Analysis of the α-Actinin/Zyxin Interaction*

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The yeast two-hybrid system was used to search for interaction partners of human zyxin. Screening of two different cDNA libraries, one prepared from human placenta, the other from human heart, yielded several positive clones that occurred in both searches, including clones coding for cyclophilin, nebulite, and α-actinin. The zyxin/α-actinin interaction was analyzed in detail. By site-directed mutagenesis, a linear motif of 6 amino acids (Phe-Gly-Pro-Val-Val-Ala) present at the N terminus of zyxin was found to play a critical role. Replacement of a single amino acid within this motif abolished binding to α-actinin in blot overlays as well as in living cells. On the other hand, the interaction site in α-actinin was mapped to a conformational determinant present in the center of the protein as demonstrated by a fragment deletion analysis. This binding site involved a tandem array of two complete spectrin-like domains. Only fragments that were able to dimerize in yeast also bound to zyxin, suggesting that dimerization of α-actinin is essential for zyxin binding.

The actin cytoskeleton of a eukaryotic cell plays a central role in a variety of cellular processes, including cell motility, migration, phagocytosis, intracellular transport, and maintenance of cell polarity. To accomplish such critical functions, the assembly and disassembly of the actin filaments must be tightly controlled, both with respect to time and space. Regulatory adapter proteins harboring specific interaction domains (SH2, SH3, PDZ domains) seem to play an important role in the control of actin filament assembly.

One of the adapter proteins that has attracted considerable attention is zyxin (1). Zyxin was originally identified in chicken fibroblasts as a protein associated with focal adhesions, stress fibers, and cell-cell adherence junctions (2). It represents a monomeric protein with an apparent molecular mass of 62 kDa that is phosphorylated at multiple sites. The mammalian homologue of chicken zyxin has recently been cloned from a subtracted cDNA library by virtue of its reduced expression in SV40-transformed human fibroblasts (3). At the same time, human and mouse zyxin cDNA clones were isolated from normal cDNA libraries by cross-hybridization (4). Sequencing studies demonstrated that the avian and the mammalian zyxin display a similar, modular structure with a proline-rich N terminus, a nuclear export signal, and three C-terminal LIM domains, although they share less than 60% sequence identity. Zyxin has been localized to the barbed ends of actin filaments, especially in lamellipodia, filopodia, and focal adhesions, suggesting that it is involved in the organization of the actin cytoskeleton (5). This notion is supported by its structural relationship with the protein ActA from the bacterial pathogen Listeria monocytogenes (6). This microorganism invades eukaryotic cells and exploits the actin cytoskeleton of the host for its own motility (7). Reorganization of the cytoskeleton seems to be accomplished by the bacterial surface protein ActA, which enhances the nucleation of new actin filaments. The structural and functional similarities of zyxin with the central and C-terminal region of ActA suggest that zyxin might also be involved in the assembly and control of the actin cytoskeleton (6).

Zyxin interacts with a variety of cytoskeletal and regulatory proteins, and some of these interactions have been mapped to individual domains of the zyxin polypeptide. It binds to α-actinin, an actin cross-linking protein enriched in focal adhesion plaques (8). The interaction site has recently been identified as the extreme N terminus of zyxin by deletion analysis and peptide inhibition studies (9, 10). When the α-actinin binding site was deleted, the association of zyxin with focal adhesion plaques was largely impaired, emphasizing the physiological role of the zyxin/α-actinin interaction (10). Zyxin also binds to members of the Ena/VASP family of proteins, which are known to control microfilament organization (11, 12). A cluster of four proline-rich motifs present in the N-terminal domain of zyxin seems to be responsible for this VASP-zyxin interaction (13). The proline-rich region of zyxin has also been demonstrated to act as a docking site for the proto-oncogene product Vav (14). Vav is a regulatory protein controlling the activity of small GTP-binding proteins in blood cells. Finally, zyxin binds to members of the cysteine-rich protein family CRP (5). This interaction is accomplished by the first of the three LIM domains found at the C terminus of zyxin (15).

The two-hybrid system offers an elegant approach to analyze protein-protein interactions in yeast (16). It is based on the modular structure of the yeast transcription factor GAL4, which, if reconstituted by interaction of two fusion proteins, will activate transcription of suitable reporter genes (HIS3, lacZ). The advantage of the system is that large numbers of cDNA clones for potential ligands can be screened and that the interactions are tested under physiological conditions within eukaryotic cells.

We have made extensive use of the yeast two-hybrid system to search for additional binding partners of zyxin. In this way we were able to identify a number of potential ligands, including proteins from the cytoskeleton and the transcription machinery, which might be directly or indirectly involved in the formation of actin filaments and focal adhesion plaques.

**EXPERIMENTAL PROCEDURES**

Yeast Two-Hybrid System—Yeast two-hybrid screenings were performed utilizing the matchmaker 2 system of CLONTECH Laboratories (Palo Alto, CA). Selected regions of the human zyxin cDNA (GenBank®
α-Actinin/Zyxin Interaction

| No. | Sequence 5′→3′ | Position | Purpose |
|-----|----------------|----------|---------|
| For zyxin (accession number X95735) | | | |
| U1 | GGCGGCGGCGGCGGCGGCGGCGGCGG | 151–174 | Zyx(V29S) |
| U2 | GGCGGCGGCGGCGGCGGCGGCGGCGG | 127–150 | Zyx(V29S), Zyx(A31S) |
| U3 | GGCGGCGGCGGCGGCGGCGGCGGCGG | 134–160 | Zyx(K4S) |
| U4 | GGCGGCGGCGGCGGCGGCGGCGGCGG | 110–133 | Zyx(K4S) |
| U5 | GGCGGCGGCGGCGGCGGCGGCGGCGG | 156–182 | Zyx(K3S) |
| U6 | GGCGGCGGCGGCGGCGGCGGCGGCGG | 132–158 | Zyx(K3S) |
| U7 | GGCGGCGGCGGCGGCGGCGGCGGCGG | 107–133 | Zyx(P17S) |
| U8 | GGCGGCGGCGGCGGCGGCGGCGGCGG | 83–106 | Zyx(P17S) |
| L9 | GGCGGCGGCGGCGGCGGCGGCGGCGG | 123–149 | Zyx(P22S) |
| L10 | GGCGGCGGCGGCGGCGGCGGCGGCGG | 99–122 | Zyx(P22S) |
| L11 | GGCGGCGGCGGCGGCGGCGGCGGCGG | 134–160 | Zyx(P36S) |
| L12 | GGCGGCGGCGGCGGCGGCGGCGGCGG | 110–133 | Zyx(P62S) |
| U13 | GGCGGCGGCGGCGGCGGCGGCGGCGG | 151–177 | Zyx(A31S) |
| U14 | GGCGGCGGCGGCGGCGGCGGCGGCGG | 170–194 | Zyx(F39S) |
| L15 | GGCGGCGGCGGCGGCGGCGGCGGCGG | 146–169 | Zyx(F39S) |
| U16 | GGCGGCGGCGGCGGCGGCGGCGGCGG | 58–83 | Zyx(GST) |
| L17 | GGCGGCGGCGGCGGCGGCGGCGGCGG | 187–212 | Zyx-GST |
| U18 | GGCGGCGGCGGCGGCGGCGGCGGCGG | 386–408 | Zyx-(110–191) |
| L19 | GGCGGCGGCGGCGGCGGCGGCGGCGG | 1143–1168 | Zyx-(318–362) |
| U20 | GGCGGCGGCGGCGGCGGCGGCGGCGG | 1097–1117 | Zyx-(345–366) |
| L21 | GGCGGCGGCGGCGGCGGCGGCGGCGG | 1215–1236 | Zyx-(398–416) |
| U22 | GGCGGCGGCGGCGGCGGCGGCGGCGG | 1010–1036 | Zyx-(318–362) |
| U23 | GGCGGCGGCGGCGGCGGCGGCGGCGG | 918–941 | Zyx-(289–368) |
| L24 | GGCGGCGGCGGCGGCGGCGGCGGCGG | 1093–1113 | Zyx-(289–346) |
| L25 | GGCGGCGGCGGCGGCGGCGGCGGCGG | 189–209 | Zyx-(42–127) |
| L26 | GGCGGCGGCGGCGGCGGCGGCGGCGG | 448–468 | Zyx-(43–127) |
| L27 | GGCGGCGGCGGCGGCGGCGGCGGCGG | 1781–1807 | Zyx-(370–572) |

For α-actinin (accession number X15804) | | | |
| U28 | GGCGGCGGCGGCGGCGGCGGCGGCGG | 1237–1355 | Actn-(183–630) |
| L29 | GGCGGCGGCGGCGGCGGCGGCGGCGG | 2872–2895 | Actn-(183–892) |
| U30 | GGCGGCGGCGGCGGCGGCGGCGGCGG | 1674–1697 | Actn-(1497–892) |
| L31 | GGCGGCGGCGGCGGCGGCGGCGGCGG | 972–996 | Actn-(1264–315) |
| L32 | GGCGGCGGCGGCGGCGGCGGCGGCGG | 1734–1758 | Actn-(1264–515) |
| L33 | GGCGGCGGCGGCGGCGGCGGCGGCGG | 2975–3103 | Actn-(1289–630) |
| L34 | GGCGGCGGCGGCGGCGGCGGCGGCGG | 2356–2379 | Actn-(1497–725) |
| U35 | GGCGGCGGCGGCGGCGGCGGCGGCGG | 1433–1458 | Actn-(411–630) |
| L36 | GGCGGCGGCGGCGGCGGCGGCGGCGG | 1976–2001 | Actn-(1383–597) |
| U37 | GGCGGCGGCGGCGGCGGCGGCGGCGG | 1548–1573 | Actn-(415–597) |
| L38 | GGCGGCGGCGGCGGCGGCGGCGGCGG | 1815–1841 | Actn-(417–543) |

* Nucleotides that do not correspond to the original cDNA sequences are underlined. Such changes were introduced into the primers to mutate the codons for individual amino acids or to create additional restriction sites.

acccession number X95735 (3)) were prepared by restriction enzyme digestion or by PCR utilizing the primers outlined above in Table I and cloned into the bait vector pAS2-1. The plasmids were transfected by the lithium acetate method into the yeast reporter strain Y190 together with a cDNA library prepared from human placenta (CLONETECH, HL4025AH) or human heart (HL4042AH) in the prey vector pACT2. Selection for HIS3 reporter gene activation was performed on agar plates lacking histidine, tryptophan, and leucine. Colonies appearing after 5–10 days at 30 °C were assayed for β-galactosidase activity utilizing the colony-lift filter assay. For quantitative data, the colonies were grown in liquid medium and assayed for β-galactosidase activity using O-nitrophenyl β-D-galactopyranoside as substrate. The plasmid DNA of positive colonies was isolated with phenol and glass beads as suggested by the supplier of the system. Positive two-hybrid protein interactions were verified by transfection of the plasmids back into the yeast strain Y190. The plasmids of positive colonies were amplified in XL-1 blue and sequenced by the dideoxy chain termination method of Sanger et al. (1977). Sequences were analyzed with the GCG computer program package of the Wisconsin University.

Selected fragments of the α-actinin cDNA (GenBank® accession number X15804 (17)) were prepared in a similar way by PCR (Table I) and cloned into the prey vector pACT2. Two-hybrid interactions with zyxin were assayed as described above. To test for self-interaction (dimerization), the cDNAs for several α-actinin fragments were cloned into the bait vector pAS2-1 and cotransfected into yeast with the prey vector pACT2 carrying the same fragment.

Site-directed Mutagenesis—The codons for individual amino acids were mutated by the ExSite PCR-based mutagenesis method (18) employing the cDNA of zyxin ligated into pUC19 as a template. The forward primer harbored the desired mutation, whereas the reverse primer was phosphorylated at its 5′-end and selected in a way that it annealed directly adjacent to the 5′-end of the forward primer (Table I). After amplification by PCR, the maternal DNA was removed by digestion with the restriction enzyme DpnI (Roche Molecular Biochemicals, Switzerland). The ends of the linear products were joined by ligation with T4 DNA ligase (Roche Molecular Biochemicals) and the nicked plasmids were transfected into competent bacteria (E. coli XL-1 blue). Authenticity and reading frame of all mutated clones were verified by DNA sequencing.

GST Fusion Protein Expression and Blot Overlays—The cDNA sequences for wild type and mutated zyxin (residues 1–42) were subcloned into the BamHI/XhoI restriction site of the expression vector pGEX-5X-2 (Amersham Pharmacia Biotech) downstream of the gst gene and transfected into competent bacteria (E. coli BL21). Fusion proteins were expressed after induction with isopropylthio-galactoside as suggested by the supplier of the GST fusion system (Amersham Pharmacia Biotech). The bacteria were collected by centrifugation and lysed by sonication. Fusion proteins were purified from the lysates by affinity chromatography on reduced glutathione-Sepharose and analyzed on 15% SDS-polyacrylamide gels. After transfer to nitrocellulose by electroblothing, the polypeptides were detected with the GST detection module (Amersham Pharmacia Biotech) using goat anti-GST antibodies, followed by alkaline-phosphatase-conjugated secondary antibodies (Sigma Chemical Co.). The color reaction was performed with bromochlorindolyl phosphate and nitroblue tetrazolium as substrate. A similar blot prepared in parallel was blocked with bovine serum albumin and incubated with radiolabeled α-actinin in 10 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.1% 2-mercaptoethanol, 20 mM HEPES, pH...
Fig. 1. Autonomous transactivation of various zyxin fragments in the yeast two-hybrid system. DNA sequences coding for the zyxin fragments as indicated were ligated into the bait vector pAS2-1. The resulting plasmids were transfected into the yeast reporter strain Y190. Reporter gene activation was analyzed by growth on histidine-, tryptophan-, and leucine-deficient plates and by the colony-lift filter assay.

RESULTS

Two-Hybrid Screening—Initial studies utilizing the complete zyxin cDNA for two-hybrid analysis suggested that zyxin itself must have transcription-activating properties. Yeast cells transfected only with the bait plasmid coding for the DNA binding domain of GAL4 fused to the full-length zyxin cDNA activated transcription of the HIS3 and the lacZ reporter genes and grew on selective agar plates. The zyxin sequence was tested separately for its autonomous activating potential (Fig. 1). Two regions were found to induce autonomous reporter gene activation. One region was situated between residues 110 and 167 and contained two proline clusters. The four fragments of zyxin that did not cause autonomous activation were used to screen a cDNA library prepared from human placenta in the prey vector pACT2. With the N-terminal fragment spanning residues 1–42, a large number of positive colonies were obtained that grew on histidine-deficient agar plates. In contrast, no meaningful clones were obtained with the other three fragments spanning residues 43–127, 289–366, and 370–572.

We therefore focused on the N terminus (residues 1–42) of human zyxin. Screening of $3 \times 10^6$ transformants with this bait led to the isolation of 68 putative positive colonies that grew on histidine-deficient plates and that transcribed the lacZ reporter gene as demonstrated by the colony-lift filter assay. The corresponding pACT plasmids were isolated and transfected back into competent cells along with the original bait plasmid, or alternatively, with an unrelated control plasmid that coded for lamin 5 fused to the DNA binding domain of GAL4. Of the 68 candidates tested, 47 induced reporter gene activation exclusively when cotransfected with the zyxin bait plasmid, but not the lamin 5 plasmid, suggesting that they represent truly positive clones.

Sequencing studies demonstrated that the 47 plasmids coded for a total of 12 independent proteins. These proteins could be grouped according to their putative function and their subcellular distribution in eukaryotic cells (Table II). The four, namely α-actinin 1, nebulette, and the zyxin-related protein ZRP, are involved in the assembly of the cytoskeleton and the formation of focal adhesion plaques. One protein, GRIP 1, is associated with the plasma membrane. Two proteins, fibulin 2 and fibronectin, are typical members of the extracellular matrix. Cyclophilin B is a peptide isomerase that assists in the cis-trans isomerization of proline-rich segments. Sorting nexin 5 is involved in intracellular protein trafficking. Finally, PPAR

KEVEEQL, which was previously demonstrated to play a role in the nuclear-cytoplasmic shuttling of zyxin (20). The other region was situated between residues 110 and 167 and contained two proline clusters.

7.5, as described previously (8, 10). After 4 h at room temperature, the blot was washed twice with the same buffer and exposed to BioMax MS film (Eastman Kodak Co.).

Cell Culture and GFP Fusion Protein Expression—Primary chicken fibroblasts were prepared from tendons of 17-day-old chicken embryos with the help of collagenase (Roche Molecular Biochemicals) as described previously (19). COS-1 cells (CRL-1650) were purchased from the American Type Culture Collection (Manassas, VA). The cells were cultivated under an atmosphere of 5% CO2 in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin.

Wild type and mutated zyxin sequences were cloned into the EcoRI/BamHI site of the expression vector pEGFP-C3 (CLONTECH) downstream of the GFP reporter gene. The plasmids (1 µg/well) were mixed with 100 µl of Opti-MEM I (Life Technologies) containing 3 µl of FuGENE-6 reagent (Roche Molecular Biochemicals) and added to the cells grown to 60% confluence in 6-well plates. One day after transfection, the cells were washed with phosphate-buffered saline and inspected under a Zeiss Axiovert microscope equipped with epifluorescence optics.

Transfected cells were examined for the distribution of endogenous zyxin, VASP, and vinculin by indirect immunofluorescence as described previously (10). A monoclonal antibody against human vinculin was purchased from Sigma (St. Louis, MO) and used at a dilution of 1:500 and 1:100, respectively. Rabbit antisera against human VASP (M4) and porcine zyxin (11) were kindly provided by Dr. M. Reinhard (University of Würzburg, Germany) and used at a dilution of 1:500 and 1:100, respectively. A monoclonal antibody against human vinculin was purchased from Sigma (St. Louis, MO) and used at a dilution of 1:400.

RESULTS

Two-Hybrid Screening—Initial studies utilizing the complete zyxin cDNA for two-hybrid analysis suggested that zyxin itself must have transcription-activating properties. Yeast cells transfected only with the bait plasmid coding for the DNA binding domain of GAL4 fused to the full-length zyxin cDNA activated transcription of the HIS3 and the lacZ reporter genes and grew on selective agar plates. The zyxin sequence was therefore cut into several fragments, and each fragment was tested separately for its autonomous activating potential (Fig. 1). Two regions were found to induce autonomous reporter gene activation. One region was situated between amino acid residues 345 and 362 and harbored the nuclear export signal KEVEEQL, which was previously demonstrated to play a role in the nuclear-cytoplasmic shuttling of zyxin (20). The other region was situated between residues 110 and 167 and contained two proline clusters.

The four fragments of zyxin that did not cause autonomous activation were used to screen a cDNA library prepared from human placenta in the prey vector pACT2. With the N-terminal fragment spanning residues 1–42, a large number of positive colonies were obtained that grew on histidine-deficient agar plates. In contrast, no meaningful clones were obtained with the other three fragments spanning residues 43–127, 289–366, and 370–572.

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**Table II**

| Proteins interacting with zyxin-(1–42) | Localization or protein family | Total clones | Independent clones | GenBank™ number |
|--------------------------------------|--------------------------------|--------------|-------------------|----------------|
| Placenta library                     |                                |              |                   |                |
| α-Actinin 1                          | Cytoskeleton                   | 5            | 3                 | M95178         |
| Nebullette                           | Cytoskeleton                   | 1            | 1                 | AF047368       |
| ZBP                                  | Cytoskeleton                   | 3            | 2                 | AF009974       |
| Fibulin 2                            | Extracellular matrix           | 11           | 8                 | X82491         |
| Fibronectin                          | Extracellular matrix           | 6            | 5                 | X002761        |
| Cyclolin B                           | Fibrillar matrix               | 8            | 5                 | M60857         |
| Sorting nexin                        | Protein sorting                | 1            | 1                 | AF121855       |
| GRIP 1                               | Membrane-associated            | 4            | 1                 | AJ33439        |
| PPAR γ2                              | Transcription factor           | 1            | 1                 | U73912         |
| BSRPY                                | Transcription factor           | 1            | 1                 | AJ276691       |
| Novel 26                             |                                 | 7            | 1                 |                |
| Heart library                        |                                |              |                   |                |
| α-Actinin 2                          | Cytoskeleton                   | 1            | 1                 | M86406         |
| Nebullette                           | Cytoskeleton                   | 4            | 3                 | AF009974       |
| Fibulin 2                            | Extracellular matrix           | 1            | 1                 | X82494         |
| Cyclolin A                           | Fibrillar matrix               | 1            | 1                 | X32851         |

**Fig. 2.** Mutational analysis of the zyxin/α-actinin interaction. Yeast reporter strain Y190 was cotransfected with the bait vector pAS2-1 containing the sequence for zyxin residues 1–42 and the prey vector pACT2 containing the sequence for α-actinin 1. The codons for some residues of wild type zyxin were replaced by codons for serine by site-directed mutagenesis as indicated. Colonies that grew on tryptophan- and leucine-deficient plates were analyzed for transcription of the β-galactosidase reporter gene using a quantitative, colorimetric assay. The results are expressed relative to the wild type zyxin sequence (100%) and represent the means with standard deviation from three independent determinations.

**Fig. 3.** Verification of the two-hybrid protein interactions by the blot overlay assay. Wild type (Zyxin) or mutated (Phe-26 → Ser or Phe-39 → Ser) zyxin sequences for amino acids 1–42 were expressed in bacteria as GST fusion proteins. The fusion proteins were resolved on polyacrylamide gels and blotted onto nitrocellulose. The blots were either stained with antibodies against GST (Immunoblot) or probed with radiolabeled α-actinin (Blot Overlay). The migration position of globular protein standards is indicated in the left margin.

**Fig. 4.** Influence of mutations on the subcellular distribution of zyxin. COS cells and chicken tendon fibroblasts (CTF) were transfected with various zyxin cDNA sequences ligated into the GFP expression vector pEGFP. One day after transfection, the cells were mounted for direct fluorescence microscopy. The vector contained either the wild type (WT) or the mutated zyxin sequences as indicated (F26S, Phe-26 → Ser; F39S, Phe-39 → Ser). Bar, 20 μm.
relative transcriptional activity. These results indicate that amino acids 26–31 (Phe-Gly-Pro-Val-Val-Ala) represent the critical residues involved in the zyxin/α-actinin interaction.

Independent Verification — The results obtained by the two-hybrid system were confirmed by the blot overlay technique (Fig. 3). The wild type N terminus of zyxin and its mutated forms were expressed as GST fusion proteins in bacteria, resolved on a polyacrylamide gel, and transferred to nitrocellulose. When incubated with radiolabeled α-actinin, the probe bound specifically to the wild type zyxin fusion protein, but barely to the mutated form in which Phe-26 had been replaced by Ser (Fig. 3). Consistent with the results from the quantitative two-hybrid analysis, mutation of Phe-39 to Ser appeared to enhance the relative binding activity to α-actinin. Thus, replacement of a single amino acid (Phe-26 → Ser) inhibits the zyxin/α-actinin interaction not only in yeast cells but also in a direct, biochemical assay.

To examine whether a single amino acid substitution would abolish the interaction also under physiological conditions, we prepared full-length fusion constructs with green fluorescent protein (GFP), which allowed the visualization of the subcellular distribution of zyxin in living cells (Fig. 4). Transfection of the wild type zyxin sequence into chicken fibroblasts yielded a positive signal along stress fibers and at focal adhesion sites consistent with the published distribution of zyxin (5, 10). When Phe-26 was replaced by Ser, the specific staining was abolished and the fusion protein distributed evenly throughout the cytoplasm of the cells (Fig. 4). Analogous results were obtained with COS cells. When Phe-39 was mutated to Ser, results similar to those obtained with the wild type sequence were obtained. In some experiments the staining at focal contacts appeared to be more strongly pronounced in agreement with the enhanced interaction of this construct with α-actinin in blot overlays. Thus, replacement of a single amino acid at the N terminus of zyxin (Phe-26 → Ser) not only abolishes the binding of zyxin to α-actinin but also impairs the recruitment of zyxin to its normal subcellular sites.

Binding Site in α-Actinin — Next we focused on the other binding partner and analyzed the interaction site in the α-actinin molecule. A full-length cDNA that had been obtained during the initial two-hybrid screening was successively truncated from the 5′-end or the 3′-end, and the resulting constructs were analyzed for their potential interaction with zyxin by the two-hybrid system (Fig. 5). α-Actinin has a modular structure consisting of two calponin homology (CH) domains, four spectrin like (SPEC) repeats, and two C-terminal EF hands. A cDNA construct coding for the N-terminal half of the molecule but lacking SPEC domains 3 and 4 as well as the C-terminal EF hands was inactive. In contrast, a construct lacking the two CH domains but containing all the SPEC domains 1 and 2 was active. Positive interactions (+) were verified by the colony filter-lift assay. CH, calponin homology domain; SPEC 1, spectrin-like repeat 1; EF, calmodulin-like domain containing EF hands. The boundaries of these domains were determined with the Simple Modular Architecture Research Tool (SMART (34)).
zyxin interacted exclusively with the complete tandem array of SPEC domains 2 and 3, but not with any of the truncated forms derived thereof. As soon as a short piece of SPEC domain 2 or SPEC domain 3 was deleted, binding to zyxin was abolished. Consequently, it is unlikely that zyxin recognizes a sequence situated in the central region of the SPEC domains, because this sequence was preserved in each of the subfragments tested. On the other hand, the full tandem array of SPEC domains 2 and 3 was the only construct that formed stable dimers in yeast as indicated by the quantitative β-galactosidase assay (Fig. 6). When the first α-helix of SPEC domain 2 or the third α-helix of SPEC domain 3 was deleted, dimerization was grossly impaired. These results demonstrate that zyxin binds specifically to the dimeric assembly of the central α-actinin rod but not to any of the monomeric fragments derived thereof.

**DISCUSSION**

Zyxin is a cytoskeletal protein that plays an important role in the organization of actin filaments at focal adhesion sites. To fulfill this function, zyxin makes multiple contacts with structural and regulatory proteins, but thus far only the interactions with the actin-cross-linking protein α-actinin, the regulatory protein VASP, and the LIM protein CRP have been described in some detail.

In this study, we have made extensive use of the yeast two-hybrid system to search for additional interaction partners of zyxin. During the initial experiments it became evident that zyxin is a difficult bait to work with, because it has autonomous transactivation properties. By a fragment analysis approach, we could identify two regions of the protein that are responsible for this autonomous activation. One region contains two proline clusters but otherwise does not exhibit any structural motif that would explain its interaction with nuclear proteins of the RNA polymerase complex. The other region contains a nuclear export signal that has previously been identified by Nix and Beckerle (20).

Our search had therefore to be restricted to fragments that did not induce autonomous transactivation in the two-hybrid system. Searches with fragments comprising the three LIM domains or portions of the proline-rich N terminus have not been fruitful so far. Our search, however, was successful with the N-terminal fragment of human zyxin spanning residues 1–42. To facilitate the identification of truly positive clones, two different cDNA libraries, one prepared from human placenta, the other from human heart, were screened in parallel. Among all the positive clones obtained, there were four that coded for the same or the homologous protein in the two tissues, namely fibulin, cyclophilin, nebulette, and α-actinin. Fibulin is a typical protein of the extracellular matrix. Because zyxin is normally not found in the extracellular matrix, this two-hybrid interaction must be fortuitous, although the majority of independent clones coded for fibulin. Cyclophilin is a cis/trans isomerase that promotes the isomerization of peptide bonds at prolyl residues. This protein might fulfill an important function during the folding of the proline-rich N terminus of zyxin. It is therefore plausible that zyxin possesses a docking site for cyclophilin at its N terminus where the enzyme is actually needed. Five independent clones for cyclophilin B were obtained from the placenta library, whereas one clone for cyclophilin A was retrieved from the heart library. Studies are now in progress to verify this interaction by biochemical experiments. Nebulette is a cytoskeletal protein expressed in heart muscle. Similar to fibulin it is involved in the assembly of the sarcomere, where actin filaments are anchored to the plasma membrane and the Z-disc. Zyxin and nebulette could therefore play an important role in the anchorage of microfilaments to

**FIG. 6. Interaction of zyxin with the α-actinin dimer.** Two-hybrid interactions were analyzed in yeast as described in the legend to Fig. 5. A quantitative colorimetric assay was used to determine the expression of the reporter gene β-galactosidase. The results are expressed in enzyme units and represent the means with standard deviation from three independent determinations. The pACT2 vector contained various cDNA fragments for the second and third SPEC domain of α-actinin as indicated. The pAS2-1 vector contained the same fragments (when self-interaction was analyzed) or the cDNA for zyxin residues 1–42 (when the α-actinin/zyxin interaction was analyzed). Helices 1–3 refer to the α-helices of the SPEC domains as described (21). n.d., not done.
the plasma membrane, an attractive hypothesis that can be
tackled now by biochemical experiments.

The last binding partner of zyxin that was identified in both
screens was α-actinin. From the placenta library, α-actinin 1
was obtained, whereas from the heart library α-actinin 2
was recovered. This is consistent with the relative expression of the
isofoms in the two tissues. The zyxin/α-actinin interaction is
thus far the only interaction that has been verified by biochem-
ical experiments (8, 10) and that has also been documented in
living cells under physiological conditions (9, 10). Because sev-
eral full-length cDNAs for α-actinin were obtained during our
screenings, we were able to map the interaction site in minute
detail.

Our results in combination with data from the literature
provide compelling evidence that a linear epitope of zyxin binds
to a conformational epitope of α-actinin. By site-directed muta-
genesis the critical amino acids of zyxin that are involved in
the interaction were identified as residues 26–31 (Phe-Gly-Pro-
Val-Val-Ala). Replacement of a single amino acid (Phe-26 →
Ser) within this motif abolished α-actinin binding in the two-
hybrid system and in blot overlays. The same mutation also
prevented the normal subcellular distribution of zyxin in fibro-
basts, emphasizing the physiological importance of the bind-
ing motif. The critical six residues are part of a larger domain
that has previously been identified as the major α-actinin bind-
ing site. Using a crude deletion analysis (10) we have mapped
this site to a fragment spanning zyxin residues 21–42 and
demonstrated that this fragment is both necessary and suffi-
cient for α-actinin binding. On the other hand, Drees et al. (9)
have developed a specific peptide inhibitor comprising zyxin
residues 16–33 that blocked the interaction of zyxin with α-
actinin. Injection of this peptide displaced zyxin from its normal
subcellular location and perturbed cell migration. Taken to-
gether all these data suggest that zyxin contains a linear epitope
exposed at its surface that is specifically recognized by
α-actinin. The most critical amino acids of this epitope are
represented by the sequence motif Phe-Gly-Pro-Val-Val-Ala.
This motif also occurs in LPP (23) but not in the related protein
ZRP (24). It will therefore be of interest to investigate whether
α-actinin does also interact with LPP but not with ZRP.

The situation seems to be quite different in the case of the other
interaction partner, α-actinin. Deletion analyses indi-
cated that zyxin binds to the central region of α-actinin made
up of SPEC domains 2 and 3. There is good evidence that a
conformational, rather than a linear determinant is recognized
in this case. When the tandem array of the two SPEC domains
is truncated by deletion of short pieces from the N terminus or
from the C terminus, binding to zyxin is abolished. The size of
the zyxin fragment used in our studies does not allow it to bind
simultaneously to the N terminus and to the C terminus of the
tandem array. It is therefore likely that the deletions cause a
major change in the conformation of the tandem repeat, which
is no longer compatible with binding. In this context, it is
important to remember that α-actinin forms rod-like dimers in
which two anti-parallel molecules align in register. The tandem
array of SPEC domains 2 and 3 appears to be the minimal
fragment that forms stable dimers in vitro (21). As soon as a
short piece is removed from the N terminus or from the C
terminus of this tandem array, dimerization is prevented as
demonstrated by our two-hybrid analysis. The loss of dimeriza-
tion might therefore explain the loss of zyxin binding. Thus, we
propose that zyxin binds into a groove formed by the α-actinin
dimer where it interacts simultaneously with both anti-parallel
chains. This interpretation is consistent with our preliminary
findings that α-actinin does not interact with zyxin in blot
overlays after heat denaturation.2

Our results are at variance with the findings of Crawford et al. (8),
who demonstrated binding of zyxin to the N-terminal
CH domain of α-actinin. When thermolysin-derived fragments
of α-actinin were blotted onto nitrocellulose and probed with
native, radiolabeled zyxin, the 27-kDa fragment corresponding
to the N-terminal domain reacted with the probe but not the
53-kDa fragment corresponding to the SPEC repeats. One pos-
sible way to reconcile these conflicting results is that α-actinin
contains more than one binding site for zyxin. One site would
be the conformational determinant in the center of α-actinin as
identified in our report, the other would be an unrelated deter-
minant at the N terminus of α-actinin. Another possibility is
that native zyxin is extremely sticky and produces unspecific
staining when used as a radiolabeled probe in blot overlays. At
any rate, binding of radiolabeled zyxin to the dimeric form of
α-actinin as proposed in our report cannot be demonstrated by
the blot overlay technique, because α-actinin will not form
dimeric assemblies after denaturation and separation on SDS-
polyacrylamide gels.

A great variety of molecules are now known that interact
with α-actinin (reviewed in Ref. 22). Some of these molecules,
including actin (22), CRP (25), and ERK (26), interact with
the N-terminal CH domain of α-actinin. Other proteins such as
titin (27) and ZASP (28) have been reported to interact specif-
ically with the C-terminal domain containing the EF hands.
Again some other proteins bind, in a way similar to zyxin, to
the central SPEC repeats of α-actinin, namely α-catenin, the
Z-disc protein ALP, and its related protein CLP-36, the protein
kinase PKN and the methyl-aspartate receptor NMDA. Bind-
ing of α-catenin involves the central region of SPEC domains
2 and 3 (residues 479–529) and does not seem to depend on
the dimerization of α-actinin (29). ALP binds via its PDZ domain
to SPEC repeat 3, which by itself does not form dimers (30). The
interaction of CLP-36 with α-actinin has not yet been analyzed
in detail (31). Based on its structural homology with APL, it is
likely that CLP-36 binds to SPEC repeat 3, too. The same
domain has also been identified as the major target for protein
kinase PKN (32). In the case of the methyl-aspartate receptor
NMDA, SPEC domain 4 appears to be essential, although a
longer fragment is required for strong binding (33). Thus, all
the interactions listed above do not seem to depend on the
integrity of the tandem array of SPEC domains 2 and 3. In
contrast, zyxin is an example that requires the dimeric, anti-
parallel conformation of α-actinin for interaction. It would be
interesting to investigate whether this interaction is affected
by binding of other ligands in close vicinity. This is conceivable
for the regulatory proteins ALP (31) and CLP-36 (30), because
these proteins possess, in addition to their PDZ domain, a
C-terminal LIM domain that could interact with the C-termi-
nal LIM domains of zyxin. In this way, ALP and CLP-36 could
modulate the interaction of zyxin with α-actinin and control the
recruitment of zyxin to focal adhesion sites.

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