

# Molecular Dissection of Zyxin Function Reveals Its Involvement in Cell Motility

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**Abstract.** Spatially controlled actin filament assembly is critical for numerous processes, including the vectorial cell migration required for wound healing, cell-mediated immunity, and embryogenesis. One protein implicated in the regulation of actin assembly is zyxin, a protein concentrated at sites where the fast growing ends of actin filaments are enriched. To evaluate the role of zyxin *in vivo*, we developed a specific peptide inhibitor of zyxin function that blocks its interaction with  $\alpha$ -actinin and displaces it from its normal subcellular location. Mislocalization of zyxin perturbs cell migration and spreading, and affects the behavior of the cell edge, a structure maintained by assembly of actin at sites proximal to the plasma membrane. These results sup-

port a role for zyxin in cell motility, and demonstrate that the correct positioning of zyxin within the cell is critical for its physiological function. Interestingly, the mislocalization of zyxin in the peptide-injected cells is accompanied by disturbances in the distribution of Ena/VASP family members, proteins that have a well-established role in promoting actin assembly. In concert with previous work, our findings suggest that zyxin promotes the spatially restricted assembly of protein complexes necessary for cell motility.

**Key words:** zyxin •  $\alpha$ -actinin • cell motility • Ena/VASP

**M**ANY cellular processes depend on cytoskeletal rearrangements involving actin filaments. For example, cell locomotion and membrane extension appear to be largely driven by the spatially restricted addition of actin monomers to free filament ends in the lamellipodial leading edge (Small et al., 1978, 1995; Wang, 1985; Theriot and Mitchison, 1991). Moreover, cellular responses to extracellular signals often involve extensive rearrangement of the actin cytoskeleton, including localized increases in actin polymerization (Zigmond, 1996; Chan et al., 1998). Because so many important cell functions depend on the actin cytoskeleton, it is important to understand how the timing and spatial restriction of actin assembly is achieved.

One protein that has been postulated to play a role in the regulation of the actin cytoskeleton is zyxin (Beckerle, 1997). Zyxin displays a subcellular distribution that would be compatible with such a function. In particular, zyxin has been shown to colocalize with the actin cytoskeleton at sites where the fast-growing ends of actin filaments are en-

riched, including lamellipodia and focal adhesions (Beckerle, 1986; Crawford and Beckerle, 1991). Moreover, zyxin's binding partner repertoire, which includes several proteins with well-established roles in control of actin function (Beckerle, 1997), suggests a role in cytoskeletal regulation. Further evidence for the involvement of zyxin in some aspect of actin assembly or organization comes from studies in which zyxin has been targeted to the inner face of the plasma membrane. Membrane-targeted zyxin produces dramatic cytoskeletal rearrangements, including loss of endogenous actin stress fibers and induction of actin-rich cell surface projections (Golsteyn et al., 1997). These studies indicate that zyxin, in the proper subcellular context, has the ability to direct cytoskeletal reorganization.

Among zyxin's notable structural features are an extensive proline-rich NH<sub>2</sub>-terminal domain, a nuclear export signal, and the presence of three COOH-terminal copies of the LIM motif (Freyd et al., 1990; Sadler et al., 1992; Macalma et al., 1996; Nix and Beckerle, 1997). The NH<sub>2</sub>-terminal 381 amino acids of zyxin, including its proline-rich region, has been shown to be sufficient to direct reorganization of the actin cytoskeleton when targeted to the plasma membrane (Golsteyn et al., 1997). In this region, zyxin contains sequences that act as binding sites for members of the Ena/VASP protein family (Niebuhr et al., 1997; Purich and Southwick, 1997). Ena/VASP family members

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serve as ligands for the actin monomer binding partner, profilin, and have themselves been shown to modulate actin assembly and/or organization (Gertler et al., 1996; Huttelmaier et al., 1999; Laurent et al., 1999). Interestingly, the Ena/VASP binding sites present in zyxin are very similar to proline-rich repeats found in the ActA protein of *Listeria monocytogenes*, a bacterial species that infects mammalian cells, and whose pathogenicity relies on its ability to assemble actin filaments on its surface. The interaction of Ena/VASP proteins with *Listeria* ActA is essential for their recruitment to the site of actin assembly at the bacterial surface (Chakraborty et al., 1995; Pistor et al., 1995; Smith et al., 1996; Niebuhr et al., 1997). Thus, like *Listeria* ActA, zyxin may act to dock members of the Ena/VASP family at particular subcellular locations and may facilitate spatially restricted changes in the actin cytoskeleton within eukaryotic cells.

Zyxin also interacts with  $\alpha$ -actinin (Crawford et al., 1992), an actin binding and cross-linking protein that is enriched at the termini of stress fibers in focal adhesions, which are sites of close membrane-substratum contact (Maruyama and Ebashi, 1965; Lazarides and Burridge, 1975; Podlubny et al., 1975). Through its interaction with the cytoplasmic domain of  $\beta$ 1 integrins (Otey et al., 1990),  $\alpha$ -actinin is in a position to contribute to membrane-cytoskeletal interactions by linking actin stress fibers directly to the integrin transmembrane receptors. The interaction of zyxin with  $\alpha$ -actinin has been proposed to be important for zyxin localization and association with the cytoskeleton (Crawford et al., 1992).

In this study, we have employed a custom peptide library to map the binding site of  $\alpha$ -actinin on zyxin, and have used this information to design a synthetic peptide inhibitor of this protein-protein interaction. We used this peptide as a tool to further define the physiological role of zyxin and the importance of its subcellular distribution by introducing it into living cells and determining the effects on cell behavior and morphology. Introduction of the peptide inhibitor into cells causes the mislocalization of zyxin and its binding partner, Mena, a mammalian member of the Ena/VASP family. Zyxin displacement also results in retraction of the cell edge and adversely affects cell spreading and cell migration. Our results illustrate that zyxin plays an important role in cell motility. We postulate that zyxin functions in some aspect of cytoskeletal regulation and microfilament dynamics, possibly by promoting the assembly of protein complexes that stimulate actin polymerization.

## Materials and Methods

### Synthetic Peptides

Peptides were synthesized with an  $\text{NH}_2$ -terminal cysteine at the University of Utah core facility or by Genosys, Inc. Lyophilized peptides were resuspended in sterile PBS, and the pH was adjusted to 7.5 if necessary. The sequence of the Zyx<sub>16-30</sub> peptide is APAFYAPQKKFGPVV. The sequence of the scrambled peptide used as a control is VAFAPKYKQAVPGPF.

### Protein Binding Studies

Binding studies with  $\alpha$ -actinin were performed using purified  $\alpha$ -actinin from chicken smooth muscle (Feramisico and Burridge, 1980). The site of

$\alpha$ -actinin binding on zyxin was determined using a custom-made peptide library (SPOTS) conjugated to a cellulose membrane (Frank, 1992) and was provided by Jurgen Wehland (Department of Cell Biology, Braunschweig, Germany). The library consisted of 187 overlapping peptides of 15 amino acids in length that spanned the complete human zyxin protein sequence. Iodinated  $\alpha$ -actinin was used to probe the SPOTS membrane at a concentration of 250,000 cpm/ml.

To evaluate the ability of the soluble Zyx<sub>16-30</sub> peptide to block the zyxin- $\alpha$ -actinin interaction, chicken zyxin was expressed in *Escherichia coli* as a GST fusion protein (Schmeichel and Beckerle, 1994). Lysates were prepared from both uninduced bacterial cultures and cultures induced to express GST-zyxin. Proteins were separated by SDS-PAGE and transferred to nitrocellulose. After blocking, nitrocellulose strips were overlaid with 5  $\mu\text{g/ml}$  purified  $\alpha$ -actinin, or  $\alpha$ -actinin preincubated with the Zyx<sub>16-30</sub> peptide, or a control scrambled peptide. The strips were washed in TBS and fixed briefly in 0.5% formaldehyde, followed by washing in 2.0% glycine.  $\alpha$ -Actinin binding to GST-zyxin was detected according to standard immunoblot procedures using an mAb against  $\alpha$ -actinin (Sigma Chemical Co.) and HRP-conjugated anti-mouse IgG (Amersham Life Science) as a secondary antibody, followed by chemiluminescent detection using enhanced chemiluminescence reagents (Amersham Life Science).

To test the protein binding specificity of the Zyx<sub>16-30</sub> peptide, the peptide was coupled to HRP via the  $\text{NH}_2$ -terminal cysteine residue using EZ-Link™ maleimide-activated HRP (Pierce Chemical Co.). Proteins present in a PtK2 cell lysate were separated by SDS-PAGE and transferred to nitrocellulose. 25  $\mu\text{g}$  of coupled Zyx<sub>16-30</sub> peptide was diluted in 5 ml TBS + 0.1% Tween 20 and was incubated with the membrane. HRP-Zyx<sub>16-30</sub> peptide binding to protein on the blot was detected by using chemiluminescence methods. The membrane was stripped and probed with an mAb against  $\alpha$ -actinin, followed by incubation with HRP-conjugated anti-mouse IgG and chemiluminescent detection.

### Microscopy

Video images were collected on a Leitz Fluovert inverted scope using a Micromax CCD camera (Princeton Instruments) and analyzed using Openlab image processing software (Improvision). Cells to be processed for indirect immunofluorescence were allowed to recover at 37°C for 15 min after injection. Primary antibodies used for immunofluorescence were the following: an antizyxin polyclonal (Macalma et al., 1996), an anti-Mena polyclonal (Gertler et al., 1996), the antiphosphotyrosine monoclonal PY-20 (Transduction Laboratories), antivinulin, and anti- $\alpha$ -actinin mAbs (Sigma Chemical Co.). Secondary antibodies used were Texas red goat anti-rabbit IgG, FITC goat anti-rabbit IgG, and Texas red goat anti-mouse IgG (Molecular Probes).

### Microinjection Experiments

Potoroo tridactylis kidney (PtK2)<sup>1</sup> cells were plated onto glass coverslips 18–36 h before microinjection. Cells were injected using an Eppendorf micromanipulator. In the experiments described here, peptides were injected at a needle concentration of 0.6 mM–0.6  $\mu\text{M}$ . These needle concentrations are above the threshold required for the maximal effect on cell behavior. At a needle concentration of 0.06  $\mu\text{M}$ , a smaller percentage of cells displayed effects of the injected peptide. If cells were to be processed for immunofluorescence, FITC-BSA was coinjected with peptides. For injections in which the Zyx<sub>16-30</sub> peptide was preincubated with  $\alpha$ -actinin, the molar ratio of  $\alpha$ -actinin to peptide was 15:1. For myosin inhibition studies, a fresh 0.5 M stock of diacetyl monoxime (ICN Laboratories) was prepared in sterile H<sub>2</sub>O and added to cell medium for a final concentration of 10 mM. Quantitative data regarding the effects of peptide injection on protein localization was obtained by visual observation of cells injected and processed for immunofluorescence. In each case, over 100 cells were injected and scored for localization of zyxin, Mena, or vinculin; analysis was performed blind and in parallel by two independent investigators.

### Cell Spreading Experiments

PtK2 epithelial cells were grown to 80–90% confluency. Cells were trypsinized, washed, and resuspended in sterile PBS + 10 mM MgCl at a concentration of  $2.5 \times 10^6$  cells/ml. Peptide (400  $\mu\text{M}$ ) and a 3,000-D Texas red dextran (0.1 mg/ml) were added to the cell suspension before electroporation. An aliquot of cell suspension (0.5 ml) was placed in 2-mm gap

1. *Abbreviation used in this paper:* ptK2, Potoroo tridactylis kidney.

cuvettes and electroporated using an electrocell manipulator 600 (BTX Inc.). Cells were incubated on ice for 15 min, washed, and plated onto fibronectin (Sigma Chemical Co.) -coated coverslips. 3 h after plating, cells were fixed and stained using FITC-phalloidin (Molecular Probes). After electroporation, virtually all cells examined were observed to have taken up this marker and, thus, can be assumed to have taken up peptide as well. Two independent experiments were performed; between 150 and 200 measurements were taken for each population of cells. Cell area was determined using NIH Image software, and statistical analysis was performed using InStat software (GraphPad Software, Inc.). Cellular area measurements of  $<100 \mu\text{m}^2$  were not included in our calculations to eliminate the possibility of including data from nonviable cells in the analysis. Data are reported as the mean area  $\pm$  SEM.

### Cell Migration Experiments

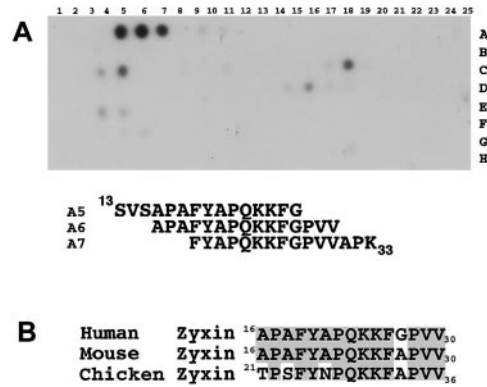
Normal immortalized human mammary epithelial cells (184A) were cultured in DFCI media containing 12.5 ng/ml EGF (Band and Sager, 1989). Cells were plated onto 60-mm optically clear tissue culture dishes at a density of  $2\text{--}4 \times 10^5$  cells/ml 18–24 h before injection. Cells were injected with the  $\text{Zyx}_{16\text{--}30}$  peptide and the control scrambled peptide using a needle concentration of 1.2 mM. Cell movement was recorded for a 3-h period after injection using video microscopy. Migration distances were measured using Openlab imaging processing software (Improvision). Four separate experiments were performed for each group of injections and measurements were determined for 10–15 cells in each group. Statistical analysis was performed using InStat software (GraphPad Software, Inc.). Data are reported as the mean distance migrated per hour  $\pm$  SEM.

### Results

#### $\alpha$ -Actinin Interacts with Sequences in the $\text{NH}_2$ -terminal Region of Zyxin

Zyxin is concentrated at sites proximal to the plasma membrane, including the focal adhesions and the lamellipodial leading edge, where the fast-growing ends of actin filaments are clustered. To define further the physiological role of zyxin, we sought to develop specific inhibitors of zyxin function. Our initial effort has been directed toward designing an inhibitor of the zyxin- $\alpha$ -actinin interaction (Crawford et al., 1992). Zyxin and  $\alpha$ -actinin are extensively colocalized in cells (Fig. 1) and we reasoned that inhibiting this interaction might perturb zyxin's subcellular distribution.

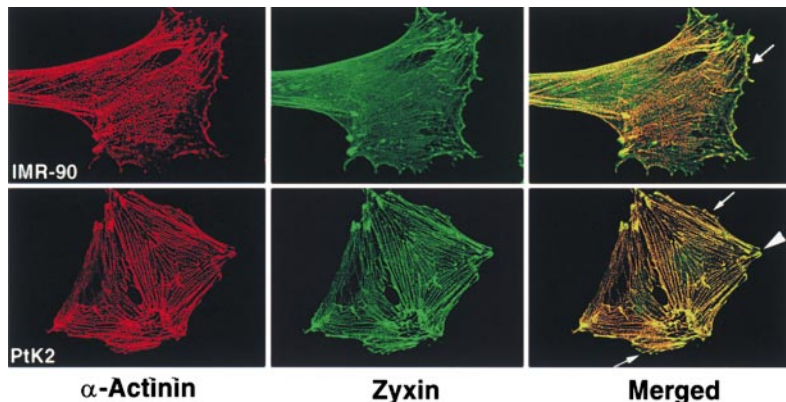
We precisely mapped the site of  $\alpha$ -actinin binding on zyxin using a custom synthesized SPOTs peptide library (Frank, 1992) that spanned the entire human zyxin sequence. Radiolabeled  $\alpha$ -actinin was used to probe this library and was found to interact most prominently with a series of three peptides corresponding to sequences in



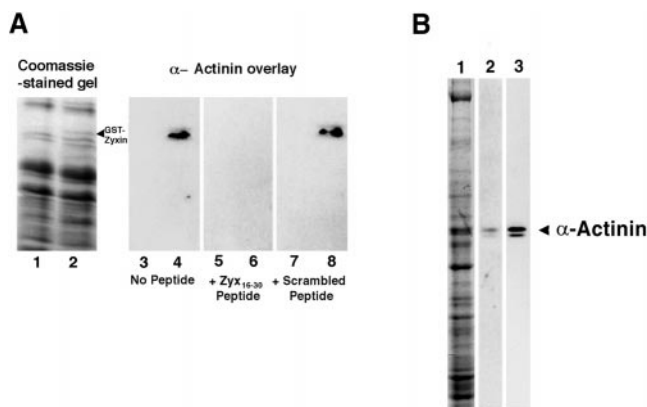
**Figure 2.** Identification of the  $\alpha$ -actinin binding site on zyxin. (A) <sup>125</sup>I-labeled  $\alpha$ -actinin was used to probe a library of zyxin-derived peptides and was found to interact most prominently with three adjacent peptides that correspond to amino acids 13–33 in the  $\text{NH}_2$ -terminal region of human zyxin (peptides A5, A6, and A7). The sequences and alignment of these peptides are shown. (B) A comparison of this region in human, mouse, and chicken zyxin shows that it is highly conserved.

amino acids 13–33 of human zyxin (Fig. 2 A). Our determination of the  $\alpha$ -actinin binding site on zyxin using the library of zyxin-derived peptides refines the recently published domain mapping performed by deletion analysis (Reinhard et al., 1999). Quantitative analysis revealed that the  $\text{Zyx}_{16\text{--}30}$  peptide (APAFYAPQKKFGPVV) interacted most strongly with  $\alpha$ -actinin. Comparison of this region in human, mouse, and chicken zyxin shows that it is highly conserved (Fig. 2 B). While other peptides in the library also interacted with  $\alpha$ -actinin, their relative strength of binding was at least 50% less than that of the  $\text{Zyx}_{16\text{--}30}$  peptide. For this reason, we focused on the testing of the  $\text{Zyx}_{16\text{--}30}$  peptide as a possible inhibitor of the interaction of zyxin with  $\alpha$ -actinin.

The ability of a synthetic  $\text{Zyx}_{16\text{--}30}$  peptide to compete with zyxin for  $\alpha$ -actinin binding was tested. Chicken zyxin was expressed as a GST fusion protein in bacteria (Fig. 3 A, lane 2). Proteins were transferred to nitrocellulose and overlaid with purified  $\alpha$ -actinin (Fig. 3 A, lanes 3–8).  $\alpha$ -Actinin interacted specifically with the zyxin fusion protein (Fig. 3 A, lane 4). The  $\text{Zyx}_{16\text{--}30}$  peptide was demonstrated to block the interaction of  $\alpha$ -actinin with zyxin



**Figure 1.** Colocalization of zyxin and  $\alpha$ -actinin in PtK2 and IMR-90 cells by confocal microscopy.  $\alpha$ -Actinin (red) and zyxin (green) are colocalized in focal adhesions (large arrow), along actin stress fibers, and at the leading edge (small arrow).



**Figure 3.** Characterization of the effectiveness and specificity of the zyxin-derived peptide as an inhibitor of the zyxin- $\alpha$ -actinin interaction. (A) A peptide corresponding to the site of  $\alpha$ -actinin interaction with zyxin inhibits the interaction of zyxin and  $\alpha$ -actinin in vitro.  $\alpha$ -Actinin binding to a GST-zyxin fusion protein was tested using a blot overlay with purified  $\alpha$ -actinin. Lanes 1, 3, 5, and 7 contain uninduced bacterial cell lysate; lanes 2, 4, 6, and 8 contain lysates from cells in which expression of the GST-zyxin fusion was induced.  $\alpha$ -Actinin binds to GST-zyxin in the blot overlay (lane 4). In the presence of the Zyx<sub>16-30</sub> peptide, binding of  $\alpha$ -actinin to GST-zyxin is inhibited (lane 6). Addition of a scrambled peptide with identical amino acid composition had no effect on the interaction (lane 8). (B) The HRP-coupled Zyx<sub>16-30</sub> peptide interacts specifically with  $\alpha$ -actinin. Lane 1 shows a Coomassie-stained gel of proteins present in a PtK2 cell lysate. HRP-conjugated Zyx<sub>16-30</sub> peptide interacts specifically with  $\alpha$ -actinin (lane 2). Lane 3 shows the same membrane after stripping and probing with an anti- $\alpha$ -actinin mAb.

(Fig. 3 A, lane 6), whereas a scrambled peptide with an identical amino acid composition did not (Fig. 3 A, lane 8). This experiment demonstrated the ability of the Zyx<sub>16-30</sub> peptide to act as an inhibitor of the zyxin- $\alpha$ -actinin interaction.

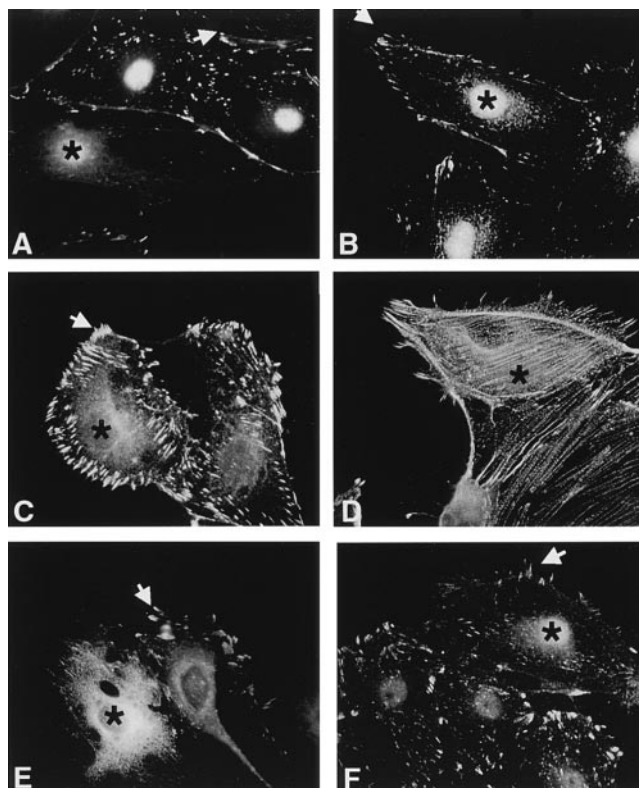
To test the specificity of the peptide for binding to  $\alpha$ -actinin, we used the Zyx<sub>16-30</sub> peptide to probe the complex mixture of proteins present in a PtK2 cell lysate (Fig. 3 B, lane 1). PtK2-derived proteins were electrophoretically resolved and transferred to nitrocellulose and were probed with HRP-conjugated Zyx<sub>16-30</sub> peptide. The Zyx<sub>16-30</sub> peptide interacted specifically with a protein that migrated at an apparent molecular mass of 100,000 D (Fig. 3 B, lane 2). To confirm the identity of this protein as  $\alpha$ -actinin, the membrane was stripped and probed with an mAb directed against  $\alpha$ -actinin, which recognized the same protein profile as that bound by the zyxin-derived peptide (Fig. 3 B, lane 3). These studies indicated that the Zyx<sub>16-30</sub> peptide is likely to be a useful reagent for examining the importance of zyxin's interaction with  $\alpha$ -actinin in vivo and for further dissection of zyxin function.

### The Zyx<sub>16-30</sub> Peptide Causes Mislocalization of Zyxin In Vivo

Once we had established the effectiveness and specificity of the Zyx<sub>16-30</sub> peptide reagent as an inhibitor of the interaction of zyxin with  $\alpha$ -actinin in vitro, we examined the effect of intracellular peptide on zyxin's subcellular distribu-

tion. For these studies, we used PtK2 cells, an epithelial cell line derived from rat kangaroo. This cell type has been used in several previous studies of cytoskeletal processes, including cell spreading and myosin-dependent activities (Sanger, 1975; Sanger et al., 1984, 1989; Cramer and Mitchison, 1995, 1997). It has been shown recently that the initial sites of actin assembly in PtK2 cells are the borders of lamellipodia and the focal adhesions (Turnacioglu et al., 1998), which were sites where  $\alpha$ -actinin is enriched and where zyxin is also found. In addition, cells of this type are especially amenable to microinjection and the localization of cytoskeletal proteins by indirect immunofluorescence.

In PtK2 cells injected with the Zyx<sub>16-30</sub> peptide, zyxin is substantially mislocalized; the protein is no longer concentrated in focal adhesions or at cell borders (Fig. 4 A, an as-



**Figure 4.** Injection of the Zyx<sub>16-30</sub> peptide causes mislocalization of zyxin and its binding partner Mena. Cells were injected with a fluorescent marker, and peptides were subsequently fixed and prepared for indirect immunofluorescence with specific antibodies. FITC-BSA was used as a marker for identification of injected cells, which are indicated by the asterisks. Injection of the Zyx<sub>16-30</sub> peptide results in mislocalization of zyxin (A). Injection of a scrambled version of the peptide does not result in zyxin mislocalization (B). Vinculin is still localized normally in cells injected with the Zyx<sub>16-30</sub> peptide, indicating that focal adhesion structure is still intact (C).  $\alpha$ -Actinin remains associated with actin microfilaments that appear intact, albeit somewhat reorganized, in the peptide-injected cells. However, many retraction fibers are evident and linear aggregates of filaments are found parallel to the cell periphery (D). Interestingly, mislocalization of zyxin is accompanied by a significant depletion of zyxin's binding partner, Mena, from focal adhesions (E). Phosphotyrosine-containing proteins remain enriched in focal adhesion structures, although there is a reproducible reduction in signal intensity compared with neighboring, uninjected cells (F).

terisk marks the injected cell which was identified by the presence of a fluorescent marker that was introduced with the peptide). In contrast, injection of a scrambled peptide with identical amino acid composition did not adversely affect zyxin localization (Fig. 4 B). Although zyxin was substantially displaced from its normal subcellular location by the introduction of the Zyx<sub>16-30</sub> peptide, the structural integrity of the focal adhesions is retained. Injection of the Zyx<sub>16-30</sub> peptide did not alter the distribution of another focal adhesion constituent, vinculin (Fig. 4 C), and focal adhesions could still be visualized using interference reflection contrast microscopy (data not shown). Thus, appropriate subcellular localization of zyxin does not appear to be essential for the short-term maintenance of preexisting focal adhesions. In addition, actin-rich stress fibers remain assembled in injected cells (data not shown) and  $\alpha$ -actinin exhibits its normal periodic distribution along stress fibers (Fig. 4 D). However, as will be discussed in greater detail below, actin-rich retraction fibers and dense bundles of actin filaments that run parallel to the cell edge are evident at the borders of cells that have been injected with the zyxin-derived peptide, illustrating that disturbance of the zyxin- $\alpha$ -actinin interaction has some adverse effects on cell morphology and cytoskeletal organization (Fig. 4 D).

Interestingly, mislocalization of zyxin is accompanied by a striking depletion of the zyxin binding partner, Mena, a member of the Ena/VASP family from its normal subcellular locations (Fig. 4 E). In particular, we noted a reduction in the accumulation of Mena and VASP in focal adhesions with a concomitant increase in cytoplasmic labeling of the peptide-injected cells. In addition to the loss of Mena, mislocalization of zyxin results in a noticeable reduction in the phosphotyrosine content of the focal adhesion (Fig. 4 F). Although antigen levels cannot be reliably quantified by immunocytochemical methods, the apparent reduction in phosphotyrosine content in the focal adhesions of the peptide-injected cells may reflect a loss of Ena/VASP family members from these sites since these proteins are known to be tyrosine phosphorylated (Gertler et al., 1996; Comer et al., 1998).

In an effort to define more quantitatively the effect of peptide injection on the protein composition of focal adhesions, cells were injected with either the Zyx<sub>16-30</sub> peptide or the control scrambled peptide, and scored for correct localization of zyxin, Mena, and vinculin (Table I). For each focal adhesion component considered, a minimum of 200 cells was injected and scored for protein localization by immunofluorescence. In cells injected with the scrambled peptide, less than a quarter of the cells (from 8 to 24%, depending on the protein evaluated) displayed any noticeable alteration in the normal subcellular distributions of zyxin, Mena, or vinculin. In contrast, the majority of cells injected with the Zyx<sub>16-30</sub> peptide showed mislocalization of zyxin and Mena; in both cases  $\sim$ 70% of the cells displayed a clear loss of these proteins from the focal adhesions. At a minimum, these results illustrate that injection of the Zyx<sub>16-30</sub> peptide causes the concurrent mislocalization of zyxin and Mena in  $\sim$ 40% of the cells examined. Only 25% of cells injected with the Zyx<sub>16-30</sub> peptide showed mislocalized or reduced vinculin at the focal adhesion; this level is similar to what we observed for the con-

Table I. Effect of Peptide Injection on Localization of Adhesion Plaque Proteins

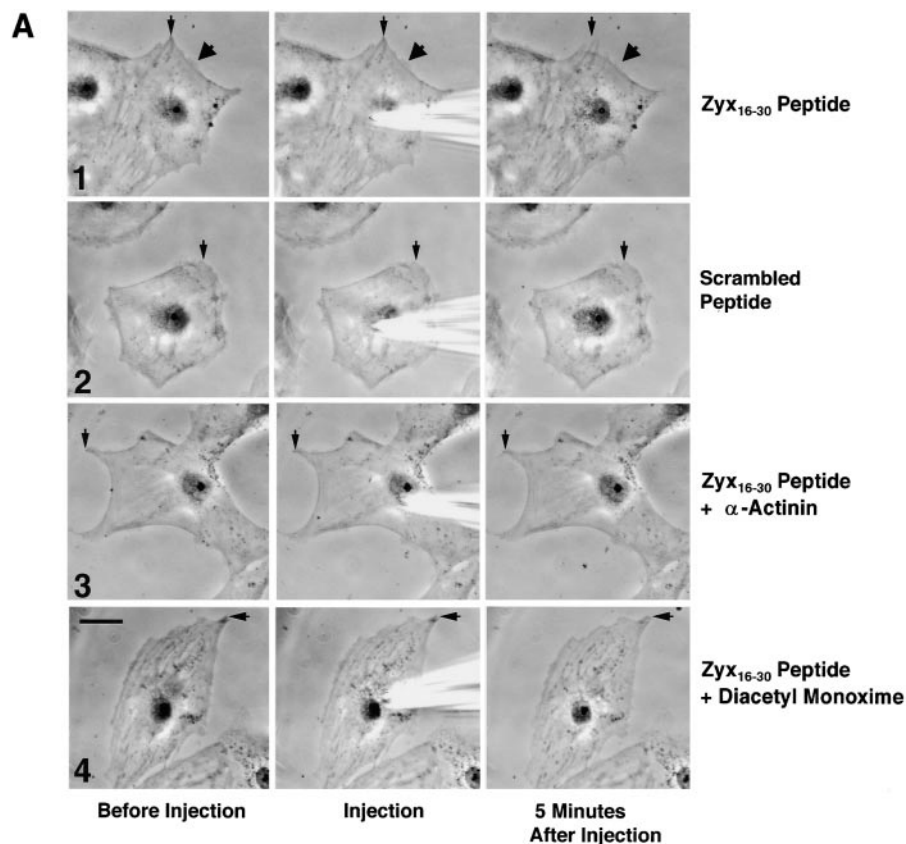
Adhesion plaque component	Injected peptide	Percentage of cells showing mislocalized protein*
Zyxin	Zyx <sub>16-30</sub>	67%
	Scrambled	8%
Mena	Zyx <sub>16-30</sub>	72%
	Scrambled	24%
Vinculin	Zyx <sub>16-30</sub>	25%
	Scrambled	19%

\*At least 200 cells were injected and scored in each category.

rol peptide. Our observation that vinculin remains largely concentrated in the focal adhesion after peptide injection also indicates that the loss of zyxin and Mena is unlikely to be a result of nonspecific disruption of focal adhesion structures.

### Introduction of the Zyx<sub>16-30</sub> Peptide into PtK2 Cells Affects the Position of the Cell Edge

If zyxin plays a role in actin dynamics, focal adhesion assembly, or cell adhesion, mislocalization of zyxin in living cells could be expected to alter cell morphology and behavior. We examined the effect of zyxin mislocalization on cell behavior at a higher resolution by microinjection of the Zyx<sub>16-30</sub> peptide into PtK2 cells and monitoring the cellular response in real time using video microscopy. Introduction of the Zyx<sub>16-30</sub> peptide into cells produced a rapid retraction of the cell edge (Fig. 5, panel 1). A quantitative view of the results from these experiments, illustrating the dynamic behavior of the cell edge for several representative cells is shown in Fig. 6. The average rate of rearward displacement for cells injected with the Zyx<sub>16-30</sub> peptide was 2.7 microns/min during the 5-min observation period. Numerous retraction fibers were evident at the borders of these injected cells. Injection of cells with buffer solutions alone or with solutions containing BSA or  $\alpha$ -actinin did not produce this response, indicating that it is not a result of the microinjection technique (data not shown). In addition, injection of a scrambled version of the peptide had no effect on cell morphology (Fig. 5, panel 2, and Fig. 6), and preincubation of the Zyx<sub>16-30</sub> peptide with  $\alpha$ -actinin before microinjection inhibited the effects of the bioactive peptide (Fig. 5, panel 3, and Fig. 6). Table II shows the results of several microinjection experiments in which cells were observed for several minutes after microinjection and scored for response. Nearly 90% of cells injected with the Zyx<sub>16-30</sub> peptide exhibited retraction of the cell edge after microinjection, whereas  $<$ 12% of the cells injected with control solutions displayed any evidence of retraction. The observation that the percentage of cells exhibiting edge retraction is apparently greater than the percentage of cells showing mislocalization of zyxin (compare Tables I and II) may be a reflection of the relatively stringent criteria we used to define loss of zyxin localization by immunofluorescence; some cells that we scored as lacking a disturbance in zyxin localization in response to peptide injection may indeed have sustained a disturbance in zyxin distribution sufficient to produce cell retraction, but insufficient for us to score it as mislocalized.



**Figure 5.** Injection of the Zyx<sub>16-30</sub> peptide induces lamellipodial retraction. Small arrows show the original position of the cell edge before injection. For the cell shown in the figure, the cell margin retracted by 12 microns during the 5-min interval after microinjection of the Zyx<sub>16-30</sub> peptide (panel 1). A lamellipodial extension that retracted rapidly after injection of the Zyx<sub>16-30</sub> peptide is highlighted (large arrow), and numerous retraction fibers are evident at the cell border. Injection of a scrambled peptide does not produce this effect (panel 2). Preincubation of the Zyx<sub>16-30</sub> peptide with  $\alpha$ -actinin before injection blocks the ability of the peptide to induce this response (panel 3). In the presence of the myosin inhibitor diacetyl monoxime, retraction of the cell edge fails to occur upon injection of the zyxin-derived peptide (panel 4).

Cell shape is controlled in part by the balance between the opposing activities of actin assembly, which drives membrane protrusion, and myosin-powered retrograde movement, which causes retraction (Theriot and Mitchison, 1991; for review see Welch et al., 1997). For example, in certain neuronal growth cones, an inverse relationship between actin-assembly driven membrane extension and myosin-powered retraction has been precisely characterized (Lin et al., 1996). To determine whether the retraction of the cell edge that we observed was dependent on myosin activity, PtK2 cells were injected with Zyx<sub>16-30</sub> peptide in the presence of diacetyl monoxime, an inhibitor of nonmuscle myosins (Cramer and Mitchison, 1995). Myosin inhibition was found to prevent retraction of the cell edge in response to microinjection (Fig. 5, panel 4, and Fig. 6).

### **The Zyx<sub>16-30</sub> Peptide Inhibits Cell Spreading on Fibronectin**

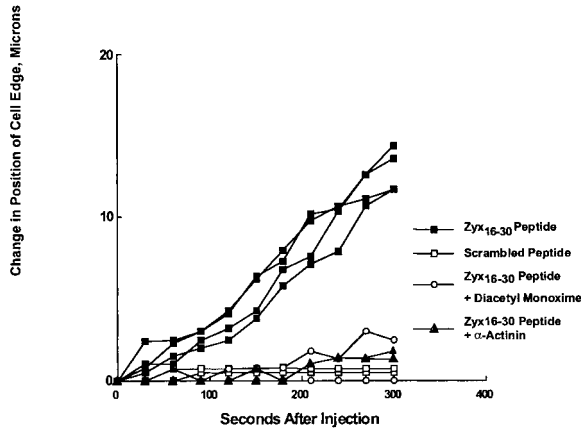
An attractive model for zyxin function is that of a molecular scaffold that functions to promote site-specific actin assembly. This hypothesis is consistent with zyxin's ability to promote assembly of actin-rich structures when targeted to the plasma membrane (Golsteyn et al., 1997), as well as its interaction with proteins known to be involved in cytoskeletal regulation (Beckerle, 1997). If zyxin plays a role in the spatial control of actin assembly, mislocalization of zyxin might be expected to affect processes that are dependent on localized actin assembly, such as cell spreading and cell migration. To explore the effect of zyxin mislocal-

ization on these processes, we first examined the effect of peptide-dependent displacement of zyxin on the rate of cell spreading.

In parallel experiments, the Zyx<sub>16-30</sub> peptide or a scrambled peptide of identical amino acid composition was introduced into PtK2 cells in suspension via electroporation. Texas red dextran was simultaneously introduced as a marker for successful electroporation; virtually all cells examined were observed to have taken up this marker (data not shown) and, thus, can be assumed to have internalized peptide as well. After electroporation, cells were plated onto fibronectin-coated coverslips (Bereiter-Hahn et al., 1990; Mooney et al., 1995), and allowed to spread for 3 h before fixation and staining with FITC-phalloidin. Cell areas were determined for 150–200 cells per experimental condition. Cells into which the scrambled peptide was introduced were well spread within 3 h after plating (Fig. 7 A) and achieved an average area of nearly 417  $\mu\text{m}^2$ . In contrast, cells into which the Zyx<sub>16-30</sub> peptide was intro-

**Table II.** Effect of Peptide Injection on Position of the Cell Edge

Needle contents	No. cells injected	Percentage of cells in which retraction of the cell edge was observed
Zyx <sub>16-30</sub> peptide	80	89%
Scrambled peptide	60	5%
$\alpha$ -Actinin + Zyx <sub>16-30</sub> peptide	65	9%
0.5 mg/ml FITC-BSA	66	12%

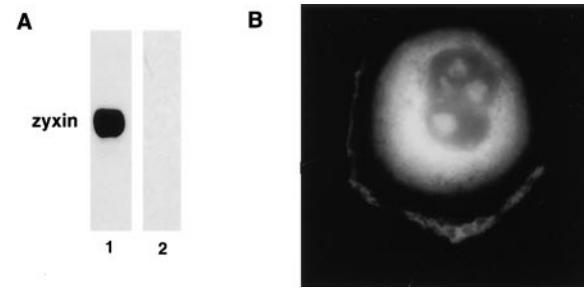


**Figure 6.** Quantitative analysis of cell behavior after injection of the Zyx<sub>16-30</sub> peptide. The change in position of the cell edge relative to its original position before injection is shown for representative individual cells injected with the Zyx<sub>16-30</sub> peptide (closed squares), the scrambled version of the peptide (open squares), Zyx<sub>16-30</sub> peptide preincubated with  $\alpha$ -actinin (triangles), and cells injected with the Zyx<sub>16-30</sub> peptide in the presence of 10 mM diacetyl monoxime (open circles). The average rate of cell edge retraction upon injection of the Zyx<sub>16-30</sub> peptide was determined to be 2.7 microns/min.

duced exhibited an average area of only 235  $\mu\text{m}^2$ . The average cell area was reduced by nearly 44% for cells harboring the inhibitory Zyx<sub>16-30</sub> peptide relative to cells harboring the scrambled peptide (Fig. 7 C).

#### **Injection of the Zyx<sub>16-30</sub> Peptide Inhibits Cell Migration**

To explore further the role of zyxin in cell motility, we examined the effect of introducing the Zyx<sub>16-30</sub> peptide into a highly migratory cell type. We used 184A cells, immortalized normal mammary epithelial cells (Band and Sager, 1989), which exhibit an increased rate of cell migration in response to EGF (Matthay et al., 1993). Immunoblot analysis confirmed that zyxin is expressed in this cell type (Fig. 8 A). Indirect immunofluorescence reveals that zyxin is present in the cytoplasm as well as the leading edges of

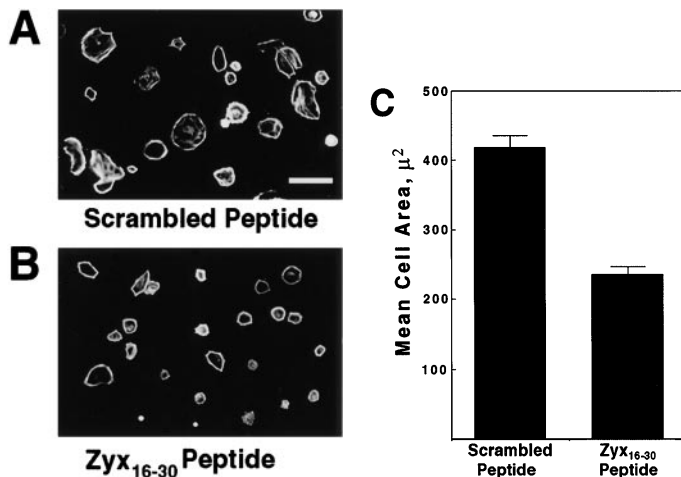


**Figure 8.** Zyxin is present in 184A normal immortalized mammary epithelial cells. (A) Proteins present in a 184A cell lysate were transferred to nitrocellulose and probed with antizyxin polyclonal antisera (lane 1) and preimmune sera (lane 2). A protein of apparent molecular mass of 82 kD is recognized by the antizyxin antisera. (B) Indirect immunofluorescence localization of zyxin in 184A cells shows that zyxin is enriched in the leading edge of these motile cells.

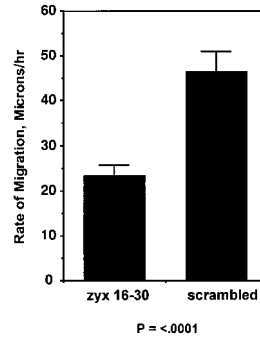
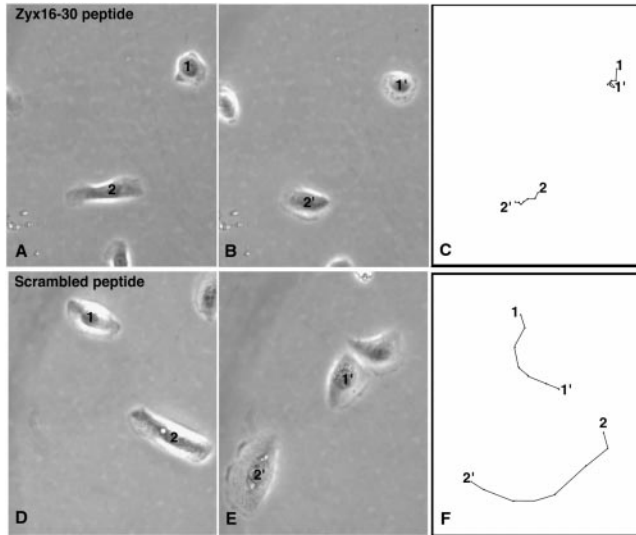
these highly motile cells (Fig. 8 B). Either the Zyx<sub>16-30</sub> peptide or the control scrambled peptide was introduced into individual cells via microinjection. After microinjection, cellular movements were recorded and measured over a 3-h observation period using time-lapse video microscopy. Cells into which the Zyx<sub>16-30</sub> peptide was injected (Fig. 9, A–C) showed a significantly decreased rate of migration relative to cells injected with the scrambled peptide (Fig. 9, D–F). The average rate of migration for the control cells was 46  $\mu\text{m}/\text{h}$ , whereas cells injected with the Zyx<sub>16-30</sub> peptide traveled at a rate of 23  $\mu\text{m}/\text{h}$ , a 50% reduction in motility (Fig. 9 G). No statistically significant difference in the rate of migration was observed in cells injected with the scrambled peptide when compared with uninjected cells (data not shown).

#### **Discussion**

Zyxin was discovered as a component of focal adhesions >10 yr ago, however, its physiological role has remained relatively obscure. An important insight into the possible function of zyxin within cells came with the recognition of



**Figure 7.** The Zyx<sub>16-30</sub> peptide inhibits cell spreading. Peptides were introduced into cells in suspension via electroporation. Cells were fixed and stained with FITC-phalloidin 3 h after plating onto fibronectin-coated coverslips, and the average cell area was determined. Compared with cells into which scrambled peptides were introduced (A), cells electroporated in the presence of the Zyx<sub>16-30</sub> peptides (B) showed a reduction in mean cell area of 43.5% (C). Data are reported as the mean area  $\pm$  SEM.



**Figure 9.** Injection of the Zyx<sub>16-30</sub> peptide into 184A cells has an adverse effect on the rate of cell migration. Cells were injected with the Zyx<sub>16-30</sub> peptide (A–C) or the control peptide (D–F). Cells are shown immediately after injection (A and D) at their initial positions (1 and 2) and 3 h after injection (B and E) at their final positions (1' and 2'). Comparison of the path of migration for injected cells over the 3-h period (C and F) shows that cells injected with the Zyx<sub>16-30</sub> peptide have substantially reduced motility compared with control cells. The difference in mean migration rates for cells injected with the Zyx<sub>16-30</sub> peptide compared with cells injected with the scrambled peptide is shown in G; a minimum of 10 cells was analyzed for each condition. Data are reported as the mean distance migrated per hour  $\pm$  SEM.

the relationship between zyxin and the ActA protein of *Listeria monocytogenes* (Reinhard et al., 1995; Golsteyn et al., 1997). Both zyxin and ActA display proline repeats capable of docking members of the Ena/VASP family (Pistor et al., 1995; Niebuhr et al., 1997). In the case of *Listeria*, the association of Ena/VASP proteins with ActA present on the bacterial surface is clearly important for the organization of the actin comet tail and for the motility of the bacterium (Smith et al., 1996; Laurent et al., 1999). The demonstration that zyxin and ActA share the capacity to bind Ena/VASP proteins led to the suggestion that zyxin may represent an important regulator of actin arrays within eukaryotic cells. This notion gained some support when it was shown that zyxin induces the elaboration of actin-rich cell surface extensions when the protein is targeted to the inner leaflet of the plasma membrane by the presence of a COOH-terminal CAAX sequence (Golsteyn et al., 1997).

Here, we have endeavored to test further the role of zyxin within living cells. Since the appropriate subcellular localization of a protein is often critical to its function, we reasoned that if we could develop a peptide inhibitor that interfered with the normal subcellular distribution of zyxin in living cells, the behavior of the cells might clarify the physiological role of zyxin, and/or protein complexes that depend on zyxin. The interaction of zyxin with  $\alpha$ -actinin has been proposed to be important for zyxin's association with the cytoskeleton (Crawford et al., 1992). We have focused on characterization of this interaction in our efforts to further define the physiological role of zyxin and explore the functional significance of zyxin's subcellular distribution.

In this study, we have mapped the region of zyxin that interacts with  $\alpha$ -actinin to a region in the extreme NH<sub>2</sub> terminus of zyxin, to amino acids 16–30, and have used this information to design a peptide inhibitor of this protein–protein interaction. In cells injected with the Zyx<sub>16-30</sub>

peptide, zyxin is substantially mislocalized. This mislocalization of zyxin is correlated with a disturbance in the distribution of the zyxin binding partner, Mena. The ability of Ena/VASP family members to interact with vinculin (Brindle et al., 1996; Reinhard et al., 1996) may account for the retention of a residual amount of Mena often detected in focal adhesions or may reflect incomplete displacement of zyxin by the peptide competitor. In either case, our results support a model in which the normal subcellular localization of Mena depends to a large extent on its interaction with zyxin.

The mechanism by which the Zyx<sub>16-30</sub> peptide interferes with the normal subcellular localization of zyxin is not completely clear. A simple interpretation would be that zyxin's interaction with  $\alpha$ -actinin is important for its subcellular distribution. Although this may indeed be the case, our preliminary results demonstrate that the LIM regions of zyxin are sufficient to target zyxin to the focal adhesions (Nix, D., S. Bockholt, and M.C. Beckerle, unpublished observations). Also, although zyxin and  $\alpha$ -actinin are substantially colocalized, the presence of  $\alpha$ -actinin alone cannot be sufficient to cause the recruitment of zyxin to specific cellular locations, since there are situations in which  $\alpha$ -actinin occurs in sites that lack zyxin. Therefore, in our microinjection experiments, it appears likely that the presence of the Zyx<sub>16-30</sub> peptide introduces some steric interference that is incompatible with the presence of full-length zyxin.

Introduction of the Zyx<sub>16-30</sub> peptide into cells produced a clear and statistically significant decrease in the rate of cell spreading on fibronectin and in the rate of cell migration in a motile cell type. In addition, our results suggest that zyxin plays a role in maintenance of the integrity and position of the cell border. For example, in PtK2 cells, mislocalization of zyxin produces a retraction of the cell edge that is dependent on a myosin motor activity. Such effects on cell behavior would be expected if zyxin contributed to



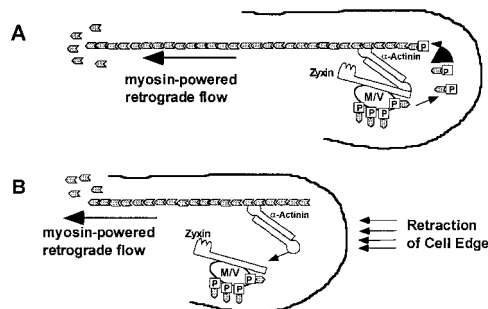
lamellipodial integrity or function. Alternatively, although some focal adhesion components, such as vinculin, still appear to be localized appropriately after microinjection of the zyxin-derived peptide, the cell behavior we observe could result from a decrease in cell adhesiveness because of partial dissolution of established focal adhesions or perturbation of nascent adhesion sites. Additional investigation will be required to evaluate whether the effects of zyxin mislocalization on cell motility results from disturbances in the function of the leading edge, substratum adhesion machineries, or both. In either case, it is intriguing to consider the possibility that a primary consequence of the mislocalization of zyxin is a reduction of localized actin polymerizing activity. Consistent with this view, it is clear that in PtK2 cells, the sites where monomeric actin is being most rapidly incorporated into existing filaments are at the focal adhesions and the edges of lamellipodia, sites where zyxin is normally detected (Fig. 1; Turnacioglu et al., 1998). Furthermore, as will be discussed in greater detail below, the now well-established relationship between zyxin and the ActA protein of *Listeria* suggests a role for zyxin in actin assembly dynamics in vivo.

Several lines of evidence illustrate the specificity and effectiveness of the Zyx<sub>16-30</sub> peptide as an inhibitor of zyxin function. Biochemical tests have illustrated the ability of the zyxin-derived peptide to compete for zyxin docking sites on  $\alpha$ -actinin, and have shown that  $\alpha$ -actinin is the sole ligand for the peptide that is detected in cell lysates. The displacement of zyxin that we observe in living cells that have been injected with the zyxin-derived peptide suggests that this peptide can dock on  $\alpha$ -actinin in vivo and prohibit simultaneous binding of endogenous zyxin to these sites. The fact that  $\alpha$ -actinin retains its ability to associate with filamentous actin in cells injected with the zyxin-derived peptide, at least as evaluated by immunocytochemical methods, illustrates that the binding of the Zyx<sub>16-30</sub> peptide to the globular head domain of  $\alpha$ -actinin does not cause a global disturbance of  $\alpha$ -actinin function in vivo. Thus, the zyxin-derived peptide appears to be exerting a relatively localized effect on the  $\alpha$ -actinin globular head domain that serves as a docking site for zyxin (Crawford et al., 1992). To demonstrate unequivocally that the effects observed with the zyxin-derived peptide are due exclusively to effects on zyxin, rather than a disturbance of  $\alpha$ -actinin function, it would be necessary to perform complementary peptide competition experiments in which a peptide corresponding to the zyxin binding site on  $\alpha$ -actinin was employed. Unfortunately, these experiments are not possible since the zyxin binding site on  $\alpha$ -actinin does not map to a discrete collection of contiguous amino acids (data not shown). However, further support that the peptide effects on motility are specifically due to effects on zyxin comes from the use of an alternative approach to probe zyxin function in vivo. Inhibition of zyxin function by chromophore-assisted laser inactivation also perturbs cell spreading and migration, which is consistent with the results reported here (Benson, B., F. Wang, D.G. Jay, and M.C. Berkerle, manuscript submitted for publication).

The evidence that zyxin is important for optimal cell motility is particularly interesting in light of the relationship established between zyxin and the ActA protein of *L. monocytogenes* (Golsteyn et al., 1997; Niebuhr et al.,

1997). *Listeria* has long been a favorite model system for understanding the mechanisms that drive actin-based motility, including the manner in which actin assembly sites are specified. Rapid addition of actin monomers to free filament ends enriched at the bacterial cell surface provides the force that powers bacterial motility (Tilney and Portnoy, 1989; Tilney et al., 1992; Sanger et al., 1992; Theriot et al., 1992). In a similar manner, the free ends of actin filaments are concentrated at the leading edge of lamellipodia, and filament elongation appears to be the driving force behind membrane protrusion (Small et al., 1978, 1995; Wang, 1985). Our results are consistent with the view that the physiological role of zyxin is somewhat analogous to that of the *Listeria* ActA protein, in particular with the idea that zyxin contributes to the specification of actin assembly zones. In this regard, it is interesting that zyxin was recently identified as one of the proteins that is recruited to sites of cdc42-induced actin polymerization (Castellano et al., 1999).

The precise mechanism by which zyxin enhances cell motility is not clarified by our studies. However, an attractive model for zyxin function is that of a molecular scaffold, which functions to facilitate the formation of a molecular complex that promotes site-specific actin assembly. This view is consistent with zyxin's ability to interact with several proteins known to be involved in cytoskeletal regulation (Beckerle, 1997) as well as its ability to promote the assembly of actin-rich structures when targeted to the plasma membrane (Golsteyn et al., 1997). Our results suggest that zyxin plays a significant role in the targeting of Ena/VASP family members to specific regions of the cell. This conclusion is consistent with genetic studies showing that lethal point mutations in the *Drosophila ena* (*enabled*) gene display both loss of ability to bind zyxin and



**Figure 10.** A model for zyxin function. We postulate that zyxin functions as a molecular scaffold to facilitate the assembly of a multiprotein complex that promotes actin assembly at specific sites within the cell, including the lamellipodial leading edge (A). The position of the leading edge is in part the result of a balance between the rate of actin polymerization and the rate of myosin-powered retrograde flow of actin filaments. According to this model, induction of zyxin mislocalization using a specific peptide inhibitor (B) results in a loss or reduction of actin polymerizing activity in the leading edge. Myosin-powered retrograde flow becomes the dominant force affecting the actin filament, resulting in retraction of the cell edge. Because the NH<sub>2</sub>-terminal region of zyxin has been shown to be sufficient for its ability to promote actin assembly at the plasma membrane (Golsteyn et al., 1997), we have focused on binding partner interactions known to occur in this area of the protein. (M/V, Mena/VASP; P, profilin).

loss of ability to localize properly within cells (Ahern-Djamali et al., 1998). The interaction of Ena/VASP family members with the *Listeria* ActA protein appears to be crucial for producing the high rate of actin assembly required for optimal bacterial motility (Smith et al., 1996; Laurent et al., 1999). By analogy, an important feature of zyxin function may be to provide localized docking sites for Ena/VASP family members at sites destined for actin assembly within the eukaryotic cytoplasm (Fig. 10). It is also likely that other zyxin binding partners, both known and as yet unidentified, participate in motile cell processes.

In conclusion, our efforts to probe the physiological role of zyxin demonstrate that the activity and positioning of zyxin is important in actin-dependent processes, including cell migration and cell spreading. Cell movement is a complex process that requires extensive cytoskeletal rearrangements, including spatially controlled actin assembly. This process clearly requires many participants, including nucleating factors, proteins that regulate the availability of filament ends, and proteins that facilitate filament elongation. We speculate that zyxin facilitates the recruitment of actin assembly machinery to specific subcellular domains, and that the concentration of zyxin and its partners at these sites is important for creation of a cytoplasmic zone that supports accelerated actin assembly.

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