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 α-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 support 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 • α-actinin • cell motility • Ena/VASP

Many 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 enriched, 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 NH₂-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 NH₂-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 family (Niebuhr et al., 1997; Purich and Southwick, 1997). Ena/VASP family members...
serve as ligands for the actin monomer binding partner, profilin, and have themselves been shown to modulate actin assembly and/or organization (Everitt et al., 1996; Huttelmaier et al., 1999; Laurent et al., 1999). Interestingly, the E na/VA SP binding sites present in zyxin are very similar to proline-rich repeats found in the A ctA 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 E na/VA SP proteins with Listeria A ctA 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 A ctA, zyxin may act to dock members of the E na/VA SP family at particular subcellular locations and may facilitate spatially restricted changes in the actin cytoskeleton within eukaryotic cells.

Zyxin also interacts with α-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; Podlubnaya et al., 1975). Through its interaction with the cytoplasmic domain of β3 integrins (Otey et al., 1990), α-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 α-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 α-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 E na/VA SP 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 NH2-terminal cysteine at the University of Utah core facility or by Genosys, Inc. Lyophilized peptides were re-suspended in sterile PBS, and the pH was adjusted to 7.5 if necessary. The sequence of the Zyx16-30 peptide is A P A F Y A P Q K F G P V V. The sequence of the scrambled peptide used as a control is V A F A K P Y - K Q A V P G P F.

Protein Binding Studies

Binding studies with α-actinin were performed using purified α-actinin from chicken smooth muscle (Feramisco and Burridge, 1980). The site of α-actinin binding on zyxin was determined using a custom-made peptide library (SPOTs) conjugated to a cellulose membrane (F. Rank, 1992) and was provided by Jürgen Weiland (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. Oligonucleotide-α-actinin was used to probe the SPOTs membrane at a concentration of 250,000 cpm/ml.

To evaluate the ability of the soluble Zyx16-30 peptide to block the zyxin-α-actinin interaction, chicken zyxin was expressed in Escherichia coli as a GST fusion protein (Schmeichel and Beckerle, 1994). Lysates were prepared from both unduced bacterial cultures and cultures induced to express GST-zyxin. Proteins were separated by SDS-PAGE and transferred to nitrocellulose. A filter blocking, nitrocellulose strips were overlaid with 5 μg/ml purified α-actinin, or α-actinin preincubated with the α-actinin peptide, or a scrambled peptide. The strips were washed in TBS and fixed briefly in 0.5% formaldehyde, followed by washing in 2.0% glycine. α-Actinin binding to GST-zyxin was detected according to standard immunoblot procedures using an mAb against α-actinin (Sigma Chemical Co.) and HRP-conjugated anti-mouse IgG (Amer sham Life Science) as a secondary antibody, followed by chemiluminescent detection using enhanced chemiluminescence reagents (Amer sham Life Science).

To test the protein binding specificity of the Zyx16-30 peptide, the peptide was coupled to HRP via the NH2-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 μg of coupled Zyx16-30 peptide was diluted in 5 ml TBS + 0.1% Tween 20 and was incubated with the membrane. HRP-Zyx16-30 peptide binding to protein on the blot was detected by using chemiluminescence methods. The membrane was stripped and probed with an mAb against α-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 (Otey et al., 1996), the antiphosphotyrosine monoclonal PY-20 (Transduction Laboratories), antivinculin, and anti-α-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)1 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 μM. These needle concentrations are above the threshold required for the maximal effect on cell behavior. At a needle concentration of 0.06 μM, a smaller percentage of cells displayed effects of the injected peptide. If cells were to be processed for immunofluorescence, FITC-BSA was cojugated with peptides. For injections in which the Zyx16-30 peptide was preincubated with α-actinin, the molar ratio of α-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 H2O and added to cell medium for a final concentration of 10 μM. 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 re-suspended in sterile PBS + 10 mM MgCl2 at a concentration of 2.5 × 10^5 cells/ml. Peptide (400 μM) 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. A abbreviation used in this paper: PtK2, Potoroo tridactylis kidney.
Results

α-Actinin Interacts with Sequences in the NH2-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-α-actinin interaction (Crawford et al., 1992). Zyxin and α-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 α-actinin binding on zyxin using a custom synthesized SPOTs peptide library (Frank, 1992) that spanned the entire human zyxin sequence. Radiolabeled α-actinin was used to probe this library and was found to interact most prominently with a series of three peptides corresponding to sequences in amino acids 13-33 of human zyxin (Fig. 2 A). Our determination of the α-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 Zyx16-30 peptide (APA FY A P Q K K F P V V) interacted most strongly with α-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 α-actinin, their relative strength of binding was at least 50% less than that of the Zyx16-30 peptide. For this reason, we focused on the testing of the Zyx16-30 peptide as a possible inhibitor of the interaction of zyxin with α-actinin.

The ability of a synthetic Zyx16-30 peptide to compete with zyxin for α-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 α-actinin (Fig. 3 A, lanes 3-8). α-A ctinin interacted specifically with the zyxin fusion protein (Fig. 3 A, lane 4). The Zyx16-30 peptide was demonstrated to block the interaction of α-actinin with zyxin.
interaction with zyxin inhibits the interaction of zyxin and interaction. (A) A peptide corresponding to the site of peptide interacts specifically with the zyxin-derived peptide as an inhibitor of the zyxin–
-actinin interaction.

Characterization of the effectiveness and specificity of Figure 3.

Effect on the interaction (lane 8). (B) The HRP-coupled Zyx16-30 scrambled peptide with identical amino acid composition had no effect on the interaction (lane 8). The HRP-coupled Zyx16-30 peptide interacts specifically with -actinin. Lane 1 shows a Coomassie-stained gel of proteins present in a PtK2 cell lysate. HRP-conjugated Zyx16-30 peptide interacts specifically with -actinin (lane 2). Lane 3 shows the same membrane after stripping and probing with an anti--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 Zyx16-30 peptide to act as an inhibitor of the zyxin–-actinin interaction.

To test the specificity of the peptide for binding to -actinin, we used the Zyx16-30 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 Zyx16-30 Peptide. The Zyx16-30 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 -actinin, the membrane was stripped and probed with an mAb directed against -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 Zyx16-30 peptide is likely to be a useful reagent for examining the importance of zyxin’s interaction with -actinin in vivo and for further dissection of zyxin function.

The Zyx16-30 Peptide Causes Mislocalization of Zyxin In Vivo

Once we had established the effectiveness and specificity of the Zyx16-30 peptide reagent as an inhibitor of the interaction of zyxin with -actinin in vitro, we examined the effect of intracellular peptide on zyxin’s subcellular distribu-

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 -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 Zyx16-30 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 Zyx16-30 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 Zyx16-30 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 Zyx16-30 peptide, indicating that focal adhesion structure is still intact (C). -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 Zyx16-30 peptide, the structural integrity of the focal adhesions is retained. Injection of the Zyx16-30 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 α-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–α-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 Zyx16-30 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 (8 to 24%) displayed any noticeable alteration in the normal subcellular distributions of zyxin, Mena, or vinculin. In contrast, the majority of cells injected with the Zyx16-30 peptide showed mislocalization of zyxin and Mena; in both cases ~70% of the cells displayed a clear loss of these proteins from the focal adhesions. At a minimum, these results indicate that injection of the Zyx16-30 peptide causes the concurrent mislocalization of zyxin and Mena in ~40% of the cells examined. Only 25% of cells injected with the Zyx16-30 peptide showed mislocalized or reduced vinculin at the focal adhesion; this level is similar to what we observed for the control 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 Zyx16-30 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 Zyx16-30 peptide into PtK2 cells and monitoring the cellular response in real time using video microscopy. Introduction of the Zyx16-30 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 Zyx16-30 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 α-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 Zyx16-30 peptide with α-actinin before microinjection inhibited the effects of the bioactive peptide (Fig. 5, panel 3, and Fig. 6). Table I 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 Zyx16-30 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.

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

| Adhesion plaque component | Injected peptide | Percentage of cells showing mislocalized protein* |
|---------------------------|------------------|-----------------------------------------------|
| Zyxin                     | Zyx16-30         | 67%                                           |
|                           | Scrambled        | 8%                                            |
| Mena                      | Zyx16-30         | 72%                                           |
|                           | Scrambled        | 24%                                           |
| Vinculin                  | Zyx16-30         | 25%                                           |
|                           | Scrambled        | 19%                                           |

*At least 200 cells were injected and scored in each category.
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 Zyx16-30 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 Zyx16-30 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 mislocalization on these processes, we first examined the effect of peptide-dependent displacement of zyxin on the rate of cell spreading.

In parallel experiments, the Zyx16-30 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. 7A) and achieved an average area of nearly 417 μm². In contrast, cells into which the Zyx16-30 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 |
|----------------------------------|--------------------|---------------------------------------------------------------------|
| Zyx16-30 peptide                 | 80                 | 89%                                                                 |
| Scrambled peptide                | 60                 | 5%                                                                  |
| α-Actinin + Zyx16-30 peptide     | 65                 | 9%                                                                  |
| 0.5 mg/ml FITC-BSA               | 66                 | 12%                                                                 |
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 Zyx16-30 peptide relative to cells harboring the scrambled peptide (Fig. 7 C).

Injection of the Zyx16-30 Peptide Inhibits Cell Migration

To explore further the role of zyxin in cell motility, we examined the effect of introducing the Zyx16-30 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 these highly motile cells (Fig. 8 B). Either the Zyx16-30 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 Zyx16-30 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 \( \mu \text{m/h} \), whereas cells injected with the Zyx16-30 peptide traveled at a rate of 23 \( \mu \text{m/h} \), 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. A n important insight into the possible function of zyxin within cells came with the recognition of...
the relationship between zyxin and the A c t A protein of Listeria monocytogenes (Reinhard et al., 1995; Golsteyn et al., 1997). Both zyxin and A c t A 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 A c t A 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 A c t A 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 α-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 α-actinin to a region in the extreme NH2 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 Zyx16-30 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 (Brindile 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 Zyx16-30 Peptide interferes with the normal subcellular localization of zyxin is not completely clear. A simple interpretation would be that zyxin’s interaction with α-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 α-actinin are substantially colocalized, the presence of α-actinin alone cannot be sufficient to cause the recruitment of zyxin to specific cellular locations, since there are situations in which α-actinin occurs in sites that lack zyxin. Therefore, in our microinjection experiments, it appears likely that the presence of the Zyx16-30 peptide introduces some steric interference that is incompatible with the presence of full-length zyxin.

Introduction of the Zyx16-30 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 Ptk 2 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 A ctA protein of 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 A ctA 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/V A 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...
loss of ability to localize properly within cells (A hern-Djamali et al., 1998). The interaction of Ena/VASP family members with the Listeria A ctA 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 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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