-
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reservation between different family members. This central part mediates interactions of murine Ab1 and Src SH3 domains with Ena and Mena (2, 28) and Mena binding to the WW domain of the neuronal adapter protein FE65 (29). Moreover, distinct proline-rich motifs, which are present in multiple copies in the VASP and Mena and once in the Evl central segment, have been shown to be involved in profilin binding to VASP and Mena (7, 30, 31). Similarly, the proline-rich central segment of Ena is also involved in profilin binding (28). Microinjection of a profilin binding VASP peptide into Listeria-infected cells arrests bacterial motility (31).

In contrast to other EVH1 domain containing proteins (32), a hallmark of all Ena/VASP family proteins is the presence of a C-terminal EVH2 region, about 160–190 amino acids in length, with a repetitive mixed charge cluster at its C-terminal end (1, 13). Despite some preliminary observations, the function of the EVH2 segment has remained elusive. Thus, focal adhesion localization of human VASP lacking part of the EVH2 region (residues 285–380) was markedly affected, and association with stress fibers differed from that of the wild-type protein (1). A recessive lethal nonsense mutation of Drosophila ena leads to a truncated protein lacking the C-terminal 49 amino acids. This mutant Ena not only failed to form oogonies but was also impaired in its zyxin and Ab1-SH3 domain binding ability and subcellular distribution (11).

We have shown previously that VASP can bind to filamentous actin (F-actin) (12), a function that appears also to reside within the EVH2 region, adding an additional level of complexity. Therefore, this study was initiated to define the possible role of the EVH2 segment in tetramerization and F-actin binding. Here we demonstrate that two distinct sites within the EVH2 region are essential for tetramerization and F-actin binding, respectively. We further show that the EVH2 fragment comprising both sites is required and sufficient to confer actin cross-linking activity to the protein.

**EXPERIMENTAL PROCEDURES**

**Preparation of Expression Vectors—** Constructs used for transient expression in eukaryotic cells (see Fig. 7A as a summary) were as follows.

Plasmids based on the expression vector pCDNA3 (Invitrogen) were constructed to encode four different C-terminal VASP fragments encompassing the complete EVH2 region (residues 225–380) or amino acids 259–380 (containing block B, the C' linker region 1, and block C of the EVH2 region), 277–380 (essentially lacking block B), and 259–342 (essentially lacking block C), respectively. The respective proteins were expressed by transient transfection into B cells in 96-well tissue culture plates. Cells were harvested after overnight induction. For the purification of the highly expressed C-terminal VASP fusion proteins His-EVH2, His-BIC, His-IC, and His-BL, cell pellets were resuspended in lysis buffer (50 mM NaP, pH 8.0, 300 mM NaCl, 0.5 mM EDTA, 2 μg/ml leupeptin, 20 units/ml Trasylol®, 5 mM benzamidine, and 1 mM phenylmethylsulfonyl fluoride) and lysed by sonication after incubation with the lysis buffer on ice (30 min on ice). After incubation (30 min) with DNase (5 μg/ml) and RNase (10 μg/ml), cell debris was pelleted. The resulting supernatant was applied to nickel-nitrotriacetic acid-Sepharose, which had been pre-equilibrated with wash buffer (50 mM NaP, pH 8.0, 300 mM NaCl), supplemented with 10 mM imidazole (for the purification of His-BIC and His-IC) or 5 mM imidazole (for the purification of His-BL). The matrix was washed and subsequently eluted with a step gradient of 10–500 mM imidazole in wash buffer. Proteins were dialyzed against PBS and stored at 4 °C.

Because of much lower expression levels, His-VASPΔC was purified as modified protocol. The lysis buffer contained 50 mM NaCl and was supplemented with 5% glycerol. After cell lysis and the addition of 1 mM imidazole and 0.5% Triton X-100 (final concentrations), the respective proteins were extracted by stirring on ice for 20 min followed by centrifugation. The supernatant was adjusted to 300 mM NaCl, cleared by centrifugation, and loaded onto a HiTrap® Chelating column (Amersham Pharmacia Biotech), pre-equilibrated with NiSO4. The column was washed with lysis buffer containing 1 mM imidazole and eluted with a step gradient of 1–500 mM imidazole in 50 mM NaP, pH 8.0 and 300 mM NaCl. Proteins were further purified by gel filtration on Superdex 75. Peak fractions were determined by absorbance measurement at 205 nm (35) or by Coomassie Blue staining. In both cases, bovine serum albumin was used as a standard (A280 mg/ml = 0.66).

**Purification of Skeletal Muscle Actin—** Actin was purified from porcine skeletal muscle acetone powder essentially as described (36). Actin was stored in G buffer (2 mM Tris-HCl, pH 8.0, 0.2 mM ATP, 0.5 mM dithiothreitol, 0.2 mM CaCl2) at 4 °C. The actin concentration was calculated from the absorbance at 290 nm (37).

**Cross-linking of Proteins—** His-BIC, His-IC, and His-BL proteins were cross-linked in PBS at final concentrations of 70 μM and 7 μM protein. His-VASPΔC was cross-linked at a protein concentration of 10 μM. The reactions were started by the addition of 100 μM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC; Molecular Probes) and 10 mM N-hydroxysulfosuccinimide (NHSS; Molecular Probes) or 500 μM ethylene glycol bis(2aminoethyl ether)-N,N'-tetraacetic acid (EGTA; Molecular Probes). After a 1-h incubation at room temperature, EDCA/NHSS-containing reactions were quenched by the addition of 100 mM Tris-HCl (pH 8.0) followed by SDS sample buffer, whereas EGS-containing samples were directly stopped by the addition of Tris-containing SDS sample buffer (pH 6.8). Equal amounts of proteins were analyzed by Tricine-SDS gel electrophoresis (38) followed by Coomassie Blue staining.

**Gel Filtration—** For determination of the Stokes radii (Rg), 1.2 ml of the respective protein solutions in PBS were chromatographed at concentrations of 0.1–1.5 mg/ml on a HiLoad 16/60 Superdex 200 gel filtration column (Amersham Pharmacia Biotech). The column had been pre-equilibrated with PBS and calibrated with standard proteins of known radii. Runs were performed at a flow rate of 0.75 ml/min and monitored by absorbance at 280 nm. Peak fractions were analyzed by SDS-PAGE and Coomassie Blue staining. Gel filtration experiments under reducing conditions were performed in the presence of 15 mM dithiothreitol in protein samples and buffers.

**Sucrose Gradient Centrifugation—** For determination of the sedimentation coefficients, sucrose gradient centrifugation was performed as
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**Fig. 1.** Three conserved sequence blocks within the EVH2 region of the Ena/VASP protein family. Alignment of the EVH2 regions of canine VASP (gi 1718078), murine VASP (gi 1617402), human VASP (gi 1718079), murine Evl (gi 1644453), rat RNB6 (gi 2058462), murine Mena (gi 1644455), chicken Avena (gi 3551531), and Drosophila Ena (gi 1362599). NCBI (National Center for Biotechnology Information) “gi” sequence identifiers are given in parentheses. Highly conserved segments are boxed (blocks A–C).

**RESULTS**

Transfection of Cultured PtK₂ Cells and Immunofluorescence Microscopy—Transfection of PtK₂ cells and immunofluorescence microscopy were done essentially as described (11). VASP fragments in transfected cells were specifically detected with the monoclonal VASP antibody IE273 (43, commercially available from immunoGlobe, Grossostheim, Germany). Bound monoclonal antibody and F-actin were detected by a Cy3-labeled goat anti-mouse antibody (Dianova, Germany) and Alexa® 488-labeled phalloidin (Molecular Probes), respectively.

Comparison of the human VASP EVH2 sequence with a data base of known parallel two-stranded coiled-coils yields a similarity score, which allows calculation of the probability for coiled-coil formation by comparing the distribution of scores in globular and coiled-coil proteins (44). These calculations predict a low propensity for coiled-coil formation for the sequence blocks A and B, whereas block C essentially coincides with a segment that has a high probability of forming a coiled-coil structure (data not shown).

For a detailed functional analysis with respect to VASP tetramerization and F-actin binding, the EVH2 region was molecularly dissected. Three plasmids were constructed for the expression of human VASP residues 259–380 (containing both sequence blocks B and C), 277–380 (essentially lacking block B), and residues 259–342 (lacking block C) as hexahistidine fusion proteins in E. coli. The respective VASP proteins (His-B/C, His-B, and His-C; see Fig. 7 as a summary) were expressed in E. coli at high levels and could be purified to >95% homogeneity as judged from SDS-PAGE analysis and Coomassie Blue staining (Fig. 2, lanes 2–4).
A hexahistidine-tagged amino acid segment (amino acids 343–380) was prepared. His-VASP D segment. To address this question, a hexahistidine-tagged con-
sequences in the VASP EVH1 domain or the proline-rich core Whether oligomerization could also be accomplished by se-
length VASP also required the presence of sequence block C or Therefore, we investigated whether oligomerization of full-
tial for tetramer formation of C-terminal VASP polypeptides. 
sequence block C, which probably forms a coiled-coil structure, is essen-
sequences block C, are sufficient for tetramerization. Moreover, 
addition to the monomer, His-B C and His-Bl were partially cross-linked; in 
fractionation under reducing conditions) His-B/C and His-VASP displayed properties of a dimer and monomer, respectively. 
minor bands, most likely corresponding to octamers, were observed with His-B/C and His-IC, which probably represent intermolecular cross-links. Cross-linking with 500 μM EGS yielded essentially the same results (not shown).

These experiments revealed that the C-terminal VASP amino acids 277–380, comprising the B/C-linker region plus sequence block C, are sufficient for tetramerization. Moreover, block C, which probably forms a coiled-coil structure, is essential for tetramer formation of C-terminal VASP polypeptides. Therefore, we investigated whether oligomerization of full-length VASP also required the presence of sequence block C or whether oligomerization could also be accomplished by sequences in the VASP EVH1 domain or the proline-rich core segment. To address this question, a hexahistidine-tagged con-
(His-VASPDC, see also Fig. 7) lacking the C-terminal segment (amino acids 343–380) was prepared. His-VASPDC, which was only poorly expressed in E. coli, was purified from a large scale culture and subjected to cross-linking. Neither the combination of EDAC and NHSS nor EGS was able to cross-link this protein. As a control, cross-linking of His-B/C was successful both in a separate reaction and in a mixture containing both proteins (not shown). Consequently, sequence block C is required for tetramerization of both full-length VASP and the various EVH2 fragments.

Gel Filtration and Sucrose Gradient Centrifugation—A combination of gel filtration chromatography and sucrose gradient centrifugation was used to determine the molecular masses of the different VASP constructs and to confirm the results ob-
tained by chemical cross-linking. The calibration curves and the Stokes radii and sedimentation coefficients obtained for His-B/C, His-IC, His-Bl, and His-VASPDC are shown in Fig. 3 and Table I. With the partial specific volumes estimated from the amino acid composition (41), the molecular masses of the polypeptides can be calculated from the Stokes radii and sedimentation coefficients (Ref. 40; Table I).

Comparison of the experimentally determined molecular masses and those calculated from the amino acid composition indicated that His-B/C and His-IC formed tetramers, whereas (under these experimental conditions) His-Bl and His-VASPDC displayed properties of a dimer and monomer, respectively.

Surprisingly, His-VASPDC appeared as a monomer in gel filtration/sucrose gradient sedimentation, whereas His-Bl (which corresponds to the C-terminal part of the former construct) behaved as a dimer in both the hydrodynamic and cross-linking analyses. To resolve this issue, we used denaturing gel electrophoresis under nonreducing conditions. In these experiments, the three C-terminal VASP polypeptides migrated as dimers (not shown), indicating that Cys-334 (the only cysteine present in these mutants) is responsible for this in vitro dimerization. However, this interaction is apparently not relevant for tetramerization because gel filtration under reducing conditions indicated that reduction of the disulfide bond of His-B/C led to dissociation of the dimer, whereas His-IC remained a tetramer. Therefore, hexahistidine-tagged C-terminal VASP polypeptides expressed in E. coli are recovered as disulfide-linked peptides, whereas Cys-334 of His-VASPDC is apparently not engaged in disulfide formation. Most likely, this

**Figure 2. Analysis of cross-linked VASP mutants by SDS-PAGE.** His-B/C, His-IC, and His-Bl were cross-linked with 100 mM EDAC/10 mM NHSS as described under “Experimental Procedures,” and equal amounts of protein were analyzed by SDS-PAGE followed by Coomassie Blue staining. Lanes 1 and 5, marker proteins; lanes 2–4, control (without cross-linker); lanes 6–8, cross-linking at protein concentration of 70 μM; lanes 9–11, cross-linking at a protein concentration of 7 μM.

**Figure 3. Gel filtration (A) and sucrose gradient sedimentation (B) of VASP mutants.** A, the Stokes radii of VASP fragments were determined by gel filtration on a Superdex-200 column. Calibration proteins were: thyroglobulin (8.59 nm), apoferritin (6.4 nm), aldolase (4.64 nm), bovine serum albumin (3.64 nm), ovalbumin (2.9 nm), chymotrypsinogen A (2.1 nm), and cytochrome c (1.65 nm). B, the sedimentation constants of VASP fragments were determined by sucrose gradient (5–20%) centrifugation in the absence and presence of the following calibration proteins: ovalbumin (3.55 S), carbonic anhydrase (3.06 S), and cytochrome c (1.9 S). ■, calibration proteins; □, VASP fragments.
accounts for the appearance of dimeric versus monomeric polypeptides when block C sequences are deleted. It should be noted that His-BI was not consistently recovered as a dimer. Thus, in some cases the polypeptide could also be purified either in a monomeric form or as a mixed population of monomers and dimers (as judged by cross-linking, gel filtration, and nonreducing SDS-PAGE).

Taken together, both cross-linking and hydrodynamic data demonstrate that VASP residues 277–380 (lacking sequence block B) are sufficient for tetramerization, with an essential determinant of the oligomerization reaction located within residues 343–380 (representing block C plus three additional C-terminal residues). As judged from its hydrodynamic properties, all of the VASP present in crude human platelet extracts is in the tetrameric form (data not shown), indicating that within cells there is no equilibrium between tetramers and significant amounts of lower degree oligomers.

**F-actin Binding of C-terminal VASP Mutants in Vitro**—VASP is localized to stress fibers in a variety of cell types and VASP purified from human platelets co-sediments with F-actin (12), suggesting that it directly binds to F-actin. Initial experiments, designed to define this interaction more precisely, showed that the complete VASP EVH2 region (residues 225–380 in the human protein; expressed as a hexahistidine fusion protein in *E. coli*) binds to F-actin in a co-sedimentation assay (data not shown). To reveal a possible contribution of the conserved sequence blocks B and C to F-actin binding, His-B/C, His-I/C, and His-BI proteins were tested for co-sedimentation with F-actin in a high speed centrifugation step (Fig. 4A). In this assay, salt concentrations of 33 mM and 150 mM KCl were applied, and centrifugation was carried out at 4 and 22 °C. In the presence of 33 mM KCl, His-B/C was exclusively detected in the pellet fraction, independent of the temperature. However, in the presence of 150 mM KCl (4 °C) about half of the protein remained in the supernatant (not shown). In contrast, His-I/C did not co-sediment, and co-sedimentation of His-BI was strongly dependent on the temperature and salt conditions. At 22 °C, His-BI was never found in the pellet, whereas the protein was partially recovered in the pellet fraction at 4 °C. Also, in contrast to His-B/C, which bound to F-actin even in the presence of 150 mM KCl, the F-actin binding capacity of His-BI was completely abolished under these conditions.

To test whether these truncated VASP proteins were able to cross-link actin filaments, identical assays were performed using low speed centrifugation at 4 °C (Fig. 4B). Under these conditions, pure F-actin incubated with buffer remained in the supernatant (Fig. 4B, control). In contrast, a mixture containing F-actin and His-B/C was nearly quantitatively pelleted, whereas the combination of F-actin and His-I/C predominantly remained in the supernatant. The sedimentation behavior of the F-actin/His-BI mixture was again variable and salt-dependent. Under low salt conditions, F-actin and His-BI mostly co-sedimented, whereas part of F-actin and His-BI remained in the supernatant at high salt concentration. Note that in the particular experiment shown in Fig. 4B, F-actin pelleted quantitatively in the presence of His-BI, whereas part of the VASP polypeptide remained in the supernatant.

Consequently, block B is essential for F-actin binding, and VASP amino acids 259–380 are sufficient to mediate both F-actin binding and cross-linking. The observation that the hexahistidine-tagged construct lacking block B (His-I/C) does not bind to F-actin in the co-sedimentation assay excludes the tag as a mediator of F-actin binding. This is of particular relevance because poly-cations such as poly-histidine and poly-lysine have been reported to bind and bundle filamentous actin relevant because poly-cations such as poly-histidine and poly-lysine have been reported to bind and bundle filamentous actin.
His-B mutants. Aliquots of the F-actin/His-B containing sample showed thick F-actin bundles forming extensive mesh- (Fig. 5A) or rope-like (Fig. 5A′) suprastructures. In contrast, F-actin incubated with His-IC (Fig. 5B), like the ovalbumin (not shown) or buffer control (Fig. 5D), was characterized by a tiny, diffuse web-like appearance and was completely devoid of comparable actin filament bundles. Only occasionally, few thin filaments were formed in the presence of this mutant (not shown). Bundles observed in the F-actin/His-B′ samples (Fig. 5C) were markedly shorter and thinner than those found after co-incubation of F-actin and His-B/C. Interestingly, monomeric His-B′ recovered from the same protein preparation as the dimeric form failed to form F-actin bundles. F-actin structures formed in the presence of this monomeric species resembled those found in the control and F-actin/His-IC co-incubation (not shown).

**Subcellular Localization of C-terminal VASP Mutants**—Because different truncated VASP EVH2-derived constructs displayed distinct in vitro F-actin binding and cross-linking activities, we investigated whether this is also reflected by the association of these polypeptides with actin containing structures in living cells. Therefore, four pcDNA3-based plasmids (pcD-EVH2, pcD-B/C, pcD-IC, and pcD-B′; see Fig. 7A) were constructed for eukaryotic expression of human VASP fragments encoding the complete EVH2 region (VASP residues 225–380) and residues 259–380, 277–380, and 259–342, respectively. These constructs were transfected into a marsupial epithelial cell line (PtK2), and expression of the proteins was confirmed by Western blot analysis (not shown). The subcellular distribution of the polypeptides was analyzed with a monoclonal antibody specific for VASP of human and some other mammalian species that does not recognize endogenous VASP in PtK2 cells (13, 43). Double staining with Alexa® 488 phalloidin revealed that the VASP EVH2 region is targeted to stress fibers (not shown). Also, a polypeptide comprising VASP residues 259–380 expressed from pcD-B/C was efficiently localized at stress fibers (Fig. 6, panels 2A and 2B) in a comparable fraction of transfected cells as full-length VASP, which served as a control (Fig. 6, panels 1A and 1B). However, both fragments displayed an increased cytoplasmic staining as compared with the wild-type protein. Except for some stress fiber association found in rare cases (Fig. 6, panels 3A and 3B), the VASP polypeptide comprising residues 277–380 expressed from pcD-IC, which failed to bind F-actin in vitro, did not localize to stress fibers. Often, in transfectants showing a high degree of expression from the pcD-IC construct, a gradual thinning or dissolution of stress fiber bundles could be observed (not shown), suggesting a dominant negative effect because of tetramer formation with endogenous Ena/VASP family proteins. The VASP polypeptide comprising amino acids 259–342 expressed from pcD-B′ was inconsistently localized at the actin cytoskeleton, and stress fiber staining appeared in fewer cells and was less pronounced when compared with the wild-type protein (Fig. 6, panels 4A and 4B). As shown above for the hexahistidine-tagged fragment, this peptide lacks the C-terminal sequence required for tetramerization and also has a compromised F-actin binding capacity. In essentially all transfected cells, polypeptides 225–380, 259–380 and 277–380 and 259–342 did not localize to focal contacts (Fig. 6, panels 2–4).

**DISCUSSION**

**Three Conserved Sequence Blocks of the EVH2 Region**—The EVH2 region comprises three highly conserved sequence blocks operationally designated as A (21 residues), B (20 residues), and C (35 residues). Except for the Ena/VASP family proper, no similar EVH2 region is present in any other known protein. The same is true for the individual segments, such as block A, which shows no clear-cut homology to known proteins, although, based on a very limited similarity between part of block A and the KLKK motif involved in G-actin binding of thymosin β4 (46), the corresponding segment within the EVH2 region has been suggested as a G-actin binding site (7). In VASP, Mena, and the avian homologue Avena (DDBJ accession number AB017437) the same basic motif is part of a consensus phosphorylation site for cyclic nucleotide-dependent protein ki-
nases, which for VASP (Ser-239) has been established as an in vitro and in vivo phosphorylation site (5).

Sequence block B is highly conserved between the EVH2 regions of all known mammalian and avian members of the Ena/VASP family. In human VASP it comprises the Thr-278 phosphorylation site for cAMP-dependent protein kinase and cGMP-dependent protein kinase (5) and 19 residues immediately preceding this site. Thr-278 is conserved in VASP of different species but is substituted by an alanine in Evl, as well as in Ena, Mena, and Avena. There is no obvious homology of block B to any known protein, except for a distant similarity with the C-terminal part of the villin 1B domain (residues 218–235 of human villin: ASPKLMEVNMHLGKRRELK (identities, bold and underlined; conservative replacements, bold) (47).

Sequence block C also appears to be unique to Ena/VASP family proteins. However, because of its heptad repeat pattern, block B bears some similarity to known coiled-coil proteins such as myosin heavy chains, tropomyosins, and collagens. Moreover, a predicted coiled-coil region within the Mena central part (7) as well as the homologous part of Avena, both comprise a tandem repeat of two block C-related segments.

The Conserved Block B within the EVH2 Region Is Essential for F-actin Binding—In an earlier study we showed that VASP binds to F-actin (12). Our present results and another very recent report (48) conclusively demonstrate that the VASP EVH2 domain mediates F-actin binding. In co-sedimentation assays presented here, the presence of block B was essential for an interaction of EVH2 fragments with F-actin. Moreover, in conjunction with the block B/C intervening sequence, block B was also sufficient for F-actin binding. The observation that a dimeric (but not a monomeric) fragment comprising VASP residues 259–342 (His-B) is able to bundle F-actin reveals that there is at least one F-actin binding site/polypeptide chain, with the 20-residue block B sequence as a plausible candidate.

Our results demonstrate strong actin filament bundle formation by EVH2 region fragments. This disagrees with the data of Laurent et al. (48), who did not find any bundles in the presence of GST-EVH2. This discrepancy may be due to different experimental conditions and/or methods used for the analysis of actin filament bundling.

Interestingly, an endogenously phosphorylated form of VASP (Ser-239) appeared either not to affect F-actin binding (12) or to show an increased affinity for F-actin (48), although the contribution of the different VASP phosphorylation sites was not investigated. As shown in Fig. 7, block B, which is involved in F-actin binding, contains the Thr-278 phosphorylation site. We therefore propose that phosphorylation of Thr-278 and perhaps also Ser-239, rather than Ser-157, regulates the F-actin binding affinity of VASP. Experiments testing this possibility are in progress.

Conserved Sequence Block C Is Required for Tetramerization—Using chemical cross-linking and by determination of the hydrodynamic properties of VASP fragments, we have shown that the mixed charge cluster of block C is required for tetramerization, probably involving coiled-coil formation. Under certain experimental conditions VASP fragments can be recovered from an E. coli expression system in a disulfide-linked form. Cys-334 of human VASP, which is the only cysteine

![Fig. 6](image-url) Subcellular localization of the C-terminal VASP mutants. PtK<sub>2</sub> epithelial cells were transfected with plasmids expressing full-length VASP (control; panels 1A and 1B), with pcD-B/C (panels 2A and 2B), pcD-I/C (panels 3A and 3B), and pcD-B/I (panels 4A and 4B). Cells were co-stained with the monoclonal anti-human VASP antibody IE273 followed by a Cy3-labeled secondary antibody (panels A) and Alexa® 488 phalloidin (panels B). Bar, 20 μm.

![Fig. 7](image-url) Functional roles of the conserved regions B and C in the EVH2 VASP domain for F-actin binding and oligomerization. A, hexahistidine-tagged VASP proteins (I) used in this study and VASP constructs transfected into PtK<sub>2</sub> cells (II). Tag sequences are as indicated under “Experimental Procedures.” B, properties of the VASP fragments with respect to oligomerization and F-actin binding, cross-linking, and intracellular localization to stress fibers. For these functions, the VASP EVH2 domain is sufficient. The conserved block B is essential for binding to F-actin. Oligomerization depends on the presence of block C which also enhances F-actin binding. *<sup>a</sup>, from Refs. 1 and 12.
residue present in these constructs, is conserved neither in
canine VASP nor in any other Ena/VASP family member.
Therefore it is quite unlikely that disulfide bond formation is
physiologically relevant in vivo. Nevertheless, occurrence of a
disulfide bond at Cys-334, i.e. in close proximity to the pre-
dicted coiled-coil segment, indicates that a putative coiled-coil
bundle must comprise at least to parallel helices. Interestingly,
two to five consecutive heptad repeats are detectable in six
of seven possible frames, suggesting a more complex bundle for-
mation, possibly comprising more than two helices.

**EVH2-mediated Tetramerization Enhances Protein-Protein Interactions of the EVH1 Domain and the Proline-rich Central Part**—VASP oligomerization is not only required for F-actin
cross-linking but also enhances the F-actin binding activity,
which (when compared with monomeric/dimeric fragments) is
less salt- and temperature-sensitive (Fig. 4A).

Most intriguingly, enhancement of F-actin binding by VASP
tetramerization closely parallels the situation observed with a
mutant ena allele encoding a protein that lacks the C-terminal
49 amino acids. Although Ena binding to the Abl-SH3 domain
and to zyxin have been attributed to the proline-rich central
region and, by inference from VASP and Mena data, to the
N-terminal EVH1 domain, respectively (2, 16), interactions of
Ena with both these proteins is compromised in the oligomer-
ization deficient mutant (11). Similarly, deletion of human
VASP residues 265–380 affects VASP localization at focal ad-
hesions (1). In contrast, a GST fusion protein comprising the
N-terminal part of Mena (residues 6–170) is targeted to focal adhesions when microinjected into PtK2 cells (7). It is tempting to
speculate that either the very C-terminal segment of this
construct, which comprises part of the predicted central coiled-
coil region (residues 155–231) (7) or the dimerization function
provided by GST (49) may lead to oligomerization of the con-
struct. Therefore, protein-protein interactions of Ena/VASP
family proteins in general appear to be enhanced or stabilized
in the oligomeric forms of these proteins. In accordance with
this view, VASP localization to focal adhesions but not its
association with stress fibers can be rescued by direct fusion of
the tetramerization inducing residues 277–380 to the VASP
N-terminal/central part (residues 1–195).2

Interestingly, with the exception of the EVH1 domain pro-
tein Homer 1a, all other currently known Homer proteins are
characterized by a C-terminal coiled-coil domain that mediates
multimerization and is thought to be required for cross-linking
of metabotropic glutamate receptors to IP3 receptors (50, 51).
 Likewise, we suggest that the tetramerization function of the
EVH2 region enables the Ena/VASP family proteins to cross-
link the EVH1 domain ligands zyxin and vinculin and binding proteins of the central proline-rich region, such as proline, SH3
domain, and WW domain containing proteins. This may have
direct consequences, e.g. for VASP-dependent regulation of in-
tegrin functions (8–10).

**VASP Localization to Focal Adhesions and Stress Fibers Differ**—VASP (12), Mena (7), and Ena (11) are associated both
with focal adhesions and with stress fibers, showing a regular
dotted staining pattern. The data reported here allow to dis-
tinguish between both types of interactions. Localization to
focal adhesions critically depends on EVH1 mediated interac-
tions (7, 16, 25), probably augmented by a C-terminal oligomer-
ization function (see above). In accordance with these results,
the EVH2 fragments investigated in this study failed to localize
to focal adhesions. In sharp contrast, however, both VASP
EVH2 fragments containing the conserved sequence block B

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2 C. Bachmann, L. Fischer, U. Walter, and M. Reinhard, our unpub-
lished observations.

that proved to be essential for F-actin binding, localized to stress fibers. Again, the tetrameric fragment was more efficient
than the monomeric/dimeric fragment. Therefore, F-actin bind-
ing appears to significantly contribute to stress fiber associa-
tion of VASP, whereas it is dispensable for focal adhesion
targeting. Taken together, the quantitative contributions of
VASP interactions with actin and with EVH1 ligands, such as
zyxin, clearly differ at focal adhesions and stress fibers. This
could not have been predicted in view of the virtually identical
subcellular distribution of VASP and its ligand zyxin as ob-
erved, e.g. in human fibroblasts (20).

In conclusion, using VASP as a prototype, this study has
shown that the EVH2 region of Ena/VASP family proteins
harbors two distinct segments involved in F-actin binding and
oligomerization, respectively. Tetramerization by the EVH2
C-terminal part augments F-actin binding and lends F-actin
bundling activity to the protein. Hence, both the regions in-
volved in F-actin binding and oligomerization appear to form a
functional entity. In conjunction with the collective occurrence
of all three conserved EVH2 sequence elements as one unit,
these results suggest that the EVH2 region represents an au-
thentic protein domain. It will be a challenging task of future
studies to unravel the function of the conserved N-terminal
part of this putative new domain as well as its regulation by
cyclic nucleotide-dependent protein kinases in different family
members.

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**Note Added in Proof**—After submission of this paper, Hüttelmaier et
al. (Hüttelmaier, S., Harbeck, B., Steffens, O., Messerschmidt, T.,
Illenberger, S., and Jockusch, B. M. (1999) FEBS Lett. 451, 68–74)
independently reported on actin filament bundling by the VASP EVH2
domain, which corroborates our present study.

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