Characterization of the Interaction between Zyxin and Members of the Ena/Vasodilator-stimulated Phosphoprotein Family of Proteins*

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Zyxin contains a proline-rich N-terminal domain that is similar to the C-terminal domain in the ActA protein of the bacterium, Listeria monocytogenes. We screened the entire amino acid sequence of human zyxin for Mena-interacting peptides and found that, as with ActA, proline-rich sequences were the sole zyxin sequences capable of binding to Ena/vasodilator-stimulated phosphoprotein (VASP) family members in vitro. From this information, we tested zyxin mutants in which the proline-rich sequences were altered. The reduction in Mena/VASP binding was confirmed by peptide tests, immunoprecipitation, and ectopic expression of zyxin variants at the surface of mitochondria. By transfection assays we showed that zyxin interaction with Mena/ VASP in vivo enhances the production of actin-rich structures at the apical surface of cells. Microinjection into cells of peptides corresponding to the first proline-rich sequence of zyxin caused the loss of Mena/ VASP from focal contacts. Furthermore, these peptides reduced the degree of spreading of cells replated after trypsinization. We conclude that zyxin and proteins that harbor similar proline-rich repeats contribute to the positioning of Mena/VASP proteins. The positioning of Ena/VASP family members appears to be important when the actin cytoskeleton is reorganized, such as during spreading.

Analysis of the human actin cytoskeleton has been greatly aided by the study of a bacterial pathogen, Listeria monocytogenes (1, 2). Listeria enter cells and then recruit actin monomers to their surface, enabling them to form F-actin-rich “comets” and move (3–5). The organization of F-actin within the comet and the generation of force at the interface between the bacterial surface and comet is strikingly similar to what occurs in actin-rich structures of human cells, such as the leading edge of the lamellipodia (6–8). Because of this property, Listeria has been very instructive in identifying proteins that function in the organization of the actin cytoskeleton of mammalian cells.

One protein whose characterization has been aided by study of Listeria is human zyxin. Zyxin is the prototype of a new family of proteins that is located at actin-rich sites in cells of higher eucaryotes (9, 10). By sequence analysis, other human zyxin-like proteins have been identified, such as the LIM-containing lipoma preferred partner (LPP) (11, 12) and the thyroid receptor-interacting protein-6 (13, 14). The hallmark of zyxin and related proteins is an N-terminal domain that is rich in prolines followed by 3 LIM domains (15, 16). The 380-amino acid proline-rich domain of zyxin has several biochemical and cell biological properties in common with the proline-rich domain of Listeria ActA (17, 18). ActA is a Listeria surface protein that is essential for its motility (19, 20). It is composed of an N-terminal domain that interacts with the Arp2/3 complex to generate actin nucleating activity (21) followed by a large proline-rich domain that is believed to be important for accelerating the rate of actin assembly (22–24). The sequence similarity between zyxin and ActA has prompted studies on their functional similarities.

Similarities between the proline-rich domains of zyxin and ActA have been confirmed by demonstrating the presence of common epitopes and by performing cell transfection assays that showed similar effects of the two proteins on cell behavior (17, 18). In addition, both ActA and zyxin bind to members of the Ena/vasodilator-stimulated phosphoprotein (VASP) family of proteins (25–28). In Listeria-infected cells, VASP and Mena (mammalian ena) are located at the site of actin polymerization on the bacteria (25, 27). In cultured mammalian cells, VASP is located at focal adhesions and the distal edge of lamellipodial extensions (9, 29, 30). VASP is implicated in the control of cytoskeletal organization because it binds F-actin (31, 32) and profilin, a 14-kDa protein that forms complexes with G-actin and regulates actin dynamics (33–35). In the case of Listeria movement, disruption of the interaction between ActA and VASP results in Listeria mutants that move more slowly (23, 24). Reconstitution of Listeria movement in vitro with purified proteins has shown that although VASP is not absolutely essential for motility, it increases the rate of bacterial movement (36). The significance of the interaction between zyxin and the Ena/VASP family of proteins has not been directly explored in mammalian cells.

Much of what is postulated regarding the function of the proline-rich region of zyxin arises by analogy to studies of ActA and has not been tested explicitly for zyxin (10). Therefore, we...
have characterized the interaction of Mena/VASP proteins with zyxin by precise mapping of the amino acids required for binding and by testing the consequences for the cell when this interaction is disrupted. Mutation of one to four key phenylalanine residues in the Ena/VASP-binding sites of zyxin progressively abrogates its ability to bind these partners in vivo. In tests in which we place zyxin at the plasma membrane, we find that the presence of VASP- and Mena-binding sites promotes the production of actin-rich structures. In addition, we show that peptides corresponding to the amino acid sequences required for Mena/VASP interaction with zyxin disturb the subcellular distribution of Mena and VASP and inhibit the rate of cell spread on fibronectin. Our results support the hypothesis that zyxin makes a critical contribution to the correct subcellular distribution of Mena and VASP and that this interaction might be important for the restructuring of the actin cytoskeleton.

EXPERIMENTAL PROCEDURES

Synthetic Peptides—Peptides were synthesized and purified at the University of Utah core facility or by Genosys (TX). Peptides were synthesized by the Fmoc solid-phase method using t-butyloxycarbonyl chemistry and resuspended in sterile phosphate-buffered saline, and the pH was adjusted to 7.5 when necessary. Peptide stocks were prepared at a concentration of 20 (120 mM) or 0.2 mg/ml (1.2 mM) and stored in aliquots at −20 °C until needed. The sequence of the Zyxin (67–81)-peptide is PPEDFPLPPPPLAGD.

Analysis of GST-Mena-(6–170) binding to zyxin was determined using a bacterially expressed GST fusion protein resuspended in sterile phosphate-buffered saline and adjusted to 7.5 when necessary. Peptide stocks were prepared at a concentration of 20 (120 mM) or 0.2 mg/ml (1.2 mM) and stored in aliquots at −20 °C until needed. The sequence of the Zyxin (67–81)-peptide is PPEDFPLPPPPLAGD.

The membrane contained three series of overlapping 15-amino acid alanine on EVH1 domain binding to the proline-rich repeats of zyxin.

A second GSTMena-(6–170) was used to probe the SPOTs membrane using a modified blot overlay procedure. Autoradiography was performed at −80 °C using an intensifying screen. Quantitative analysis of protein binding was performed using a PhosphoImager and ImageQuant software (Molecular Dynamics, CA).

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Analysis of GST-Mena-(6–170) binding to zyxin was determined using a custom-made peptide library (SPOTs) conjugated to a cellulose membrane (37); this SPOTs membrane was generously provided by Jürgen Wehland (Braunschweig, Germany). The library consisted of 187 overlapping peptides of 15 amino acids in length that spanned the complete human zyxin protein sequence. Adjacent peptides shared a 5′ overlap. A second SPOTs membrane (Genosys) was used to determine the effect of amino acid substitution for phenylalanine on EVH1 domain binding to the proline-rich repeats of zyxin.

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Construction and Expression of Zyxin Mutants—The human zyxin sequence was cloned into the pALTER1 vector (Promega), and mutagenesis reactions were performed using reagents and protocols contained in the Altered Sites II in vitro mutagenesis kit. Amino acid substitutions were made using mutagenic oligonucleotides that spanned sequences in the N-terminal Region of Human Zyxin. The human zyxin amino acid sequences capable of binding GST-Mena-(6–170) interacted with four series of zyxin-derived peptides (Fig. 3).

RESULTS

The EVH1 Domain of Mena Interacts with Proline-rich Sequences in the N-terminal Region of Human Zyxin—We were interested in probing the physiological significance of the ability of zyxin to bind Ena/VASP proteins. Therefore, as a first step, we have mapped precisely the sites in zyxin that bind to the N-terminal EVH1 domain of Mena. It was shown previously that zyxin displays several proline-rich sequences similar to those in Listeria ActA that serve as docking sites for Ena/VASP family members. However, it was not clear if these were the sole sites in zyxin that had the capacity to bind these proteins. Since our goal was to compromise the ability of zyxin to bind Ena/VASP family of proteins and to explore the physiological consequences of this interaction, we precisely mapped the zyxin amino acid sequences capable of binding GST-Mena-(6–170) using a SPOTs custom immunosynthesized peptide library of 187 peptides that spanned the entire human zyxin sequence. Each peptide was 15 amino acids in length, and adjacent peptides shared a 12-amino acid sequence overlap. This library was probed using radiolabeled, recombinant GST-Mena-(6–170), which contains the Mena EVH1 domain. GST-Mena-(6–170) interacted with four series of zyxin-derived peptides (Fig. 3).

Microscopy—Immunofluorescence analysis of injected cells was performed after a 10-min recovery period at 37 °C. Cells were fixed with 3% paraformaldehyde and permeabilized with 0.2% Triton X-100. Primary antibodies used for immunofluorescence were the B38 anti-zyxin serum (15), an anti-Mena serum (27), and a mouse anti-VASP antibody (Transduction Laboratories, KY). Secondary antibodies used were Texas Red goat anti-rabbit IgG, FITC goat anti-rabbit IgG, and Texas Red goat anti-mouse IgG. Texas Red-phalloidin was used to visualize F-actin (Molecular Probes). Cells were photographed using a Zeiss Axiohot, and developed negatives were scanned and processed by Adobe Photoshop software.

RESULTS

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These peptides correspond to the so-called “proline-rich” sequences in the N-terminal region of zyxin that had been shown previously to bind Ena/VASP family of proteins and that share high degree of similarity with EVH1 domain binding sequences in ActA (18). PhosphorImager analysis was used to quantify the amount of GST-Mena-(6–170) bound to each peptide sequence (Fig. 1C). The first proline-rich sequence of zyxin, specifically the peptide that spanned amino acids 67–81 (Fig. 1, A and B, spot A23), showed the highest degree of binding to GST-Mena-(6–170). Binding of GST-Mena-(6–170) to the fourth proline-rich motif which spanned amino acids 109–123 (Fig. 1, A and B, spot B12) was considerably weaker, although, as described later, still significant.

The zyxin peptide sequences identified by GST-Mena-(6–170) binding contained a phenylalanine residue which, in the case of ActA, had been shown to be essential for VASP and Mena binding (18). We tested the significance of this phenylalanine in the interaction between zyxin-derived peptides and GST-Mena-(6–170) by preparing a membrane in which the conserved phenylalanine residues in peptides A22–24, A23, B5, 6, 9, and 10 were replaced by alanine. Consistent with what was shown for ActA (18), this substitution resulted in a loss of GST-Mena-(6–170) binding to the zyxin-derived peptides (Fig. 1E and data not shown). Importantly, no other EVH1-binding sites were detected in zyxin. The comprehensive characterization of the Ena/VASP-binding sites in zyxin has enabled us to probe the significance of the Mena/VASP binding capacity of zyxin in vivo.

**Mutations That Affect the Proline-rich Repeats in Zyxin**

**D**

**FIG. 2.** Cytoplasmic zyxin variants that lack the first two proline-rich regions fail to displace VASP from focal adhesions. A, HeLa cells were transfected with zyxin variants composed of the N-terminal 380 amino acids of zyxin with a Myc-tag (NtWt) or zyxin in which the proline-rich regions 69–79 and 91–101 were deleted (NtΔ1Δ2). Extracts were analyzed by immunoblotting with anti-Myc antibody. NtΔ1Δ2 migrates faster in gels than would be predicted by the absence of 22 amino acids. B, HeLa cells expressing either the N-terminal 380 amino acids of zyxin (NtWt, B and C) or zyxin in which the proline-rich regions 69–79 and 91–101 were deleted (NtΔ1Δ2, D and E) were analyzed by immunofluorescence microscopy for zyxin production (B and D) and VASP localization (C and E). In cells that express NtΔ1Δ2, VASP staining was retained at focal adhesions (E), as in untransfected cells (see arrow, C), whereas in cells that express the N terminus of zyxin with proline-rich regions intact, VASP staining is displaced (C).

1. A and B). These peptides correspond to the so-called “proline-rich” sequences in the N-terminal region of zyxin that had been shown previously to bind Ena/VASP family of proteins and that share high degree of similarity with EVH1 domain binding sequences in ActA (18). PhosphorImager analysis was used to quantify the amount of GST-Mena-(6–170) bound to each peptide sequence (Fig. 1C). The first proline-rich sequence of zyxin, specifically the peptide that spanned amino acids 67–81 (Fig. 1, A and B, spot A23), showed the highest degree of binding to GST-Mena-(6–170). Binding of GST-Mena-(6–170) to the fourth proline-rich motif which spanned amino acids 109–123 (Fig. 1, A and B, spot B12) was considerably weaker, although, as described later, still significant.
expression of Nt-zyxin, which fails to accumulate in adhesion plaques (Fig. 2B), results in a loss of VASP from these sites (Fig. 2C). In contrast, VASP remained concentrated at focal adhesions in cells producing the NtΔ1Δ2 zyxin variant that lacks two of the proline repeats (Fig. 2D). These results confirm that the proline-rich repeats present in zyxin are important for interaction with an Ena/VASP family member in vivo.

We further tested the importance of the proline-rich repeats of zyxin for its interaction with Ena/VASP family members in vivo by immunoprecipitation. We showed previously that targeting zyxin to the inner leaflet of the plasma membrane, via the addition of a C-terminal CAAX sequence from the protein k-Ras, resulted in changes in actin assembly and organization (17). Based on the findings with the SPOTS membrane with zyxin (Fig. 1) and with ActA (18), we made site-specific mutations that resulted in the substitution of phenylalanine residues at positions 71, 93, 104, and 114 within the proline-rich Ena/VASP-docking sites. These substitutions were introduced into a construct engineered for the expression of the first 380 amino acids of zyxin, as well as a C-terminal Myc epitope tag and a CAAX sequence. Immunoprecipitation experiments showed that Mena exists in a complex with the zyxin variant containing unaltered proline-rich sequences (Fig. 3). Substitution of alanine at position 71 of the first proline-rich sequence resulted in a reduction in Mena association with zyxin. Mutant proteins in which two (F71A/F93A) or three (F71A/F93A/F104A) substitu-

The Effects upon the Actin Cytoskeleton Induced by Membrane-targeted Zyxin Can Be Separated into VASP-dependent and VASP-independent Types—We tested the role of Ena/VASP members in producing these rearrangements by expressing the N-terminal zyxin (F71A/F93A/F104A/F114A) mutant in which all Ena/VASP-docking sites were mutated. VASP staining was no longer detected at the mitochondria (Fig. 4, C and D). These results reveal that the EVH1-binding site containing the Phe-114 is likely active in vivo, despite that it gave the weakest signal by SPOTS analysis and did not recruit sufficient Mena or VASP for detection by Western blotting. For this analysis, it was clear that in order to test the significance of zyxin binding with Ena/VASP members it was necessary to mutate all four proline-rich sites.

Ala variants in cells by ectopically expressing zyxin at the surface of mitochondria. This method permits the examination of protein assemblages by immunofluorescence and has been previously applied to ActA, zyxin, and a zyxin-related protein, LPP (39, 42, 43). As expected, expression of zyxin Nt at the mitochondria resulted in accumulation of VASP at these organelles (Fig. 4, A and B). Interestingly, even when the triple mutant (F71A/F93A/F104A) was expressed, VASP staining could still be detected in association with the mitochondria, although the staining was weaker than when wild-type zyxin sequences were utilized (data not shown). However, when we expressed Nt(ΔF71,93,104,114) in which all EVH1-binding sites were altered, VASP staining was no longer detected at the mitochondria (Fig. 4, C and D). These results showed that Mena exists in a complex with the zyxin variant containing unaltered proline-rich sequences (Fig. 3). Substitution of alanine at position 71 of the first proline-rich sequence resulted in a reduction in Mena association with zyxin. Mutant proteins in which two (F71A/F93A) or three (F71A/F93A/F104A) substitutions were made showed very little binding of Mena by immunoblotting. Thus, the degree of Mena interaction with zyxin depended on the number of unaltered proline-rich repeats in zyxin. Similar results were obtained when these zyxin mutants were tested for their ability to interact with VASP (data not shown).

To explore further the importance of zyxin proline-rich sequences for docking of Ena/VASP proteins, we examined the interaction between VASP and wild-type zyxin or zyxin Phe-
targeting motif (NtWt, A acids) of zyxin in frame with a Myc epitope and a CAA transfected with plasmids encoding the N-terminal domain (380 amino not require Ena/VASP-docking sites.

**Actin-rich Surface Projections Are Enhanced by Nt-zyxin-CAAX That Contains Ena/VASP-docking Sites**—We further explored the importance of the capacity of zyxin to bind Ena/VASP members by comparing the effect of expression of wild-type and the N-terminal (F71A/F93A/F104A/F114A) zyxin mutant in NIH3T3 cells because these cells produce striking actin-rich projections at the apical surface upon zyxin production (17). Cells were stained with rhodamine-phalloidin to mark F-actin and with anti-Myc epitope antibody to detect transfected cells that express membrane-targeted Nt-zyxin. In these cells, actin-rich surface structures were observed in 67% of the cells counted (S.D. = 4%). These structures varied in their abundance on the surface of a cell; however, to score positive, at least half the apical surface area of the cell had to be covered with such structures (Fig. 6, A and B). In contrast to the high level of surface ruffling observed in cells expressing Nt-zyxin CAAX, actin-rich surface projections in cells producing zyxin Nt(F71A/F93A/F104A/F114A) were consistently and significantly less frequent than cells producing unaltered zyxin (Fig. 6, C and D). As with Nt-zyxin these surface structures were still occasionally present in zyxin Nt(F71A/F93A/F104A/F114A)-producing cells; however, they were consistently reduced in size and in number, and only in 32% of the cases did they cover at least half of the cell surface (S.D. = 8%). Because the presence of docking sites on zyxin for Ena/VASP members had an effect upon the actin cytoskeleton, although under artificial conditions, we continued to explore the role of Ena/VASP members in zyxin-dependent actin cytoskeleton events using other methods.

**Introduction of a Zyxin-derived Proline-rich Peptide into Cells Causes Mislocalization of Ena/VASP Members and Reduces the Degree of Cell Spreading on Fibronectin**—As described above, comparison of the effects of membrane-targeted Nt-zyxin and the Nt(F71A/F93A/F104A/F114A) mutant form of zyxin revealed a role for Ena/VASP binding capacity in the zyxin-dependent changes in actin assembly and organization that we observe by employing the CAAX assay. In an effort to study the possible importance of the ability of zyxin to associate with Mena/VASP by a more quantitative method, as the actin cytoskeleton is undergoing rearrangements, we examined the process of cell spreading. We have utilized a zyxin-derived peptide, Zyx-(67–81)-(PPEDFLPPPPLAGD), which corresponds to the amino acid sequence of the first proline-rich repeat (spot A23, Fig. 1A) and primary binding site for Ena/VASP family members in zyxin, to inhibit competitively the ability of zyxin to dock these proteins in vivo.

First we examined the effect of introducing the Zyx-(67–81)-peptide into well spread PtK2 cells. As anticipated, the introduction of the Zyx-(67–81)-peptide into cells by microinjection resulted in the reduction of Mena (Fig. 7, A and B) and VASP (data not shown) from their normal subcellular locations. Introduction of the Zyx-(67–81)-peptide into cells did not appear to affect the subcellular distribution of zyxin (Fig. 6, C and D). In contrast, introduction of the Zyx-(67–81)-(F71A) peptide had no apparent effect on Mena localization (Fig. 6, E and F). These results further confirm that the Ena/VASP interaction with zyxin detected in vitro likely occurs in vivo and is important for the normal subcellular distribution of Ena/VASP family of proteins.

Once we had confirmed the ability of the Zyx-(67–81)-peptide to reduce the amount of Ena/VASP members from their normal subcellular location, we examined the effect of perturbing the zyxin-Ena/VASP interaction on the process of cell spreading, an activity that depends on the assembly and reorganization of the actin cytoskeleton. The Zyx-(67–81)-peptide or the Zyx-(67–81)-(F71A) peptide was mixed with Texas Red-dextran to serve as a marker for uptake and was introduced into PtK2 cells in suspension by electroporation. In a typical experiment, over 90% of the cells examined displayed internal Texas Red-dextran following electroporation (data not shown). After a short recovery period, cells were plated onto fibronectin-coated coverslips and allowed to spread. Cells into which the control (non-inhibitory) Zyx-(67–81)-(F71A) peptide was introduced were well spread by 1.5 h after plating (Fig. 8A) and achieved an average area of 409 μm² by 3 h (Fig. 8C). These cells spread at a rate comparable to cells subjected to the electroporation protocol in the absence of peptide (data not shown). In contrast, cells into which the Zyx-(67–81)-peptide was introduced displayed an average area of only 203 μm² by 1.5 h and 260 μm² by 3 h, which represents a 36% reduction in cell area compared with controls (Fig. 8, B and C). Since the Zyx-(67–81)-peptides have been shown to disrupt the interaction between zyxin and Ena/VASP family members, these results suggest that this interaction is important during cell spreading.

**DISCUSSION**

One strategy for defining the precise role(s) of zyxin in vivo is to characterize the functional significance of the ability of zyxin to bind to its partners. In this report, we have focused our attention on the interaction of zyxin with Ena/VASP family members, proteins implicated in actin assembly processes. We describe the zyxin amino acid sequences required for binding to Mena and VASP, and we examine the consequences of disrupting this interaction in vivo.

We have scanned the entire zyxin amino acid sequence by SPOTs analysis using the EVH1 domain of Mena to identify sequences capable of binding Ena/VASP family members (27). We identified four proline-rich peptides in human zyxin that bind to the EVH1 domain. These regions of zyxin represent the sequences that exhibit high similarity to the ActA protein of Listeria (18), which have also been named ABM-1 sites (44, 45). As was described for the Mena-binding peptides in the Listeria
ActA protein, the conserved phenylalanine residue serves as a critical predictor of Mena-binding capacity (18). Detailed analysis of the EVH1 domain of VASP has identified two conserved hydrophilic regions that are necessary for the interaction with the proline-rich sequences, such as those found in ActA and zyxin (46). Resolution of EVH1 domain by x-ray crystallographic studies confirms the presence of a pocket that accepts the phenylalanine in proline-rich sequences of zyxin and ActA (47, 48).

Because Listeria ActA and zyxin display four proline-rich-binding sites for Ena/VASP, it is possible that they have the capacity to amplify the local concentration of these proteins. Ena/VASP proteins can homo-oligomerize and are thought to form tetramers, thus if each proline repeat could dock a tetrameric Ena/VASP protein, 16 Ena/VASP monomers would be expected to be docked on each fully occupied ActA or zyxin. Our results illustrate that as the proline repeats of zyxin are compromised, less Ena/VASP can be co-immunoprecipitated with zyxin. Indeed, by the mitochondria targeting assay, which is more sensitive than immunoprecipitation assays, we were able to show that all four EVH1-binding sites must be altered to compromise maximally the interaction between zyxin and VASP. However, it should be pointed out that the precise molar ratio of zyxin to Ena/VASP protein in the complex is not known. Although it is clear from studies using the SPOTs membrane and individual zyxin-derived proline-rich peptides that these short sequences are sufficient to bind Ena/VASP proteins in vitro, it is also plausible that the zyxin protein may cooperatively wrap around a single Ena/VASP tetramer with each proline-rich sequence docking one Ena/VASP monomer. In this regard, it is interesting that a mutation in Drosophila ena that results in lethal nervous system disorganization affects the ability of the Ena protein to homo-oligomerize and to bind zyxin (28).

In an effort to evaluate the role of zyxin in binding of Ena/VASP family members within living cells, we examined the possibility that expression of a cytosolic zyxin variant would cause Ena/VASP members to be displaced from focal adhesions. Consistent with biochemical findings showing that zyxin harbors multiple binding sites for Ena/VASP members (this report

![Image](image_url)
and Refs. 18 and 26–28), VASP was lost from focal adhesions of transfected cells that overproduced the N terminus of zyxin, a construct that would be expected to compete with endogenous zyxin for Ena/VASP partners. Overexpression of zyxin (N1Δ132), which lacks two of the four proline-rich docking sites for Ena/VASP members, does not lead to detectable mislocalization of VASP. Furthermore, the interaction of Ena/VASP members with zyxin was also disrupted by injection of peptides that mimic the amino acids 67–81 of zyxin that encompass one proline-rich sequence. The displacement of Mena and VASP from focal adhesions has also been described in experiments in which similar proline-rich peptides derived from ActA sequences were injected into cells (18, 27, 39). These results illustrate that the interaction between zyxin and Ena/VASP family of proteins that occurs via the proline rich in vitro also occurs in vivo and is a property common to this motif. Genetic evidence from studies in Drosophila has revealed that mutations that disturb the ability of Ena to bind zyxin, and presumably adversely affect its proper localization, lead to failure of nervous system development (28). It should be pointed out, however, that there may be other proline-rich ligands that contribute to the targeting of Ena/VASP partners to particular subcellular locations. Indeed, both vinculin and robo have Ena/VASP-docking sites (49–52) and Fyn-binding protein (Fyb/SLAP) has also been identified as a ligand for Ena/VASP protein in T-cells (53).

Our studies also suggest that the placement of zyxin within a cell can affect the distribution of the Ena/VASP family of proteins. Zyxin targeted to mitochondria, for example, appears to recruit VASP proteins away from focal adhesions. Moreover, in cells injected with the Zyx-(67–81)-peptide, zyxin localization remained unchanged, revealing that zyxin likely directs Mena and VASP to F-actin-rich sites rather than the converse possibility that Mena or VASP directs zyxin to particular subcellular domains. The ligand that directs zyxin to focal adhesions is not yet known. Determination of how zyxin is targeted to the focal adhesions is likely to be of interest for understanding cell motility.

One experimental system to test the role of zyxin in cells is by transfection assays in which zyxin and zyxin variants are placed at the inner leaflet of the plasma membrane via an in-frame CAAX motif (17). This approach was used to characterize the functional similarities between the C-terminal domain of ActA and the N-terminal domain of zyxin, domains that contain similar proline-rich sequences. Previously we had proposed that a variety of actin cytoskeleton defects caused by the ectopic expression of the N-terminal domain of zyxin might be due to VASP binding. In experiments in which we tested zyxin mutants mutated at the four EVH1-binding sites, F71A/F93A/F104A/F114A, we were able to separate zyxin-induced changes in the actin cytoskeleton that were dependent upon Ena/VASP docking from those that were not. For example, disruption of stress fibers and generation of homogenous phalloidin staining did not require Ena/VASP-docking sites, suggesting that other zyxin-binding partners might be responsible for this effect.

In contrast, the CAAX assay had revealed a zyxin-specific phenotype, the production of actin-rich structures at the dorsal face of cells. Despite that this is an artificial system for producing actin-rich structures, it points toward an Ena/VASP-independent role for zyxin in the control of F-actin because cells expressing the zyxin mutant F71A/F93A/F104A/F114A produced these structures at a significantly lower frequency. The precise origin of these structures is not known; however, it might resemble another system in which actin-rich structures were produced by receptor-mediated clustering (54). In that system by induction of WASP/CDC42 actin-rich filopodia were produced and zyxin, and VASP were recruited. We noted that Ena/VASP binding might not be absolutely required for the zyxin-induced surface structures; rather, Ena/VASP members may cooperate with other zyxin-binding partners. One candidate for involvement in cytoskeletal events that require the N-terminal domain of zyxin is α-actinin, which binds directly to zyxin via a motif near the N terminus (42, 55, 56).

To examine the interaction of zyxin with Ena/VASP family members by another approach, we introduced the peptides Zyx-(67–81) and Zyx-(67–81)-(F71A) into cells and measured their rate of spreading after trypsinization. With peptides that represent the first proline-rich repeat of zyxin but not those in which a phenylalanine was substituted for an alanine, we observed a reduction in the rate of spreading after plating on a
substrate. It is possible that by disrupting the delivery of Ena/VASP members to zyxin-containing sites, the capacity of a cell to generate F-actin at specific sites might also be reduced. There are several observations that support this hypothesis. During spreading of trypsinized cells, there is a global increase in F-actin, including at the edge of the cell (57, 58). Injection of ActA-related peptides, which are very similar to those derived from zyxin, will arrest Listeria movement in infected cells (59). In addition, the peptides had no effect upon adherent cells in which the actin cytoskeleton was at steady state, as shown here, and as has been previously reported for injection of ActA-derived peptides (27). Spreading of trypsinized cells might offer new opportunities to test processes that require actin polymerization.

Proline-rich sequences similar to those found in zyxin are also present in proteins such as LPP and vinculin (43, 49, 50); therefore, we cannot rule out the possibility that mimicking peptides would disrupt these interactions as well as that of zyxin. Recent evidence suggests that such a broad approach might be necessary to detect the consequences of zyxin and VASP binding upon cells. In mice in which the VASP gene is disrupted, fibroblast cells show no obvious abnormalities in adhesion or migration (60, 61). It is only in cells, such as platelets from the same animals where Mena is not expressed, that any differences can be detected (60, 61). The possibility of overlapping function between VASP and related proteins is also suggested from studies of transgenic flies in which human VASP can functionally replace Mena and VASP members to zyxin-containing sites, the capacity of a cell to generate F-actin at specific sites might also be reduced. There are several observations that support this hypothesis. During spreading of trypsinized cells, there is a global increase in F-actin, including at the edge of the cell (57, 58). Injection of ActA-related peptides, which are very similar to those derived from zyxin, will arrest Listeria movement in infected cells (59). In addition, the peptides had no effect upon adherent cells in which the actin cytoskeleton was at steady state, as shown here, and as has been previously reported for injection of ActA-derived peptides (27). Spreading of trypsinized cells might offer new opportunities to test processes that require actin polymerization.

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Zyxin and VASP Interaction

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