cAMP-dependent Protein Kinase Phosphorylation of EVL, a Mena/VASP Relative, Regulates Its Interaction with Actin and SH3 Domains*

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Proteins of the Ena/VASP family are implicated in processes that require dynamic actin remodeling such as axon guidance and platelet activation. In this work, we explored some of the pathways that likely regulate actin dynamics in part via EVL (Ena/VASP-like protein). Two isoforms, EVL and EVL-I, were highly expressed in hematopoietic cells of thymus and spleen. In CD3-activated T-cells, EVL was found in F-actin-rich patches and at the distal tips of the microspikes that formed on the activated side of the T-cells. Like the other family members, EVL localized to focal adhesions and the leading edge of lamellipodia when expressed in fibroblasts. EVL was a substrate for the cAMP-dependent protein kinase, and this phosphorylation regulated several of the interactions between EVL and its ligands. Unlike VASP, EVL nucleated actin polymerization under physiological conditions, whereas phosphorylation of both EVL and VASP decreased their nucleating activity. EVL bound directly to the Abl, Lyn, and nSrc SH3 domains; the FE65 WW domain; and profilin, likely via its proline-rich core. Binding of Abl and nSrc SH3 domains, but not profilin or other SH3 domains, was abolished by cAMP-dependent protein kinase phosphorylation of EVL. We show strong cooperative binding of two profilin dimers on the polyproline sequence of EVL. Additionally, profilin competed with the SH3 domains for binding to partially overlapping binding sites. These data suggest that the function of EVL could be modulated in a complex manner by its interactions with multiple ligands and through phosphorylation by cyclic nucleotide-dependent kinases.

To respond properly to environmental cues, cells possess multiple complex signal transduction networks. Many pathways lead to dynamic changes of the actin cytoskeleton that form the basis for cell movement in a wide variety of biological phenomena. In recent years, many proteins participating in one or more signal transduction pathways have been identified. One group of multifunctional proteins, involved in actin-based motility, is the Ena/VASP family of proteins that include Drosophila Ena (Enabled), Mena (mammalian Ena), VASP (vaso-dilator-stimulated phosphoprotein), and EVL (Ena/VASP-like protein) (1). Ena was identified through genetic interactions with the Drosophila Abl homologue (2, 3), whereas VASP was identified as a prominent target for cAMP (PKA)1- and cGMP-dependent protein kinases in platelets (4). Mena and EVL were identified by similarity to Ena (1). Ena, Mena, and VASP are important in processes that require highly dynamic actin reorganization, including axon guidance (5), platelet aggregation (6, 7), and fibroblast motility (8). The proteins are concentrated in regions of the cell associated with movement and adhesion, including the leading edge of lamellipodia, focal adhesions, and adherens junctions (1, 9, 10).

The Ena/VASP proteins share a common domain structure that consists of an amino-terminal Ena/VASP homology (EVH) 1 domain, a carboxyl-terminal EVH2 domain, and a central proline-rich domain. The EVH1 domain is highly conserved and binds to a target sequence that has the consensus (E/D)FPPPPXDE (11, 12). Functional EVH1-binding motifs are present in the Listeria monocytogenes surface protein ActA (12); in the focal adhesion proteins vinculin and zyxin (13, 14); in Fyb/SLP (Fyn-binding protein/SLP76-associated protein), a component of the T-cell receptor pathway (15); and in the axon guidance proteins ROBO (16) and Semaphorin-6A-1. In fibroblasts, the EVH1 domain mediates focal adhesion (1, 12, 17) and leading edge targeting (8), and a functional EVH1 domain is required for Ena function in Drosophila (18). The EVH2 domain contains conserved motifs implicated in actin binding (9, 19, 20) and formation of both homo- and heteromultimers of the Ena/VASP family proteins (17, 18). In contrast to the highly conserved EVH1 and EVH2 domains, the central proline-rich domain of the different proteins contains variable lengths of consecutive polyproline clusters. Three types of ligands have been shown to bind to this region in Ena/VASP proteins: the SH3 and WW domains and the actin-binding protein profilin (1, 3, 18, 21, 22).

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1 The abbreviations used are: PKA, cAMP-dependent protein kinase; EVH, Ena/VASP homology; PBS, phosphate-buffered saline; GST, glutathione S-transferase; λ-Pase, λ-protein phosphatase.

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Several lines of evidence suggest that the interactions between profilin and Ena/VASP proteins are important in vivo. In mice, there is a potent dosage-sensitive genetic interaction between Mena and profilin I (5). Although Mena mutants are viable, displaying defects in several nerve fiber tracts in the brain, a reduction of profilin levels by 50% causes Mena mutants to exhibit a severe defect in closure of the cephalic portion of the neural tube. In Drosophila, mutations in Ena and profilin have each been shown to exhibit dosage-sensitive genetic interactions with Abl (2, 23). Despite this genetic evidence, the mechanisms that regulate the interaction and function of Ena/VASP-profilin complexes remain poorly understood (24).

The third member of the Ena/VASP family, EVL, has not been functionally characterized. EVL partially restores Listeria movement in cell-free extracts depleted of VASP and Mena (20) and shares many structural features with the other family members. The EVH1 domain and distinct parts of the EVH2 domain are homologous, but the central portion differs in both length and proline content. The number of conserved cysc nucleotide-dependent kinase phosphorylation sites also differs; whereas VASP and Mena have three and two, respectively, EVL has only one site (1). Although these structural features suggest that the three mammalian proteins have overlapping functions, we show here they may also have unique properties and mechanisms of regulation. To gain further insight in the regulatory pathways linked to actin dynamics, we studied the binding of EVL to actin, profilin, and SH3 domains and the effect of PKA phosphorylation on these interactions.

EXPERIMENTAL PROCEDURES

Protein Purification—The coding region of EVL was amplified from a mouse embryonic stem cell cDNA library. Murine VASP cDNA was a kind gift of Dr. R. Fasler. Both cDNAs were cloned into pEB-Nhis (Life Technologies, Inc.), and recombinant proteins were obtained by using the Bac-to-Bac™ baculovirus expression system (Life Technologies, Inc.) according to the manufacturer’s instructions. The protein was purified on Talon resin (CLONTECH) and subsequently dialyzed in buffer (50 mM Tris-HCl and 10 mM MgCl₂, pH 7.5) supplemented with 200 μM ATP and 10 μCi of γ-32P[ATP for 30 min at 30 °C. Subsequently, phospho-EVL was dialyzed in 50 mM Tris, 0.1 mM EDTA, 5 mM dithiothreitol, and 2 mM MnCl₂, pH 7.5, and then treated with λ-phosphatase (New England Biolabs Inc.) for 30 min at 30 °C.

Binding of EVL to SH3 Domains—Binding of EVL, phosphorylated or dephosphorylated, were added to 50 μl of GST-SH3 fusion protein bound to glutathione-agarose beads in PBS and 1% Triton X-100 and incubated for 30 min at 4 °C. After three washes, the resin was boiled in 1× SDS sample buffer for 5 min. One-tenth of the fraction was loaded onto an 8% SDS-polyacrylamide gel (Bio-Rad) and, after transfer to Hybond™, probed with monoclonal antibody 84A1 against EVL.

Analysis of the Binding Motif by the SPOTs Method—The membrane was probed as described (30).

BLAcore Analysis—The BLAcore measurements were carried out as described (31).

RESULTS

EVL Localizes to Dynamic Regions in Fibroblasts and Activated T-cells—The subcellular distributions of Mena and VASP have been well characterized in fibroblasts and several other cell types. Since fibroblasts lack detectable levels of EVL (see Fig. 2B), we examined EVL distribution in primary T-cells, a rich source of EVL protein.

When primary CD4+ T-cells were plated on control antibody (anti-CD71)-coated coverslips, EVL was mainly present in the cytoplasm of the cells (data not shown). Plating T-cells on anti-CD3 antibody-coated coverslips induced a clustering of the T-cell receptor and subsequent activation of the polarization response. EVL became localized in F-actin-rich patches that developed on the coverslip side of the cell (Fig. 1A). In addition to this actin patch localization, EVL could be detected at the distal tips of microspikes that formed in response to T-cell receptor cross-linking (Fig. 1A). Endogenous VASP (also expressed in this cell type) showed a similar re-localization (data not shown). This observation is consistent with a recent report demonstrating that Ena/VASP proteins play a critical role in the actin reorganization that occurs in activated Jurkat T-cells.
(15) and supports a model in which their localization is regulated by specific signal transduction pathways.

In fibroblasts, Mena and VASP are recruited to focal adhesions via interactions with proteins containing the EVH1-binding motif (12). To determine if EVL could be targeted by a similar mechanism, we generated Rat2 fibroblasts that express EVL. Staining of these cells revealed that EVL was concentrated in focal adhesions and at the leading edge of the lamellipodium in a pattern very similar to Mena and VASP (Fig. 1B).

**EVL Is Phosphorylated by PKA in Vitro and in Vivo—VASP and Mena have been shown to be substrates for PKA. Since only one of the possible phosphorylation sites mapped in VASP is present in EVL (Ser156) (Fig. 2A) (1), we wanted to see if EVL is a substrate for PKA in vivo and in vitro. Western blot analysis of EVL in adult mouse organ extracts revealed two protein bands (Fig. 2B) (5). Similarly, two protein bands were observed in cultured cortical neurons, whereas mainly the upper band was detected in cultured glia (Fig. 2C). In analogy with VASP and Mena (1, 32, 33), we speculated that these two bands represent dephospho and phospho forms of EVL. However, when cortical neuron and glial cell extracts were treated with the catalytic domain of PKA in vitro, Western blot analysis indicated that both protein bands showed a small shift upward rather than the expected shift of the lower form into the slower migrating form (Fig. 2C). We note that the shift seen for PKA-phosphorylated VASP in glial cells is larger than the one seen for EVL (Fig. 2D). Treatment of the extracts in vitro with λ-protein phosphatase (λ-PPase) did not cause any detectable shift in either EVL band as compared with untreated extracts (Fig. 2C). In extracts of Rat2-EVL cells, a single EVL-reactive band comigrated with the lower of the two bands found in spleen and brain (Fig. 2B), indicating that the faster migrating form is unlikely to be simply a proteolytic fragment of the slower migrating form.

We reasoned that the upper form of EVL found in spleen and cortical neurons might represent a larger isoform of EVL produced by the inclusion of an additional exon. Sequence analysis of both the human genomic locus of EVL as well as mouse expressed sequence tags predicted the existence of an extra small exon. Therefore, it seems likely that alternative splicing of the EVL mRNA produces two isoforms of EVL. The shorter isoform contains the EVL sequence as described originally (1). The larger protein, which we term EVL-I, contains an extra portion that is 21 amino acids long and is located between Ser339 and Arg340 of EVL (Fig. 2, A and G). Western blot analysis of Rat2 cells expressing a cDNA encoding EVL-I indicated that this isoform comigrated with the larger signals observed in spleen (Fig. 2B). The two isoforms displayed a tissue-specific expression pattern (Fig. 2B) (5), and both isoforms could be phosphorylated by PKA in vitro.

Although the EVH1 domain is necessary and sufficient for subcellular targeting of Ena/VASP proteins, the EVH2 domain is also thought to play a role in localization, perhaps by promoting more avid, multimeric complexes (18). Since the extra sequence included in EVL-I falls in the EVH2 domain, we expressed EVL-I in Rat2 cells to determine if subcellular targeting might be affected by the additional sequence. The staining pattern (Fig. 3) indicated that EVL-I is localized in a pattern very similar to EVL, suggesting that the extra sequence in EVL-I does not alter subcellular targeting under these conditions.

To assay whether EVL is also an in vivo substrate of PKA, we stimulated the Rat2-EVL cells with forskolin, an activator of adenyl cyclase (34). After 10 min, cells were lysed and analyzed by Western blotting. Forskolin induced a similar small band shift as PKA treatment, indicating that EVL can be phosphorylated in vivo by PKA (Fig. 2E).

For further experimental analysis of the effect of phosphorylation on ligand binding, we purified EVL as a His6-tagged protein from the baculovirus expression system. The purified protein was phosphorylated in vitro with the catalytic subunit of PKA and ATP. Typically, after 30 min, all EVL was phosphorylated, and the observed shift was identical to the one seen with native EVL in cell extracts (Fig. 2F). The shift could be reversed by subsequent treatment with λ-PPase. We used these and similar samples in the actin, profilin, and SH3 domain binding assays.

**EVL Discriminates between Several SH3 Domains, and Phosphorylation of EVL Affects Binding of Some SH3 Domains—**The central proline-rich domain of EVL contains two separate proline-rich sequences, P1VP4 and P2LP separated by a TGST sequence (Fig. 2A), that contain possible binding sites for SH3 domains (35, 36). To test whether EVL could bind to SH3 domains, several GST-SH3 fusion proteins (Fig. 4) were purified and incubated with EVL. The formed complexes were pulled down with glutathione-agarose beads; and after thor-
the number of bound profilin molecules. This is consistent with interactions outside the proline-rich core of ligands are also involved in SH3 domain binding (38). Therefore, we wondered whether phosphorylation of EVL might affect its ability to bind ligands through its proline-rich core since the PKA site is located just in front of this portion of EVL (Fig. 2A). We performed the same experiment as described above, except that the EVL protein was phosphorylated in vitro by PKA prior to addition to the GST-SH3 proteins. The results are shown in Fig. 4 (middle panel). The interaction with both the nSrc and Abl SH3 domains was almost completely abolished by PKA phosphorylation of EVL, whereas there was no or little effect on the binding of EVL to the Lyn SH3 and FE65 WW domains. We observed no effect on the non-interacting SH3 domains of Fyn, Src, Cak, and Crkl. Reversing the phosphorylation event with λ-PPase restored the binding properties of EVL to yield results identical to those of the original experiment (Fig. 4, compare upper and lower panels).

The Proline-rich Domain of EVL Binds with High Affinity to Prolin IIa—In addition to SH3 domains, profilin is also a ligand for the proline stretches found in all other members of the Ena/VASP family (1, 18, 21). VASP preferentially binds profilin IIa in bovine brain extracts containing both profilins I and IIa (39), and profilin IIa has a >500-fold higher affinity for the (GP5)3 proline sequence derived from VASP compared with profilins I and IIb (31). Therefore, we used profilin IIa to identify sequences through which profilin binds to EVL. A SPOTs filter containing overlapping 15-mer peptides that represent the entire EVL sequence was incubated with purified profilin IIa. Bound profilin IIa was detected with antiprofilin IIa antibodies (Fig. 5A). Two major binding sites could be identified that overlap with the proline-rich region: peptides 59–61 and 64–66. Interestingly, peptides 62 and 63, which also have a high proline content, did not bind profilin, suggesting the specificity of the interaction. Peptides 58 and 67 do not have enough proline residues for binding of profilin. In addition to these two sites, a few single peptides and one other non-proline region on the SPOTs filter seemed to interact with profilin as well. The sequence spanning peptides 9–13 is a conserved part in the EVH1 domain, and the same region bound to profilin on a peptide scan with the Mena sequence. However, when we tested a peptide overlapping this region on a BIAcore, we did not observe any binding to profilin IIa (data not shown).

Based on the results from the peptide scan, we synthesized peptides spanning the proline-rich region. The peptides had an amino-terminal biotin group with which the peptides were coupled to a streptavidin-coated BIAcore sensor chip. Surface plasmon resonance data were collected for each peptide with a concentration series of recombinant profilin IIa (Fig. 5B). From the surface plasmon resonance data, both stoichiometry and dissociation constants ($K_d$) could be derived. Peptide A, containing the entire polyproline sequence of EVL, bound four profilin molecules with an affinity of 0.3–0.4 μM. Peptides B and C, containing the amino- and carboxyl-terminal parts of the polyproline sequence, respectively, each bound two profilins with a lower affinity than peptide A. This indicates that there is cooperativity between the two sites on the polypolypeptide domain of EVL. Peptide B has a fast $k_{on}/k_{off}$ (data not shown), and its $K_d$ is 160 times higher than that of peptide A. Peptide D is similar to peptide C, but has the additional VP3 part. However, this extra sequence does not change the affinity or the number of bound profilin molecules. This is consistent with the results from the SPOTs filter assay, where the peptides with the valine in the middle and not more than four prolines on each side of the valine did not bind profilin. Peptide E, in which the leucine is replaced by another proline, has unexpect-
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**Fig. 3. Immunolocalization of EVL-I in transfected Rat2 cells.** Rat2-EVL-I cells were stained with monoclonal antibody 84A5 for EVL, polyclonal antibody 2197 for Mena, and Oregon Green-488 phalloidin. A, EVL-I localizes to focal adhesions and at the end of stress fibers; B, overlapping localization of EVL-I with Mena.

**Fig. 4. EVL binding to SH3 domains and effect of phosphorylation.** EVL bound to the indicated GST-SH3 fusion protein was pulled down with glutathione-agarose. *Upper panel,* recombinant EVL dephosphorylated with λ-PPase; *middle panel,* recombinant EVL phosphorylated by PKA prior to SH3 domain binding; *lower panel,* recombinant EVL phosphorylated by PKA and subsequently dephosphorylated by λ-PPase prior to SH3 domain binding. GST is a negative control with only GST bound to the glutathione-agarose. *Input* is the amount of EVL added to the different SH3 domains.

The preference of EVL to bind to profilin IIa was also observed in a solution binding assay (Fig. 5C). Immobilized dephospho- and phospho-EVL proteins were incubated with recombinant profilin I or IIa. After washing the columns, the resin was boiled and analyzed by SDS-polyacrylamide gel electrophoresis. Consistent with the BIAcore data, profilin I was not retained by the resin and therefore bound only weakly to EVL under the assay conditions. The interaction of profilin IIa was not affected by phosphorylation of EVL by PKA.

**Profilin Competes with the nSrc SH3 Domain for EVL Binding.**—Since the SH3 domains and profilin both bind to the same parts of the protein, we wondered if both could be bound simultaneously. We bound EVL to GST-nSrc SH3-glutathione-agarose and then exposed the column to increasing concentrations of profilin IIa (Fig. 6). EVL was eluted from the nSrc SH3 column when the profilin IIa concentration reached 2 μM. More EVL was eluted when higher concentrations of profilin II were used. A similar result was obtained with the Abl SH3 domain and EVL and also with the Src and Abl SH3 domains and Mena (data not shown). We conclude that EVL contains overlapping binding sites for the nSrc SH3 domain and profilin and that profilin competes with the SH3 domain for binding.

**Dephospho-EVL Is a Potent Nucleator of Actin Polymerization.**—Ena/VASP family proteins have been implicated in the regulation of actin dynamics. The recruitment of EVL to the F-actin spots, its distinct localization at the tips of filopodia in activated T-cells, and its presumed role in actin cap formation in Jurkat T-cells (Fig. 1A) (15) suggest that EVL is also involved in actin assembly. Therefore, we assayed the ability of EVL to drive actin nucleation and polymerization in *vivo.* Polymerization of 4 μM Ca²⁺/ATP/actin in G-buffer was initiated by adding MgCl₂ and KCl to final concentrations of 2 and 100 mM, respectively. Under these conditions and at this actin concentration, polymerization was slow and had a long lag phase (Fig. 7A, curve 1). Addition of dephospho-EVL (1 μM) enhanced actin polymerization immediately by strongly reducing the lag phase and increasing the rate of polymerization. This resulted in a 3-fold higher F-actin content after 30 min (Fig. 7A, curve 2). Phospho-EVL was, however, less effective in enhancing polymerization. It partially reduced the lag phase, but the rate of polymerization was much slower then in the presence of dephospho-EVL (Fig. 7A, curve 3). Similar results were obtained in independent assays with different EVL and actin protein preparations. The effect was concentration-dependent, as shown for dephospho-EVL in Fig. 3B. We synthesized a peptide containing amino acids 261–283 of EVL. This sequence in EVL corresponds to a region in the EVHZ domain of VASP shown to be important for actin binding in *vivo.* (19). Preincubation of actin with 100 μM peptide-(261–283) inhibited the nucleating activity of dephospho-EVL (Fig. 7A, curve 4). We conclude that dephospho-EVL nucleates and enhances actin polymerization more effectively than phospho-EVL and that nucleation can be inhibited by adding a peptide mimicking the actin-binding region of the EVL EVH2 domain.

We tested the ability of dephospho- and phospho-VASP proteins to nucleate and enhance actin polymerization to obtain a direct comparison of the ability of VASP and EVL to influence actin assembly in *vivo.* Initially, dephospho-VASP was assayed under the same conditions as we used for EVL (4 μM Ca²⁺/ATP/actin in G-buffer supplemented with 2 mM MgCl₂ and 100 mM KCl); however, significant polymerization of actin was not observed (Fig. 7C, inset). This result is in agreement with published data showing that VASP nucleates polymerization of Mg²⁺/ATP/actin only at low, nonphysiological salt concentrations (20, 40). Under these low salt conditions (2 mM MgCl₂ and 15 mM KCl), dephospho-VASP had strong nucleating activity, whereas phospho-VASP did not nucleate actin polymerization (Fig. 7C). Note, however, that the ratio of VASP to actin was 1:1, whereas for EVL, this ratio was 1:4, indicating that EVL nucleates actin polymerization more efficiently than...
Regulation of EVL Function

During the course of this work, we discovered an alternatively spliced variant (EVL-I) containing an insertion of 21 amino acids in the EVH2 domain of EVL. The expression of the two EVL isoforms is regulated in a tissue-dependent manner, with EVL being the major form in adult brain and EVL-I enriched in T-cells. Although alternatively spliced variants of Mena have been identified, the resulting Mena isoforms vary in the amino-terminal half of the protein (1). The inclusion of an alternate exon in the EVH2 domain suggests that EVL-I might cause alterations in the EVH2 domain functions such as oligomerization and actin binding. It is also possible that the additional insert in EVL-I might provide additional sites for protein-protein interaction or regulation. In this regard, it is interesting to note that the alternately included sequence contains two KSP motifs that match the consensus for phosphorylation by Cdk5, a kinase implicated in the regulation of neuronal migration (42–46). Experiments to determine the functional differences between EVL and EVL-I are in progress.

The Actin-nucleating Capacities of EVL and VASP Are Different—Our peptide inhibition assay indicated that actin binding by EVL requires region B of the EVH2 domain, as expected from sequence similarity to VASP (19). EVL and VASP differ in the actin-binding properties of these two highly related proteins. VASP induces actin polymerization only under low salt conditions (this work and Refs. 20 and 40), whereas EVL nucleates at both physiological and low salt concentrations (this work). Differences in the actin-binding activities of EVL and VASP can also be inferred from the observation that EVL can only partially restore Listeria motility in VASP-depleted platelet extracts (20).

The in vitro actin-nucleating activity of EVL is regulated by phosphorylation. Deyphoso-EVL is a better nucleator and

![Image](image-url)

FIG. 5. Profilin IIa is the preferred ligand for EVL. A, a SPOT's filter containing 15-amino acid-long peptides spanning the entire EVL sequence was probed with profilin IIa, followed by detection with anti-profilin antibody. The dark spots represent profilin IIa bound to peptides, and the sequences of these peptides are shown below. Peptides displaying strong profilin binding are in boldface. Some other peptides listed (peptides 57, 58, 62, 63, and 67) do not bind, although they have considerable proline content.

![Image](image-url)

FIG. 6. Profilin II competes with the nSrc SH3 domain for EVL binding. EVL was bound to GST-nSrc SH3-glutathione-agarose; and after washing the column, profilin II was applied in increasing concentrations as indicated. Western blotting with anti-EVL antibody showed the presence of EVL in the eluted fractions. F, flow-through; W, buffer wash.

VASP. As with EVL, the nucleating activity of dephospho-VASP was inhibited by addition of a 100-fold molar excess of peptide-(261–283). We conclude that although the nucleating activities of EVL and VASP differ, the activity of both can be regulated by phosphorylation.

**DISCUSSION**

The emerging picture from recent in vitro and in vivo data suggests there is no simple linear signal transduction cascade linking an extracellular signal to actin remodeling. Temporal and spatial fine-tuning of the regulation is necessary for the cell to respond properly to extracellular signals. Networks of proteins and protein-protein interactions are involved in this process, and many of the interactions are regulated (41). We analyzed the interactions of EVL with several of its binding partners, actin, profilin, and SH3 domains, and examined several possible mechanisms by which these interactions might be regulated within cells.

During the course of this work, we discovered an alternatively spliced variant (EVL-I) containing an insertion of 21 amino acids in the EVH2 domain of EVL. The expression of the two EVL isoforms is regulated in a tissue-dependent manner, with EVL being the major form in adult brain and EVL-I enriched in T-cells. Although alternatively spliced variants of Mena have been identified, the resulting Mena isoforms vary in the amino-terminal half of the protein (1). The inclusion of an alternate exon in the EVH2 domain suggests that EVL-I might cause alterations in the EVH2 domain functions such as oligomerization and actin binding. It is also possible that the additional insert in EVL-I might provide additional sites for protein-protein interaction or regulation. In this regard, it is interesting to note that the alternately included sequence contains two KSP motifs that match the consensus for phosphorylation by Cdk5, a kinase implicated in the regulation of neuronal migration (42–46). Experiments to determine the functional differences between EVL and EVL-I are in progress.

**The Actin-nucleating Capacities of EVL and VASP Are Different**—Our peptide inhibition assay indicated that actin binding by EVL requires region B of the EVH2 domain, as expected from sequence similarity to VASP (19). EVL and VASP can both support actin-based Listeria motility (20); interestingly, however, we found differences in the actin-binding properties of these two highly related proteins. VASP induces actin polymerization only under low salt conditions (this work and Refs. 20 and 40), whereas EVL nucleates at both physiological and low salt concentrations (this work). Differences in the actin-binding activities of EVL and VASP can also be inferred from the observation that EVL can only partially restore Listeria motility in VASP-depleted platelet extracts (20).

The in vitro actin-nucleating activity of EVL is regulated by phosphorylation. Deyphoso-EVL is a better nucleator and
enlarged view of EVL peptide-(261–283). Polymerization was started by adding MgCl₂ and KCl to 2 and 15 mM final concentrations, respectively.

Similar results were obtained for VASP (under low salt conditions). Phosphorylation does not block the nucleating activity completely. The decrease in activity of phospho-VASP was attributed to phosphorylation of serine 239 and/or threonine 278, which are located close to and in the proposed F-actin-binding region of VASP (amino acids 259–278) (19). In EVL, these residues are changed to glutamine and alanine, respectively, possibly explaining why phospho-EVL is not completely inhibited with regard to its actin-nucleating capacity. However, we noted that serine 157, in the primary structure distant from the proposed actin-binding site, still exerted some effect on actin nucleation. These data indicate that, despite their extensive sequence similarity, the proteins of the Ena/VASP family have different actin-binding properties and may be differentially regulated (see also below).

**EVL Binding to Profilin and Possible Implications for Actin Dynamics**—The function of profilin binding to Ena/VASP proteins is more enigmatic. Similar to VASP (39) and Aczonz (47), EVL is a preferred partner of profilin IIa, the major profilin II isoform in brain.³ The Ena/VASP proteins tend to form homo- and hetero-oligomers and contain multiple profilin-binding sites, possibly resulting in tethering of several profilin molecules to sites at which they concentrate. The role of profilin may be rather passive by pooling actin monomers that are then shuttled to the EVH2 domain and used for polymerization. Alternatively, a more active role may be possible. We have shown that the combined sequestering effect of high profilin IIa and thymosin β4 concentrations is reversed when profilin IIa binds the proline-rich sequence of VASP (31). Therefore, it is possible that nucleation of actin polymerization by EVL could be enhanced in the presence of profilin. Consistent with this are the observations that addition of the VASP-derived proline-rich peptide arrests or decelerates Listeria motility in PtK2 cells (48) and that profilin is recruited only to motile Listeria in an Ena/VASP-dependent manner (49). It is clear, however, that Ena/VASP proteins can promote Listeria motility independently of profilin (20, 50). Therefore, it is possible that Ena/VASP proteins affect actin dynamics by both profilin-dependent and -independent mechanisms. Preliminary experiments to clarify the function of EVL-profilin complexes in actin assembly indeed show very complex kinetics.⁴

**Regulation of SH3 Domain Binding**—SH3 domains have distinct target specificities (51–55), and the polyproline domains of the Ena/VASP proteins are the least conserved domains in these proteins. This may permit them to interact with different SH3 domain-containing proteins. The polyproline domain of EVL binds to the SH3 domains of Lyn, nSrc, and Abl and, to a lesser extent, to those of Fyn and Src. In contrast, MenA binds only the Abl and Src SH3 domains out of >30 different SH3 domains tested (1),⁵ and VASP binds the Abl and Src SH3 domains (18). It is important to note that this is the first report of a protein that binds stronger to the neuronal splice variant nSrc SH3 domain than to the Src SH3 domain. Since EVL is expressed in neuronal tissues (5), it may be a cellular partner of nSrc. At present, it is unclear whether EVL and the nSrc SH3 domain interact in a cellular context.

Profilin IIa and the Fe65 WW and Abi SH3 domains bind to overlapping regions of the polyproline-rich domain of EVL, illustrating the importance of polyproline motifs in signaling cascades (56). Interestingly, the interaction of EVL with SH3 domains, but not with the Fe65 WW domain or profilin IIa, is regulated by PKA phosphorylation on Ser156, a site close to the polyproline sequence. The phosphorylation decreases the binding of the nSrc and Abi SH3 domains, but not of the Lyn SH3 domain. These data suggest that extra regions outside the

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⁴ A. Lambrechts, C. Ampe, and F. B. Gertler, unpublished results.
⁵ F. B. Gertler, unpublished results.
proline-rich sequence are involved in this interaction (38, 52). The role of the interaction of the Ena/VASP proteins with SH3 domain-containing proteins is still unresolved. SH3 domains may function as simple recruitment factors, bringing the Ena/VASP proteins to an appropriate location in the cell, or may play an active role in signal transduction or actin modulation, similar to Grb2 and N-VASP (neural Wiskott-Aldrich syndrome protein) (57). In addition, due to overlapping binding sites, competition of profilin and SH3 domains may be an additional regulatory mechanism for providing access of SH3 domains to the polyproline domain. We suggest two possible scenarios that are not necessarily mutually exclusive. First, PKA phosphorylation of EVL promotes dissociation of the SH3 domain, whereas profilin can still bind phospho-EVL. Second, a local increase in profilin concentration may compete away bound SH3 domains. Such a system, in combination with the different regulatory mechanisms of actin binding discussed above, would allow fine-tuning of the function of the Ena/VASP proteins.

**EVL Localizes to Dynamic Actin Structures**—Immunolocalization of EVL in transfected Rat2 cells shows that the protein is targeted to the leading edge of the lamellipodium and the distal tips of stress fibers, a pattern very similar to Mena and VASP. In activated primary T-cells and Jurkat T-cells, EVL localizes to the F-actin collar and to the distal tips of microspikes, two regions of dynamic actin polymerization (this work and Ref. 15). The recruitment of EVL to the T-cell receptor complex is mediated by Fyb/SLAP130, a molecule that was recently identified as a ligand for Ena/VASP proteins (15). These data are also consistent with the recently reported observation that VASP and Mena localize to tips of embedded filopodia and to puncta that stabilize the contact with the neighboring cell during intercellular adhesion of epithelial cells (10). Together with their actin-nucleating activity, Ena/VASP proteins are ideally positioned to participate in the development of lamellipodia and to puncta that stabilize the contact with the neighboring cell during intercellular adhesion of epithelial cells (10). Together with their actin-nucleating activity, Ena/VASP proteins are ideally positioned to participate in the development of polarized actin-rich structures in several cellular processes. Depending on the cellular context, individual Ena/VASP proteins may have specific functions. Reinforcing this idea, EVL, but not Mena, could be immunoprecipitated with semaphorin-6A-1, a new isoform of semaphorin-6, a member of a family of proteins that act as ligands for axon guidance receptors. 2

**Ena/VASP Proteins: Involvement in Signaling and Actin Remodeling**—The *in vitro* data presented in this work demonstrate complex and highly regulated interactions between EVL and its partners. EVL participates in several types of protein-protein interactions involving actin, profilin, and signaling molecules such as semaphorin-6A-1 and SH3 and WW domain-containing proteins. The binding sites on EVL for profilin, WW domains, and SH3 domains overlap, and several of these interactions are selectively modulated by PKA phosphorylation of EVL. Therefore, phosphorylation of EVL *in vivo* could substantially alter both the capacity of EVL to promote actin polymerization and the composition and activity of multimeric protein complexes containing EVL. Activated PKA and cGMP-dependent protein kinase are key regulators of the guidance of migrating axonal and dendritic growth cones (58) and in inhibition of the T-cell antigen response and of platelet activation (4, 59, 60). In the latter two cases, this inhibition is accompanied by inhibition of actin polymerization (4, 61), consistent with our observation that phospho-EVL displays reduced and phospho-VASP displays no nucleation of actin polymerization. In VASP-deficient mice, inhibition of platelet aggregation is strongly reduced (7). In addition, collagen-induced aggregation and shape change of VASP null platelets occur faster than in wild-type platelets, although the extent of shape change is identical in both types of platelets. One function of VASP is therefore to inhibit the rate of platelet aggregation, and this function is enhanced by phosphorylation of VASP by PKA and cGMP-dependent protein kinase (6, 7). The role of VASP in retarding the actin-driven processes of platelet aggregation suggests that Ena/VASP proteins may not always act to promote actin assembly *in vivo*.

The function of Ena/VASP proteins in actin dynamics in living cells is likely complex and context-dependent. Studies of fibroblast motility and lamellipodial extension may support this conclusion. Mitochondrial targeting of all Ena/VASP proteins within Rat2 cells results in an increase in the rate of lamellipodial extension and cell motility. The same is observed in fibroblasts isolated from Mena−/−/VASP−− mice, whereas overexpression of Mena or VASP leads to decreased rates of lamellipodial extension and cell motility (8). It is possible either that these phenomena arise by Ena/VASP-mediated inhibition of actin assembly or that Ena/VASP proteins regulate the formation of actin in a way that inhibits cell motility, e.g. by driving actin polymerization in a manner that does not promote productive lamellipodial extension. How exactly the Ena/VASP proteins affect actin polymerization *in vivo* is a question for future experiments. The many ligands for Ena/VASP proteins and possible regulatory mechanisms we have described provide new insights that should help to elucidate the function of this protein family in controlling cell motility and establishing cell morphology.

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**Note Added in Proof**—After submission of this paper, Harbeck et al. (Harbeck, B., Hüttemaier, S., Schlüter, K., Jockusch, B., and Illenberger, S. (2000) *J. Biol. Chem.* 275, 30871–30875) demonstrate that phosphorylation of VASP regulates its interaction with actin, corroborating our present work with EVL. Using a yeast two-hybrid screen, we have identified Profilin I as a binding partner of EVL, suggesting a potential interaction between EVL and Profilin I could occur in *vivo* despite our inability to detect binding in *vivo* (A. V. Kwiatkowski, D. Serna, and F. B. Gertler, unpublished results).

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