THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 29, Issue of July 21, pp. 22503–22511, 2000
Printed in U.S.A.

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

Received for publication, February 28, 2000, and in revised form, April 28, 2000
Published, JBC Papers in Press, May 2, 2000, DOI 10.1074/jbc.M001698200

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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 bacteria, *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)[1] (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

* This work was supported in part by grants from the National Institutes of Health, the Huntsman Cancer Institute at the University of Utah, and the CNRS (Sciences de la Vie). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: LPP, LIM-containing lipoma preferred partner; VASP, vasodilator-stimulated phosphoprotein; GST, glutathione *S*-transferase; FITC, fluorescein isothiocyanate; BSA, bovine serum albumin; zyx, Zyxin; Nt-Wt, N-terminal 1–380 amino acids of wild-type zyxin.

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 spreading 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 with an N-terminal cysteine. Lyophilized peptides were 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 Zyx-(67–81)-peptide is PPEDFPLPPPPLAGD, and the sequence of the Zyx-(67–81)-F71A peptide is PPEDAPLPPPPLAGD.

Protein Binding Studies and SPOTs Analysis—Protein binding studies were performed using a bacterially expressed GST fusion protein containing amino acids 6–170 of the N terminus of Mena (kindly supplied by Frank Gertler), which includes the EVH1 domain (GST-Mena-(6–170)) (27) that was radiolabeled with ^{32}P using bovine heart muscle kinase (Sigma). Labeled GST-Mena-(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 PhosphorImager and ImageQuant software (Molecular Dynamics, CA).

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 12-amino acid sequence 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. The membrane contained three series of overlapping 15-amino acid peptides corresponding to the first three proline-rich repeats in zyxin and three corresponding series of peptides in which the amino acid substitution had been made.

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 substitution of alanine for phenylalanine at positions 71, 93, 104, and 114 in human zyxin were constructed using mutagenic oligonucleotides that produced a codon change from phenylalanine to alanine. Deletion mutants were constructed using mutagenic oligonucleotides that spanned sequences 5′ and 3′ of the deleted nucleotides. The deleted codons were those for amino acids 69–79 inclusive (Δ1) and amino acids 91–101 inclusive (Δ2). Mutagenesis reactions were verified by sequence analysis. Fragments containing the mutated sequences in human zyxin were introduced by substitution into the *NcoI*/*BglII* site of a eucaryotic expression vector containing the N-terminal 380 amino acids of zyxin, except for Nt-zyxin (F71A/F93A/F104A/F114A) (17). The expressed zyxin was tagged at the C terminus with the epitope for the mouse 9E10 anti-Myc antibody, followed by 18 amino acids containing the CAA*X* membrane-targeting motif of k-Ras (38). For expression of zyxin at the mitochondria, DNA encoding the zyxin Nt domain was placed in frame with the membrane anchor sequence of ActA (39).

HeLa cells were transfected by calcium phosphate DNA precipitation (40). Immunoblotting with anti-Myc antibody was used to confirm expression of zyxin constructs. Extracts were prepared as described (41), and epitope-tagged zyxin and associated proteins were immunoprecipitated from cell lysates with the anti-Myc antibody. Protein mixtures were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and analyzed by immunoblotting with anti-VASP or anti-Mena antibodies.

In experiments designed to determine the ability of zyxin mutants to effect the cytoskeletal changes previously shown to be caused by membrane targeting of zyxin, DNA constructs expressing zyxin variants were introduced into HeLa and NIH3T3 cells by transient transfection. Following transfection, cells were fixed and processed for fluorescent double labeling. The actin cytoskeleton was visualized using rhodamine-phalloidin (Molecular Probes, OR). Expressed membrane-targeted zyxin was localized by indirect immunofluorescence using monoclonal antibodies against the Myc tag, followed by FITC-conjugated secondary antibodies. At least 100 cells were counted for each case, in three separate experiments. Data are reported as percentage ± 1 S.D.

Microinjection Experiments—*Potoo tridactylis* kidney (PtK2) epithelial cells were plated onto glass coverslips 18–36 h prior to microinjection and grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and 2% penicillin/streptomycin. Cells were injected using an Eppendorf micromanipulator. The peptide concentration in the injection needle was 0.6 mM. FITC-BSA was co-injected with peptides in experiments where cells were also examined by immunofluorescence.

Cell Spreading Experiments—PtK2 cells were grown to 80–90% confluence in 100-mm dishes. Cells were trypsinized, washed, and resuspended in sterile phosphate-buffered saline plus 10 mM $MgCl_2$ at a concentration of 2.5×10^6 cells/ml. 0.1 mg/ml of M_r 3000 Texas Red-dextran (Molecular Probes, OR) was added to the cell suspension to serve as a marker for introduction of peptides into cells. Peptides were present at a concentration of 0.4 mM. 0.5 ml of cell suspension was placed in 2-mm gap cuvettes and electroporated using a Electrocell Manipulator 600 (BTX Inc., San Diego). Cells were placed on ice for 15 min following electroporation, washed, and plated onto fibronectin-coated coverslips. At 1.5 and 3 h after plating, cells were fixed and stained using FITC-phalloidin (Molecular Probes). Cells were photographed using a Zeiss Axiophot, and the developed negatives were scanned using Adobe Photoshop software, and cell areas were determined for a population of cells using NIH Image software. Data are reported as the mean area ± S.E. 150–200 cell area measurements were determined for each time point.

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 Axiophot, and developed negatives were scanned and processed by Adobe Photoshop software.

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 of proteins (18). 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-synthesized 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.

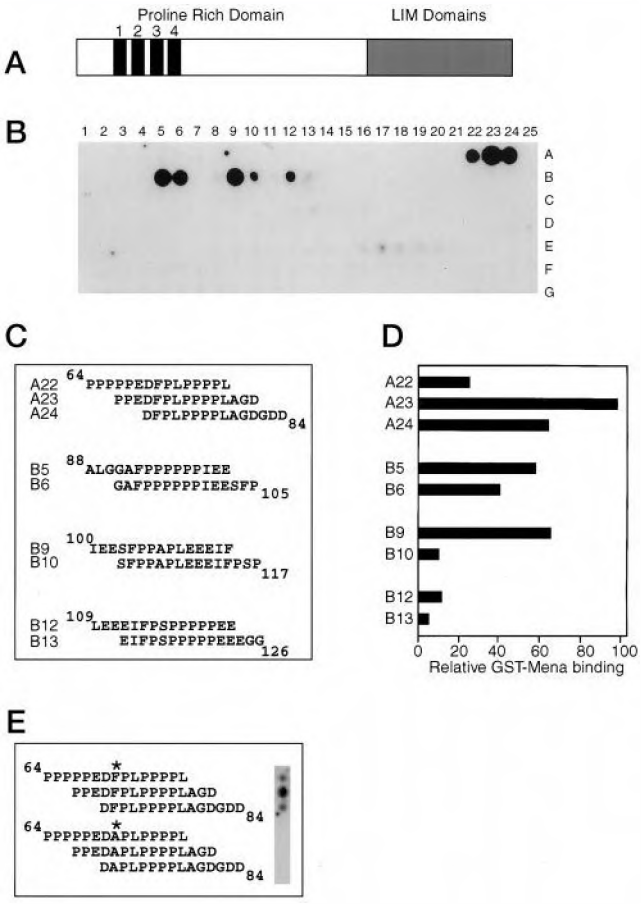

FIG. 1. **SPOTs analysis of GST-Mena-(6–170) binding to human zyxin peptide sequences.** *A*, schematic drawing of the four proline-rich repeats in zyxin is shown. Amino acid sequences are given in *C*. *B*, GST-Mena-(6–170) interacts with proline-rich sequences of zyxin. ^{32}P-Labeled GST-Mena-(6–170) was used to probe a library of zyxin-derived peptides and found to interact most prominently with four series of peptides containing proline-rich sequences. The peptides on the grid are identified by their column (*1–25*) and row (*A–H*) position. *C*, the sequences and alignment of these groups of GST-Mena-(6–170)-interacting peptides are shown. *D*, zyxin peptides vary in their binding to GST-Mena-(6–170). The degree of binding to GST-Mena-(6–170) was determined by PhosphorImager analysis. The signal obtained with the peptide Zyx-(67–81) (*A23*) in zyxin was defined as 100% of maximal binding. The grid position number on the SPOTs membrane and the amino acid numbers are shown. *E*, the conserved phenylalanine residue in the zyxin proline-rich motifs is required for Ena/VASP protein binding activity. A membrane-conjugated peptide array containing the first series of zyxin peptides, Zyx-(64–78), *A22*; Zyx-(67–81), *A23*; Zyx-(70–84), *A24*, and complementary peptides in which a phenylalanine residues at position 71 had been replaced with alanine (marked with an *asterisk*) were tested for GST-Mena-(6–170) binding. Substitution of phenylalanine by alanine resulted in a complete loss of binding activity for all peptides.

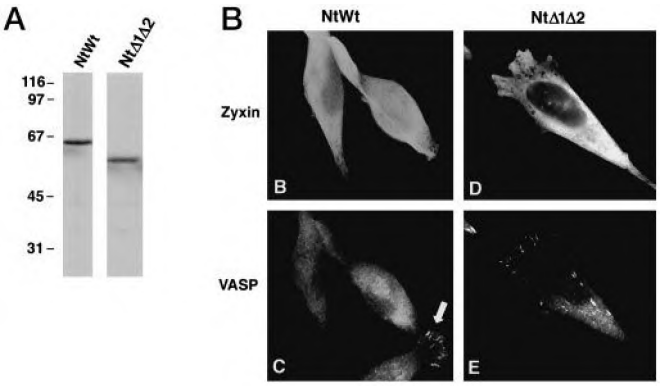

FIG. 2. **Cytoplasmic zyxin variants that lack the first two proline-rich regions fail to displace VASP from focal adhesions.** *A*, HeLa cells were transfected with zyxin variants composed of the N-terminal 380 amino acids of zyxin with a Myc-tag (*NtWt*) or zyxin in which the proline-rich regions 69–79 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 quantitate the amount of GST-Mena-(6–170) bound to each peptide sequence (Fig. 1*C*). 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, 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. 1*E* 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 Disturb Mena and VASP Binding to Zyxin in Vivo—The N-terminal domain of zyxin has previously been shown to be sufficient for interaction with Mena and VASP in overlay assays (17). Here we have examined the effects of altering the proline-rich region of zyxin *in vivo*. Site-directed mutagenesis was used to construct a zyxin plasmid (NtΔ1Δ2) encoding the N-terminal domain of zyxin in which amino acids 69–79 and 91–101 were deleted. The NtΔ1Δ2 plasmid encodes a protein product that lacks the first two proline-rich sequences that together constitute nearly 70% of the Mena binding capacity that we observed. Expression constructs encoding the wild-type N-terminal domain of zyxin (NtWt) or NtΔ1Δ2 zyxin were introduced into cells by transient transfection, and protein expression was confirmed by immunoblotting (Fig. 2*A*). Both proteins were produced in similar amounts. Consistent with the reduction in proline content, the NtΔ1Δ2 species migrated faster on gels than the unmodified Nt version (15).

If the proline repeats of zyxin play a central role in docking Ena/VASP family members, we predicted that the production of a zyxin variant that failed to localize to adhesion plaques would act as a dominant negative version of zyxin that would recruit Ena/VASP family members away from their normal residence in the focal adhesions. Consistent with that view,

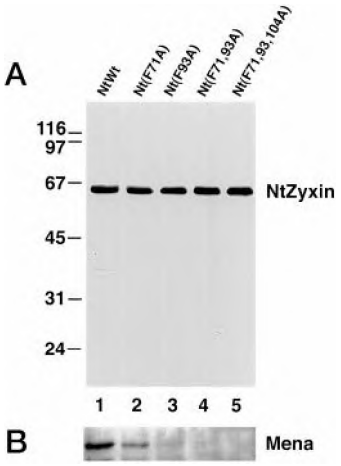

FIG. 3. **Substitution of conserved phenylalanines in zyxin reduces Mena binding *in vivo*.** *A*, the N-terminal domain (380 amino acids) of zyxin in frame with a Myc epitope tag (*lane 1*, *NtWt*) and variants harboring phenylalanine to alanine mutations (*lane 2*, *F71A*; *lane 3*, *F93A*; *lane 4*, *F71,93A*; *lane 5*, *F71,93,104A*) were expressed by transient transfection in HeLa cells. Extracts were examined by immunoblotting with anti-Myc antibody to confirm protein levels. *B*, NtWt zyxin and Nt(F-A) variants were isolated by immunoprecipitation with anti-Myc antibodies. The immunoprecipitates were then analyzed for the presence of Mena by immunoblotting with anti-Mena antiserum. A relatively strong Mena signal was detected in the NtWt zyxin (*lane 1*) sample. Mutation of phenylalanine 71 (*lane 2*) or 93 (*lane 3*) resulted in a reduced Mena binding, whereas alteration of both phenylalanine 71 and 93 (*lane 4*) or 71, 93, and 104 (*lane 5*) reduced Mena binding to levels below detection.

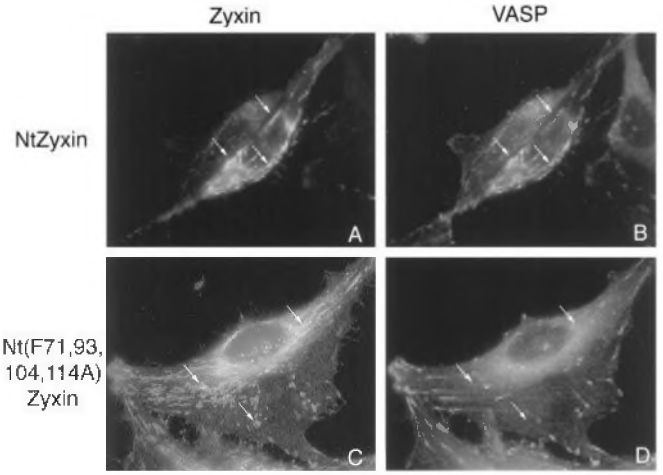

FIG. 4. **All four EVH1 binding domains must be mutated in order to disrupt zyxin VASP binding.** HeLa cells were transiently transfected with cDNAs encoding zyxin Nterm-Mito or zyxin Nterm-Mito F71A/F93A/F104A/F114A. The protein products contain a membrane anchor domain of ActA, which causes them to localize at the surface of mitochondria. Cells were double-stained with anti-zyxin (*A* and *C*) and anti-VASP (*B* and *D*) antibodies. The VASP protein was recruited to the mitochondria by wild-type zyxin N terminus but not zyxin N-terminal F71A/F93A/F104A/F114A, see *arrows*. Note that in the absence of VASP recruitment to the mitochondria, VASP staining at focal adhesion plaques is more intense (*D*).

expression of Nt-zyxin, which fails to accumulate in adhesion plaques (Fig. 2*B*), results in a loss of VASP from these sites (Fig. 2*C*). In contrast, VASP remained concentrated at focal adhesions in cells producing the NtΔ1Δ2 zyxin variant that lacks two of the proline repeats (Fig. 2*D*). 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 or at position 93 in the second 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-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-zyxin (F71A/F93A/F104A/F114A) in which all EVH1-binding sites were altered, 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.

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 compromised. In HeLa cells expressing the unaltered membrane-targeted Nt-zyxin, endogenous stress fibers were disrupted, and the cells presented a homogenous F-actin staining as had been previously reported (Fig. 5, *A* and *B*) (17). Somewhat surprisingly, we observed alterations in the actin cytoskeleton upon expression of the membrane-targeted N-terminal zyxin (F71A/F93A/F104A/F114A) mutant, even when all four Ena/VASP-binding sites are compromised (Fig. 5, *C* and *D*). As with the wild-type zyxin, stress fibers were no longer present, and F-actin was distributed throughout the cytoplasm. Thus, the N-terminal region of zyxin must harbor sequences in addition to the Ena/VASP-binding sites that have the potential to stimulate cy-

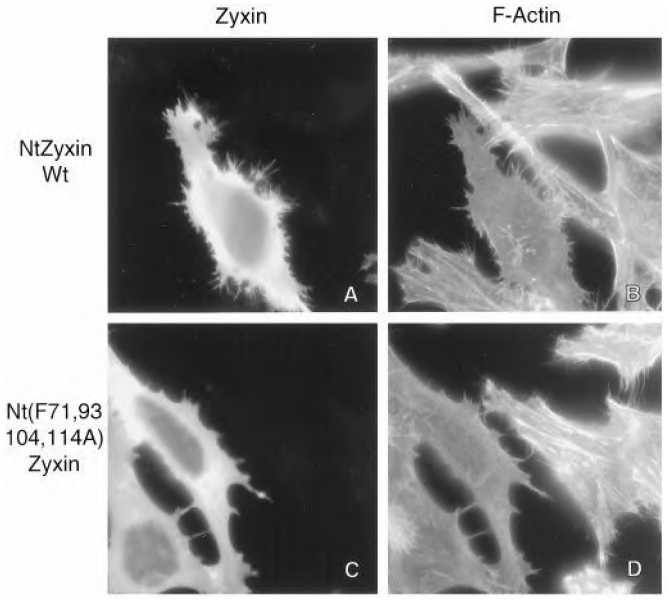

FIG. 5. **Zyxin-CAAX-mediated disruption of stress fibers does not require Ena/VASP-docking sites.** HeLa cells were transiently transfected with plasmids encoding the N-terminal domain (380 amino acids) of zyxin in frame with a Myc epitope and a CAAX membrane-targeting motif (NtWt, *A* and *B*) or a variant with phenylalanine to alanine mutations (*Nt(F71,93,104,114A)*, *C* and *D*). Membrane-targeted zyxin was detected with an anti-Myc antibody (*Zyxin*, *A* and *C*). F-actin was detected by rhodamine-phalloidin staining (*B* and *D*). The absence of VASP binding does not affect the ability of zyxin to alter the actin cytoskeleton in this manner.

toskeletal reorganization.

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)-(PPEDFPLPPPPLAGD), which corresponds to the amino acid sequence of the first proline-rich repeat (*spot A23*, Fig. 1*A*) 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. 7, *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 (M_r 3000) 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. 8*A*) and achieved an average area of 409 μm^2 by 3 h (Fig. 8*C*). 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 μm^2 by 1.5 h and 260 μm^2 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*

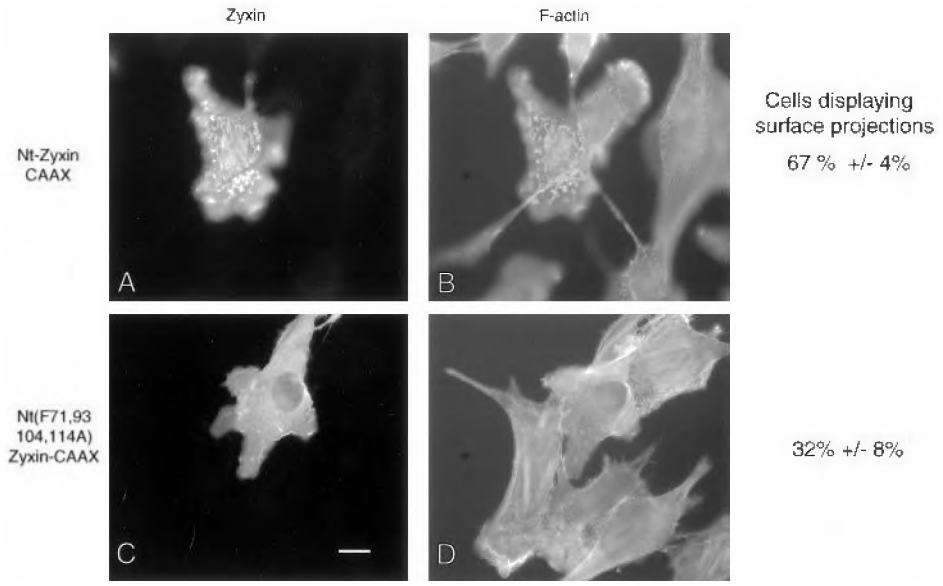

FIG. 6. **Actin-rich surface structures induced by Zyxin-CAAX are enhanced by the presence of Ena/VASP-docking sites.** NIH3T3 cells were transiently transfected with plasmids encoding the N-terminal domain (380 amino acids) of zyxin in frame with a Myc epitope and a CAAX membrane-targeting motif (NtWt, *A* and *B*) or a variant with phenylalanine to alanine mutations (*Nt(F71,93,104,114A)*, *C* and *D*). In these images, the plane of focus is at the apical surface of the cells in order to more clearly see surface structures; the remaining actin cytoskeletal structures are outside of the plane of focus. The apical face is shown of NIH3T3 cells expressing Nt-zyxin-CAAX contains many actin-rich structures that are also stained for zyxin. Of these cells, 67% display F-actin-rich structures at their surface. These structures were less frequently present in cells expressing *Nt(F71,93,104,114A) zyxin-CAAX* (*C* and *D*) as only 32% of these cells display such structures shown in *A*, whereas the majority of cells display far fewer or smaller actin-filled structures (*C*). Cell counts were made from the results of three experiments, in which 100 cells were counted each time. Standard deviations are shown. *Bar* represents 10 μm.

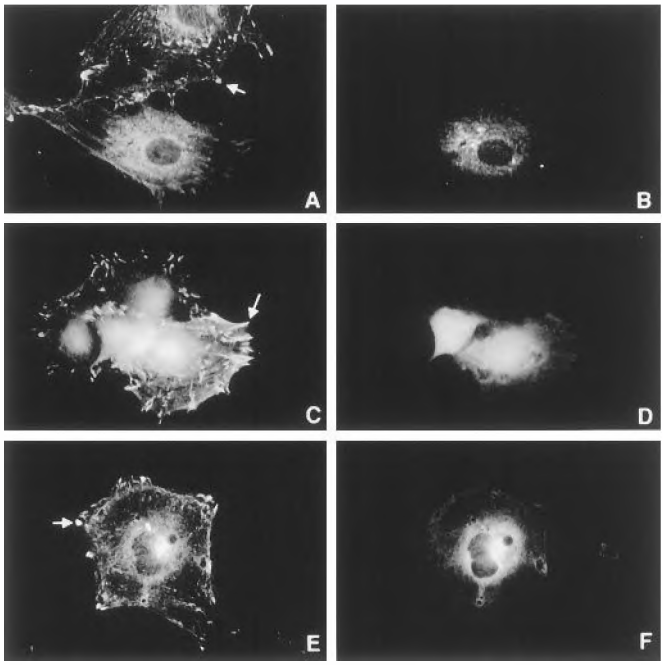

FIG. 7. **Injection of the Zyx-(67–81)-peptide into cells causes the mislocalization of Mena.** Cells were injected with a fluorescent marker (BSA-FITC) and either Zyx-(67–81)-peptide or Zyx-(67–81)-F71A peptide and were subsequently fixed and prepared for indirect immunofluorescence. FITC-BSA was used as a marker for identification of injected cells (*B*, *D*, and *F*). Injection of the Zyx-(67–81)-peptide resulted in a strong reduction of Mena at focal contacts (Mena antibody, *A*). Injection of the Zyx-(67–81)-peptide did not result in zyxin displacement (zyxin antibody, *C*), indicating that focal contacts were still intact. Injection of Zyx-(67–81)-(F71A) peptide did not result in Mena mislocalization (Mena antibody, *E*).

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

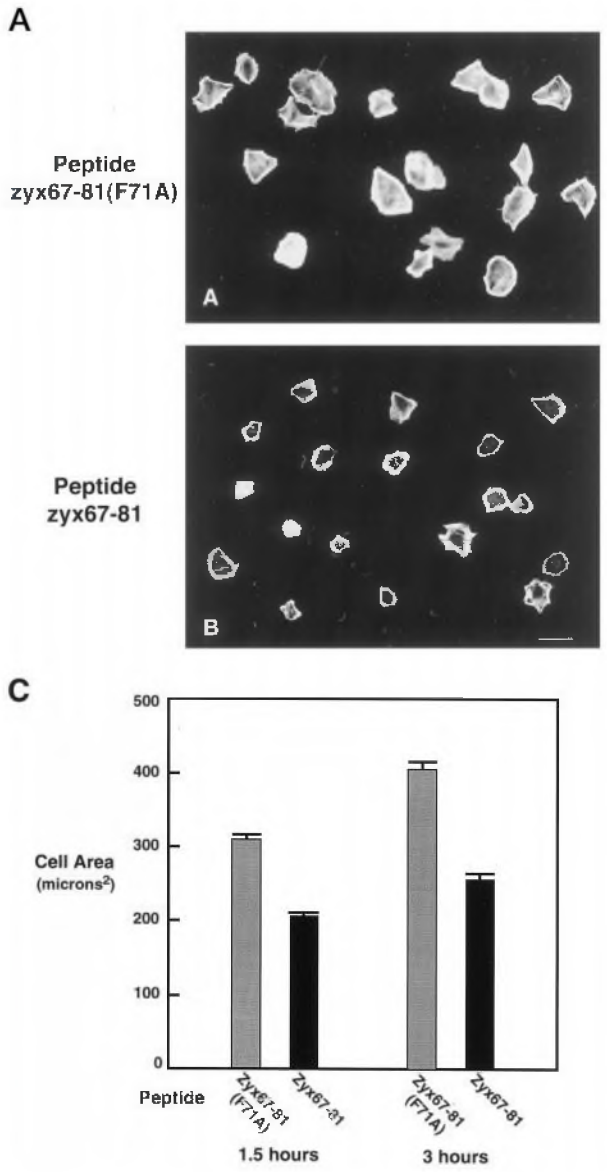

FIG. 8. **The Zyx-(67–81)-peptide inhibits cell spreading on fibronectin.** *Zyx-67–81-(F71A)* peptide (*A*) or *zyx67–81* peptide (*B*) were introduced into cells in suspension by electroporation. Cells were fixed and stained with FITC-phalloidin 3 h after plating onto fibronectin-coated coverslips, and average cell area was determined. *Bar* = 25 μm. *C*, the mean area of cells that had taken up Zyx-(67–81)-peptide was measured at 1.5 and 3 h after plating. Zyx-(67–81)-peptide-treated cells showed a reduction in mean cell area of 36% as compared with cells that had taken up Zyx-(67–81)-(F71A) peptide. Mean area ± S.E. ($p < 0.001$) is shown.

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 (NtΔ1Δ2), 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 members 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 CAA*X* 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 CAA*X* 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 significant 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 *D. ena* (28) and from experiments in which VASP and Mena can restore *Listeria* movement in cell-free extracts (32). The observation that platelets aggregate faster in the absence of VASP suggests that VASP might play a role in the inhibition of actin assembly and cell-surface extension. This interpretation is in contrast to the experiments presented here that indicate that neutralization of Mena and VASP inhibits cell spreading. This discrepancy might reflect the differences in cell types studied (fibroblasts *versus* platelets) or in experimental approach (chronic inhibition with the genetic approach *versus* transient inhibition with the peptide). Further work will be required to resolve this issue.

Studies of ActA have been important in identifying and characterizing a role for zyxin and Ena/VASP family members in eucaryotic cells. We have shown that the sequence requirements for zyxin and Ena/VASP interaction are similar to what had been predicted from studies of the ActA and Ena/VASP interaction. By mutating the critical phenylalanine residue and testing the mutants in transfection assays, we find that there are likely other functional similarities between ActA and zyxin that have yet to be characterized. In addition, we have used peptides derived from the sequence of zyxin to perturb the interaction between zyxin and Ena/VASP members *in vivo*. We found that cells showed a reduced ability to spread, suggesting that this interaction, perhaps at a level that involves entire protein families, may be necessary during cellular events where the actin cytoskeleton is remodeled. It will be a challenge to biologists to characterize the individual contribution of each member of these families in the regulation of the actin cytoskeleton of mammalian cells.

Acknowledgments—We are extremely grateful to Jürgen Wehland for providing the zyxin SPOTs membrane; to Kirsten Niebuhr for technical advice; to Frank Gertler for reagents and comments; and to members of our laboratories for valuable discussions. We are grateful to Bob Schackmann for peptide synthesis and the Huntsman Cancer Institute Sequencing core facility for DNA sequence analysis; we acknowledge the National Institutes of Health for support of the Core Facilities at the University of Utah.

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