The LIM domain protein zyxin is a component of adherens type junctions, stress fibers, and highly dynamic membrane areas and appears to be involved in microfilament organization. Chicken zyxin and its human counterpart display less than 60% sequence identity, raising concern about their functional identity. Here, we demonstrate that human zyxin, like the avian protein, specifically interacts with α-actinin. Furthermore, we map the interaction site to a motif of approximately 22 amino acids, present in the N-terminal domain of human zyxin. This motif is both necessary and sufficient for α-actinin binding, whereas a downstream region, which is related in sequence, appears to be dispensable. A synthetic peptide comprising human zyxin residues 21–42 specifically binds to α-actinin in solid phase binding assays. In contrast to full-length zyxin, constructs lacking this motif do not interact with α-actinin in blot overlays and fail to recruit α-actinin in living cells. When zyxin lacking the α-actinin binding site is expressed as a fusion protein with green fluorescent protein, association of the recombinant protein with stress fibers is abolished, and targeting to focal adhesions is grossly impaired. Our results suggest a crucial role for the α-actinin-zyxin interaction in subcellular zyxin localization and microfilament organization.

A panoply of cellular processes, such as cell-cell and cell-matrix adhesion, motility, morphogenesis, growth and differentiation, are largely dependent on the structure and dynamics of the actin-based cytoskeleton and its associated signaling proteins. Elucidation of the mutual interactions between the wide variety of known microfilament-associated proteins has shed light on the underlying organization of the multi-level protein network and also on its linkages e.g. to transmembrane receptors (for a review see Ref. 1). Due to their involvement in a variety of biological systems, special interest has been devoted to zyxin and the F-actin-binding protein α-actinin, two interacting constituents of these supramolecular protein complexes.

α-Actinin is a major constituent of diverse actin-based structures, such as stress fibers, focal adhesions, and the peripheral belt of epithelial cells (2, 3). α-Actinin is also found in the cortical actin filament webs at the periphery of highly dynamic lamellae (2). The protein forms a rod-shaped antiparallel dimer of approximately 100-kDa subunits, each subunit comprising an N-terminal actin binding domain, four central spectrin-like repeats, and two C-terminal EF-hand motifs (4). Whereas the spectrin-like repeats are thought to be involved in dimerization, the C-terminal domain confers Ca\textsuperscript{2+} sensitivity on actin binding of different nonmuscle isoforms (5). Like few other cytoskeleton-associated proteins, α-actinin constitutes a direct link between the actin cytoskeleton and the cytoplasmic domains of several cell surface receptors, including integrins β\textsubscript{1} (6), β\textsubscript{2} (7), and α\textsubscript{v}β\textsubscript{3} (6), ICAM-1 (8), ICAM-2 (9), L-selectin (10), and the N-methyl-d-aspartate receptor (11). This cytoskeletal connection has been implicated in receptor function and anchorage, cytoskeletal reorganization, and concomitant signaling events (1, 7, 11). Binding of α-actinin to PIP\textsubscript{2} and phosphatidylinositol 3-kinase, as well as a PIP\textsubscript{2}-regulated interaction with the Rho effector kinase PKN (12) and vinculin (13) are also consistent with functional roles of α-actinin in signal transduction pathways and cytoskeletal organization (5). α-Actinin is intimately linked with microfilamentous structures (5) not only by its actin cross-linking activity but also by its interaction with the cell-cell adherens junction protein α-catenin, different cysteine-rich proteins (CRPs) (14–17) and zyxin (18). Due to the substantial overlap in subcellular distribution and the emerging functions of zyxin in actin filament regulation, the α-actinin-zyxin interaction has gained special interest.

Zyxin was originally identified in chicken fibroblasts as a protein associated with focal adhesions, stress fibers, and cell-cell adherens junctions (19). A human cDNA coding for a protein closely resembling chicken zyxin but with less than 60% sequence identity has been isolated in a subtractive cDNA cloning approach by virtue of its reduced expression in SV40 transformed fibroblasts (20). Independently, an equivalent cDNA has been cloned from an umbilical vein endothelial cell library (21). Zyxin is composed of an extended N-terminal part, which is rich in prolines, and three C-terminal LIM domains. In addition to its sequence similarity, the human protein showed the same subcellular distribution (21, 23) and VASP binding activity (23) as chicken zyxin and hence was proposed to represent the mammalian homologue of avian zyxin (20, 21, 23).

There is a large body of evidence that zyxin plays a major role in the organization of actin filaments in living cells. This evidence originates from its structural and functional relationship with the bacterial protein ActA found at the surface of the intracellular bacterial pathogen *Listeria monocytogenes* (23, 25, 27, 28). This microorganism invades eukaryotic cells and...
exploits the host actin-based cytoskeleton to promote its own intracellular motility and cell to cell spread (for a review, see Ref. 29). Detailed studies have revealed that the bacterial protein ActA is both necessary and sufficient for the recruitment of actin filaments and actin-based motility (29). ActA seems to accomplish this function by enhancing the Arp2/3 complex driven nucleation of new actin filaments (30). The N-terminal region of zyxin appears to share some structural and functional similarity with the central and the C-terminal portion of the ActA protein (23, 25, 28). In particular, both zyxin and ActA harbor proline-rich binding sites for their common ligands VASP (23, 25, 27) and Mena (24, 25). The interactions with VASP and Mena are thought to contribute to efficient actin polymerization by recruiting polymerization competent profilin complexes (24, 31). Hence, zyxin may likewise regulate actin filament organization and thus represent an endogenous ActA analogue in eukaryotic cells (23, 25, 28).

By in vitro binding assays, chicken zyxin has been shown to interact with α-actinin (18); the LIM-only proteins CRP1, CRP2, and CRP3/MLP (14–17); and the Ena/VASP homology 1 domain of the microfilament organizing proteins VASP and Mena (23–25). The proto-oncogene product Vav binds zyxin in an SH3 domain-dependent manner (26). The in vitro interaction between zyxin and α-actinin is of moderate affinity and involves the N-terminal part of α-actinin (18), which includes the actin binding domain (4) and also binds to PIP2 (5) and chicken CRP1 (32). Vice versa, the α-actinin binding site has only roughly been mapped to the N-terminal region of chicken zyxin (amino acids 1–348; Ref. 15). A similar interaction with α-actinin has not yet been established for human zyxin, and also, functional in vivo evidence for a direct α-actinin-zyxin linkage is missing.

Therefore, we set out to investigate a putative interaction between human zyxin and α-actinin in more detail. In this study, we demonstrate that the human protein binds to α-actinin as described for the avian protein. We further localize the interaction site to a motif of about 22 amino acids, present in the zyxin N terminus. Using a synthetic peptide and various deletion constructs, we finally show that this motif, but not a related downstream region, is both necessary and sufficient for zyxin interaction with α-actinin in vitro. Furthermore, this α-actinin binding site is essential for zyxin-dependent α-actinin recruitment in living cells as well as for proper subcellular zyxin localization.

**EXPERIMENTAL PROCEDURES**

**GST Zyxin Fusion Proteins**

Twelve cDNA clones for human zyxin had previously been isolated from a placenta cDNA library (20). The inserts of seven clones with successively truncated 5′-ends (B91, B92, B4, 925, 835, 725, and 225) were selected and subcloned into the expression vector pGEX-5X (Amersham Pharmacia Biotech) downstream of the GST gene. Orientation and reading frame of the final constructs were verified by DNA sequencing. Shorter fusion polypeptides were prepared by expression of different restriction fragments in E. coli (BL21). These fragments were prepared from the 5′-end of the full-length zyxin cDNA by partial or complete digestion with the enzymes EcoRI, SmaI, NcoI, MspI, and HaeIII and subsequently ligated in the desired reading frame into the expression vector pGEX-5X.

**Mitochondrial Targeting of Zyxin**

An FspI fragment comprising bases 55–1318 of the human zyxin cDNA (20) was inserted into the SmaI site of the eukaryotic expression vector pSP61 (33, 34). The MscI-BamHI fragment of this construct was replaced by a different restriction fragment with the zyxin MscI-MaeII fragment and an MaeII-BamHI adapter consisting of the complementary oligonucleotides A1 and A2, resulting in the wild type zyxin mitochondrial targeting construct (targZyxinWT).

**Construction of Zyxin Deletion Mutants**

Deletion of Zyxin Amino Acids 19–41—Two PCR products were amplified with the wild type mitochondrial targeting construct as a template and the primer pairs U4589/S1L and S1U/L1708, introducing a unique SacII restriction site into each PCR product. Both products were digested with SacII and ligated. The ligation product was then used as a template for PCR amplification with the primer pair U1559/L1423. The resulting PCR product was gel purified and cloned into pcRII vector (Invitrogen) and a 267-base pair Ncol-BglII fragment of the corresponding clone was ligated into either Neo1/BglII digested targZyxinWT or wild type GST-zyxin vector.

Deletion of Zyxin Amino Acids 261–283—Two PCR products amplified from wild type GST-zyxin vector with the primer pairs S1U/S2L and S2U/L1186, respectively, were ligated after NcoI digestion. With the ligated product as a template and the primer pairs S1U and L1186, a PCR product was amplified, gel purified, and ligated into pcScript Amp SK(+) vector (Stratagene). A 589-base pair fragment, which comprises the deletion site corresponding to zyxin amino acids 261–283, was excised with BglII-DraI and inserted into the BglII-DraI sites of wild type GST-zyxin, GST-zyxin Δ19–41, targZyxinWT, and targZyxin Δ19–41, yielding the corresponding constructs GST-zyxin Δ261–283, GST-zyxin Δ19–41/Δ261–283, targZyxin Δ261–283, and targZyxin Δ19–41/Δ261–283, respectively.

Insertion of Zyxin Amino Acids 262–284 into Position 17–41—Two complementary synthetic oligonucleotides, OU and OL, were annealed, phosphorylated with T4 polynucleotide kinase, and inserted into the SacII-digested and dephosphorylated targZyxin Δ19–41 construct. To reduce the background of parental plasmids, the ligated product was digested with SacII prior to transformation into competent DH5α cells.

All junctions of different sequence inserts as well as all PCR-amplified regions were verified by DNA sequencing.

**Oligonucleotides**

The oligonucleotides used were as follows: A1, 5′-CGTTGCTTGTGCGAATGCCACCTGCAGCCCCAGCG-3′; A2, 5′-GATCCGTGCTTGGCTCTAGCTGTGGCACTTCGCAAGACGA-3′; L1423, 5′-ACTTACGGCGCTGCAGGAGATGAG-3′; D19-41, 5′-TGCACCCCGCGGGAGCCGAGACCGAAACGGAG-3′; S1L, 5′-TGACCCCCGGGGCCGAGGAGACCGAAGAACGAG-3′; S1U, 5′-CGCTCTCCGCCGGTGAGCCACGAGCGCTCCCCCGGCCAC-3′; S2L, 5′-GAATTTGGCCGCGCAAGAGCAGCCGCCG-3′; S2U, 5′-GAGTAAAAAGACCCCTGCTTTTACCACTTTGG-3′; U4589, 5′-GCACAGGAATGTTGAAAAG-3′; TTAGGCGCTGGAGCCGGAGATGAG-3′; and L1708, 5′-CTCGAGCTGTGGCAGACCTTACAGTTC-3′.

**Green Fluorescent Protein (GFP) Zyxin Fusion Constructs**

The full-length cDNA sequence of human zyxin and three deletion constructs (see above) were amplified by PCR and cloned into the EcoRI-BamHI site of the expression vector pEGFP-C3 (CLONTECH, Palo Alto, CA) downstream of the reporter gene for GFP. The final constructs harbored the zyxin sequence for amino acid residues 1–570 (wild type) or the same sequence with deletions corresponding to amino acid residues 19–41 (GFP-zyxin Δ19–41), 261–283 (GFP-zyxin Δ261–283) or 19–41 or 261–283 (GFP-zyxin Δ19–41/Δ261–283). The reading frame and the authenticity of the final constructs were verified by DNA sequencing.

**Transfection of Cells**

pEGFP-C3 plasmids (1 μg/well) were mixed with 100 μl of Opti-MEM 1 (Life Technologies) containing 3 μl of FuGENE-6 reagent (Roche Molecular Biochemicals) and were added to COS-1 cells (ATCC CRL-1650), which had grown to 60% confluence in 6 well plates. Chicken tendon fibroblasts (36) were transfected in a similar way using SuperFect reagent (Qiagen). One to 2 days after transfection, the cells were washed with PBS and inspected at an excitation wave length of 450–490 nm.

PtK2 cells were grown on coverslips in minimum essential medium with Eagle’s salts, Glutamax™ (Life Technologies, Inc.), and 10% fetal calf serum. Cells were transfected with the mitochondrial targeting constructs by the calcium phosphate method, using an overnight incubation with 5 μg of DNA per 3-cm dish. Two days posttransfection, the cells were prepared for indirect immunofluorescence microscopy.
Functional Actinin Binding Site of Zyxin

Immunofluorescence Microscopy

Immunofluorescence microscopy of formaldehyde fixed cells was done essentially as described (37). The following antibodies were used: rabbit-anti zyxin serum AS83-1 (23), mouse anti-α-actinin monoclonal antibody BM 75.2 (Sigma), Oregon Green-labeled goat anti-rabbit antitody (Molecular Probes, Eugene, OR, USA), and Cy3-labeled goat anti-mouse IgG + IgM antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). Prior to use the latter antibodies were preadsorbed to rabbit γ-globulins (Serva, Heidelberg, Germany) immobilized on a Sepharose cartridge.

Immunoblots

Proteins were resolved on SDS-polyacrylamide gels and transferred to nitrocellulose by electroblotting. The blots were incubated with various antibodies or processed for blot overlays. Goat anti-GST antibodies were purchased from Amersham Pharmacia Biotech. The preparation of the anti-human zyxin monoclonal antibody 164ID4 will be described elsewhere. A polyclonal antiserum against a synthetic peptide corresponding to human zyxin residues 134–147 was raised in rabbits. Bound antibodies were detected with 125I-labeled sheep anti-mouse antibody (Amersham Pharmacia Biotech) or alkaline phosphatase-conjugated anti-mouse antibodies (Sigma) followed by development with bromochloroindol phosphate and nitroblue tetrazolium substrate.

Solid Phase Binding Assay

For conjugation with peroxidase α-actinin was purified from chicken gizzard essentially as described (38), except that phosphocellulose P11 was substituted by fibrous phosphocellulose (Sigma, C-3145), and α-actinin was eluted with a linear gradient of 0–500 mM NaCl in equilibration buffer. The protein was >95% pure as judged from SDS-polyacrylamide gel electrophoresis analysis and Coomassie Blue staining. 300 μg of purified α-actinin was coupled to 1 mg of preactivated horseradish peroxidase (Pierce) according to the manufacturer’s instructions.

Peptides corresponding to human zyxin (20, 21) amino acids 21–42 plus an N-terminal cysteine residue (zyxin21–42) and murine VASP (EMBL accession number X98475) amino acids 313–333 including a potential interaction of human zyxin residues 28–41 are part of a major binding site for α-actinin (Fig. 1). Our probe bound to bands of 80 and 36 kDa originating from the N terminus as indicated at the top.

RESULTS

Blot Overlays of N-terminal Zyxin Deletion Mutants Reveal a Functional α-Actinin Binding Site—A potential interaction of α-actinin with human zyxin was investigated in a blot overlay assay. To this end, several cDNA clones for human zyxin were expressed as GST fusion proteins in a prokaryotic expression system. These clones coded for the full-length zyxin sequence and for fragments thereof that were successively truncated from the N terminus. Detection of the expressed fusion proteins with an anti-GST antibody showed that the full-length protein and the truncated forms could successfully be expressed in bacteria, but that a large percentage of the products was degraded into a complex mixture of fragments (not shown).

Changing the conditions (i.e. lowering the temperature for expression or utilizing a bacterial strain with minimal endogenous protease activity) did not solve the problem. We therefore transferred the entire mixture of GST fusion proteins to nitrocellulose membranes and probed this blot with radioiodinated α-actinin (Fig. 1). Our probe bound to bands of 80 and 36 kDa derived from the full-length construct (amino acids 1–572). A positive reaction with the full-length fusion protein of 110 kDa was visible only when the gel was overloaded and when the blot was exposed for a prolonged period of time (not shown; see also Fig. 6). In contrast, no specific interaction was observed with any of the successively truncated GST-zyxin fusion proteins starting at zyxin amino acid 58 or C-terminal thereof. Some unspecific binding was observed with two bacterial polypeptides of 28 and 33 kDa present in all samples, but these signals disappeared when the fusion proteins were purified on glutathione-Sepharose before being transferred to nitrocellulose.

Thus, human zyxin contains at least one major binding site for α-actinin, and this site must be located, at least in part, within the first 57 amino acids of the polypeptide.

To narrow down this site, we prepared several shorter constructs that covered different regions within the first 51 amino acids of human zyxin and expressed them in bacteria. These fusion peptides proved to be fairly resistant against endogenous proteolysis and could be immobilized on nitrocellulose in virtually intact form after purification on glutathione-Sepharose (Fig. 2). Radiolabeled α-actinin bound specifically to the fusion peptides containing residues 1–51 and 1–41, but not to those containing residues 4–18 or 4–27. These results suggest that amino acids 28–41 are part of a major binding site for α-actinin.

In an effort to verify that the protein bound to the zyxin fusion peptides was in fact α-actinin, we excised one of the radiolabeled bands from the nitrocellulose membrane and eluted the protein with SDS sample buffer. On a polyacrylamide gel the eluted protein migrated with the same mobility as α-actinin. Western blot analysis of the eluate showed that the radiolabeled bands were specific for α-actinin, and that the bands were present almost exclusively in the N-terminal region of α-actinin (Fig. 2).

For conjugation with peroxidase α-actinin was dialyzed exhaustively against 500 mM NaCl, 50 mM Tris-HCl, pH 7.6, and transferred to a test tube, which had been coated with iodogen according to the instructions of the manufacturer (Pierce). Carrier-free Na125I (0.5 mCi/200 μg of protein; final volume, 500 μl) was added, and the reaction was allowed to proceed for 10 min on ice, followed by 5 min at room temperature. Radiolabeled protein was separated from nonincorporated iodine by chromatography on Sephadex G-50.

Blot overlays were performed essentially as described (18). Residual protein-binding sites on the nitrocellulose blots were blocked overnight with 0.75% bovine serum albumin in 10 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.1% 2-mercaptoethanol, 20 mM HEPES, pH 7.5. The blots were incubated at room temperature on a rocking platform with the radiolabeled probe (5 × 105 cpm/ml). After 4 h, the blots were washed twice with the same buffer and exposed to BioMax MS film (Eastman Kodak Co.).

Maleimide activated microtiter plates (Reacti-Bind™, Pierce) and Maleimide activated microtiter plates (Reacti-Bind™, Pierce) and amine binding microtiter plates with an N-xylocylinic acid surface (DNA-BIND™, Costar) were coated with 50 or 100 μg/ml peptide solution, respectively. After washing and blocking of remaining binding sites 0.5–50 μg/ml peroxidase-labeled α-actinin (protein concentration refers to α-actinin) was added (triplicates). Bound α-actinin-peroxidase conjugate was detected by incubation with α-phenylendiamime peroxidase substrate solution (Sigma). The reaction was stopped by addition of 1 volume of 750 mM HCl. For analysis, background values obtained without peptide coating were subtracted.

Blot Overlays

Peptide corresponding to human zyxin (20, 21) amino acids 21–42 plus an N-terminal cysteine residue (zyxin21–42) and murine VASP (EMBL accession number X98475) amino acids 313–333 including a potential interaction of human zyxin residues 28–41 are part of a major binding site for α-actinin. The polypeptide conjugate was detected by incubation with an anti-GST antibody showed that the full-length protein and the truncated forms could successfully be expressed in bacteria, but that a large percentage of the products was degraded into a complex mixture of fragments (not shown).

Changing the conditions (i.e. lowering the temperature for expression or utilizing a bacterial strain with minimal endogenous protease activity) did not solve the problem. We therefore transferred the entire mixture of GST fusion proteins to nitrocellulose membranes and probed this blot with radioiodinated α-actinin (Fig. 1). Our probe bound to bands of 80 and 36 kDa derived from the full-length construct (amino acids 1–572). A positive reaction with the full-length fusion protein of 110 kDa was visible only when the gel was overloaded and when the blot was exposed for a prolonged period of time (not shown; see also Fig. 6). In contrast, no specific interaction was observed with any of the successively truncated GST-zyxin fusion proteins starting at zyxin amino acid 58 or C-terminal thereof. Some unspecific binding was observed with two bacterial polypeptides of 28 and 33 kDa present in all samples, but these signals disappeared when the fusion proteins were purified on glutathione-Sepharose before being transferred to nitrocellulose.

Thus, human zyxin contains at least one major binding site for α-actinin, and this site must be located, at least in part, within the first 57 amino acids of the polypeptide.

To narrow down this site, we prepared several shorter constructs that covered different regions within the first 51 amino acids of human zyxin and expressed them in bacteria. These fusion peptides proved to be fairly resistant against endogenous proteolysis and could be immobilized on nitrocellulose in virtually intact form after purification on glutathione-Sepharose (Fig. 2). Radiolabeled α-actinin bound specifically to the fusion peptides containing residues 1–51 and 1–41, but not to those containing residues 4–18 or 4–27. These results suggest that amino acids 28–41 are part of a major binding site for α-actinin. In an effort to verify that the protein bound to the zyxin fusion peptides was in fact α-actinin, we excised one of the radiolabeled bands from the nitrocellulose membrane and eluted the protein with SDS sample buffer. On a polyacrylamide gel the eluted protein migrated with the same mobility...
sequence derived from the 5'9 horseradish peroxidase-labeled a solid phase binding assay with increasing concentrations of sized and coupled to maleimide-activated microtiter plates. In terminal cysteine residue for coupling purposes, was synthetized and known as a sequence element within the N-terminal zyxin region amino acids submotif occurring twice in tandem (Fig. 5). The binding site of human zyxin showed that it consists of a 10-amino acid sequence located between residues 261 and 284 in human zyxin. This sequence is characterized by a repeat structure similar to that of the zyxin and is evolutionary conserved in mouse zyxin (21), but not in chicken zyxin (14) or LPP (22). This sequence is found in the N terminus of the human LIM domain protein lipoma preferred partner (LPP) (22), which shares limited similarity with human zyxin over its entire sequence.

A closer analysis of the sequence comprising the α-actinin binding site of human zyxin showed that it consists of a 10 amino acids submotif occurring twice in tandem (Fig. 5b). A more detailed search for possible repeat structures revealed a second sequence element within the N-terminal zyxin region that resembles the α-actinin binding site identified here. This sequence is located between residues 261 and 284 in human zyxin and is evolutionary conserved in mouse zyxin (21), but not in chicken zyxin (14) or LPP (22). This sequence is characterized by a repeat structure similar to that of the α-actinin binding site: it comprises one complete copy of the 10 amino acids submotif (amino acids 261–270 in human zyxin), followed by two truncated copies occurring twice in tandem (amino acids 271–277/278–284 in human zyxin). Each of the truncations lacks the first three amino acids of the 10-amino acid submotif (Fig. 5b).

Our studies so far have identified an N-terminal α-actinin binding site in human zyxin and a downstream sequence with remarkable similarity. For a functional comparison of the two sites, three deletion constructs were prepared that lacked either the sequences corresponding to the first site (Δ19–41), the second site (Δ261–283), or both sites (Δ19–41/Δ261–283). When these constructs were expressed in bacteria as GST fusion proteins and tested in a blot overlay (Fig. 6), an interaction of radiolabeled α-actinin was observed with wild type zyxin as well as with zyxin lacking the second site. No interaction was detected with zyxin lacking either the first site or both sites. The first site is therefore indispensable for α-actinin...
Figure 5. Alignment of the α-actinin binding site from human zyxin to homologous sequences from mouse and chicken zyxin and human LPP. a, alignment of the α-actinin binding site from human zyxin to zyxin of other species and human LPP. b, dissection into submotifs of the alignment shown in panel a.

The binding, whereas there is no evidence for a functional significance of the downstream sequence element.

The α-Actinin Binding Site Is Essential for Proper Subcellular Targeting of Zyxin—To address possible functions of the α-actinin binding site in living cells, the wild type sequence and the three deletion constructs, detailed above, were cloned into a GFP expression vector and transfected into COS cells. With the GFP fluorescence, the subcellular distribution of the GFP fusion proteins was visualized in living cells. The fusion protein synthesized from the wild type sequence was found to be localized at focal contacts and along stress fibers (Fig. 7). Synthesized from the wild type sequence was found to be localized at focal contacts and along stress fibers (Fig. 7). Synthesized from the wild type sequence was found to be localized at focal contacts and along stress fibers (Fig. 7). Synthesized from the wild type sequence was found to be localized at focal contacts and along stress fibers (Fig. 7).

The α-Actinin Binding Site Is Required for Zyxin-dependent α-Actinin Recruitment in Living Cells—To further analyze the α-actinin binding activity of zyxin constructs in living cells, we sought to ectopically express different zyxin constructs at discrete subcellular sites. For this, we made use of a mitochondrial targeting system described by Pistor et al. (33). Wild type zyxin and zyxin constructs bearing deletions of either the α-actinin binding site (targZyxin Δ19–41), the related sequence described above (targZyxin Δ261–283), or both (targZyxin Δ19–41/Δ261–283) were cloned into the pSPL61 vector (33, 34), a derivative of the eukaryotic expression vector pMPSVHE (35), for expression as fusion proteins with a 29 amino acid C-terminal peptide. This peptide is derived from the membrane anchor of the L. monocytes ActA protein. After transfection into eukaryotic cells, it directs the fusion protein to the mitochondrial surface (33). A major benefit of the mitochondrial targeting system is the absence of any endogenous zyxin and α-actinin at these ectopic sites. Therefore, there is no need to discriminate against a background of endogenous proteins. In addition, functionally related proteins are also absent from mitochondria and hence will not interfere with the binding assay.

Transfection of PtK2 cells with these vectors, encoding zyxin fusion proteins under the control of the myeloproliferative sarcoma virus promoter, resulted in high level protein expression, regardless of the construct. Double staining of transfected cells with an antisera to zyxin and the mitochondrial dye CMTMRos demonstrated that the zyxin fusion proteins almost exclusively colocalized with mitochondria (not shown). Also, independent of the construct used for transfection, mitochondria of transfected cells tended to form extensive clusters (Fig. 8, a–d).

Double label indirect immunofluorescence microscopy with antibodies to zyxin and α-actinin revealed that wild type zyxin fusion protein was capable of recruiting endogenous α-actinin to the mitochondria of transfected cells, a phenomenon that was especially evident at mitochondrial clusters (Fig. 8, a and a’; open arrows). In sharp contrast, a zyxin deletion construct lacking the α-actinin binding site (targZyxin Δ19–41), completely lost its α-actinin binding activity (Fig. 8, b and b’).

Several binding sites for the focal adhesion and microfilament-associated protein VASP map to a zyxin region starting from about amino acid 69 (25), i.e. in close proximity to the α-actinin binding site. Both wild type zyxin and targZyxin Δ19–41 recruited VASP to the mitochondrial surfaces (not shown). As these closely adjacent VASP binding sites were still functionally preserved, we conclude that the Δ19–41 deletion did not cause any gross structural disturbance, although we cannot rule out a possible conformational change.

Unlike targZyxin Δ19–41, deletion of the related sequence element (targZyxin Δ261–283) did not abolish mitochondrial α-actinin recruitment (Fig. 8, c and c’). Finally, constructs bearing deletions of both sites (targZyxin Δ19–41/Δ261–283) also failed to direct α-actinin to the mitochondria of transfected cells (Fig. 8, d and d’). Obviously, zyxin residues 19–41 are...
indispensable for α-actinin binding not only in blot overlays but also in transfected cells. Moreover, α-actinin binding via this site accounts for the major part, if not all, of the α-actinin binding activity of zyxin in living cells, as revealed in the mitochondrial targeting assay. The striking sequence homology between the α-actinin binding site and its related downstream sequence raised the question of whether the latter could be rendered a functional α-actinin binding site when grafted into the position of the authentic binding site within the polypeptide chain. Therefore, we made a construct, coding for peptide sequence 262–284 inserted into the deletion site of targZyxin Δ19–41. After transfection of this construct into PtK2 cells, no colocalization of zyxin and α-actinin at mitochondria was observed (not shown). This indicates that despite similarities in sequence and charge, the downstream sequence cannot functionally rescue deletion of the α-actinin binding site, even if an additional copy is introduced at the normal binding site position.

**DISCUSSION**

Using different in vitro and transfection experiments, we have mapped an α-actinin binding site in human zyxin. This binding site was found to be both necessary and sufficient for α-actinin binding in blot overlay and solid phase binding assays, respectively. The site is also involved in zyxin localization to stress fibers and focal adhesions and is required for α-actinin binding to zyxin in a mitochondrial targeting approach, i.e. in the physiological ambience of living cells. In addition, we have shown that the human protein LPP shares a sequence element closely related to the zyxin α-actinin binding site, raising the possibility that LPP is also an α-actinin-binding protein.

**The α-Actinin Binding Site of Zyxin Shares Some Features with α-Actinin Binding Sites in Proteins That Bind to the α-Actinin Central and C-Terminal Region**—Similar to the α-actinin binding site of zyxin characterized here (Fig. 5), basic and hydrophobic residues also prevail in several other α-actinin binding sites, including those of integrin β1 (39, 40), 1-selectin (10), and the NR1 subunit of the N-methyl-D-aspartate receptor (11). Integrin β1 (6) and 1-selectin (10) bind to the 53-kDa thermolysin fragment of α-actinin (which comprises both the central and C-terminal regions of the protein). In a yeast two-hybrid assay, the NR1 subunit of the N-methyl-D-aspartate receptor interacts with the region of the central spectrin-like repeats (11). This is remarkable, because zyxin binding has previously been attributed to the N-terminal 27-kDa fragment, not to the 53-kDa fragment of α-actinin (18). Apparently, different α-actinin domains mediate binding to zyxin and to the other proteins mentioned above. Therefore, we questioned whether the similarities between the α-actinin binding sites of

**FIG. 7. Subcellular distribution of GFP-zyxin fusion proteins.** COS cells (a–d) and primary chicken fibroblasts (e–h) were transfected with different zyxin cDNA constructs cloned into a GFP expression vector. One or 2 days after transfection, the cells were prepared for direct fluorescence microscopy. The expression vector contained either wild type zyxin sequence (a and c), zyxin Δ19–41 (b and f), zyxin Δ261–283 (c and g), or zyxin Δ19–41/261–283 (d and h) ligated to the 3′-end of the GFP sequence. Bar, 20 μm.
zyxin and those other proteins are only fortuitous.

Is There More Than One \( \alpha \)-Actinin:Zyxin Interface?—In resolving this issue, it is important to note that the blotted N-terminal 27-kDa proteolytic fragment of \( \alpha \)-actinin was previously shown to bind radiolabeled full-length zyxin (18). In sharp contrast to these experiments, we used an overlay with radiolabeled \( \alpha \)-actinin to identify the zyxin region involved in the interaction. Generally, in blot overlays, a labeled native protein is used as probe to detect an interaction with denatured proteins separated by SDS-polyacrylamide gel electrophoresis and blotted to a membrane. Renaturation on the blot membrane should, for example, be easier for small linear peptide motifs than for complex protein folds or complete domains. Therefore it may be unexpected that overlays probing the \( \alpha \)-actinin-zyxin interaction work in either direction. The existence of two independent binding interfaces may be an obvious explanation. This raises the attractive possibility that native zyxin interacts with a peptide motif in the 27-kDa fragment of \( \alpha \)-actinin (18), whereas another interaction may be due to binding of a zyxin peptide motif by a different \( \alpha \)-actinin region (possibly in the central or C-terminal region). In support of this view, both the 27- and 53-kDa \( \alpha \)-actinin fragments, but not the full-length protein, failed to compete for \( \alpha \)-actinin binding to zyxin in solid phase binding assays (18). This hypothesis may also explain the similarities between the \( \alpha \)-actinin binding motifs of zyxin and those of known ligands for the central/C-terminal part of \( \alpha \)-actinin.

In fact, interaction with more than one \( \alpha \)-actinin region appears to be quite common and has been suggested for several proteins, such as ICAM-2 (9), actinin-associated LIM protein (41), titin (42), and PKN (12). Vice versa, \( \alpha \)-actinin ligands often harbor two essentially nonoverlapping, apparently unrelated binding sites in close proximity, as shown for integrin \( \beta_1 \) (39, 40), Ep-CAM (40), and ICAM-2 (9).

\( \alpha \)-Actinin Binding Sites: Specific Sequence Requirements or Compositional Bias?—Besides some bias in amino acid composition, there is no obvious consensus motif for the \( \alpha \)-actinin binding sites of zyxin (this study), integrin \( \beta_1 \) (39, 40), L-selectin (10), the NR1 subunit of the N-methyl-D-aspartate receptor (11), and some other \( \alpha \)-actinin binding sites (8, 9, 40). Indeed, there are reports that even certain scrambled or reverse sequence ICAM-1 and \( \beta_1 \) integrin peptides show comparable binding activity when tested in vitro (8, 39). Contrary to \( \alpha \)-actinin binding sites of ICAM-1 and integrin \( \beta_1 \), our results suggest that the \( \alpha \)-actinin binding site of zyxin in blot overlays and in transfection experiments is strictly dependent on the sequence of the binding site. In particular, the related sequence element between residues 261 and 283 of human zyxin (Fig. 5) was neither necessary nor sufficient for \( \alpha \)-actinin binding in blot overlays or in living cells. The negative results obtained

**FIG. 8.** Recruitment of \( \alpha \)-actinin to zyxin constructs targeted to the mitochondrial surface. PtK\(_2\) cells were transfected with different zyxin cDNA constructs cloned into a mitochondrial targeting vector. Two days posttransfection, cells were prepared for indirect immunofluorescence microscopy using rabbit anti-zyxin serum AS83–1 (a–d) and mouse anti-\( \alpha \)-actinin monoclonal antibody BM 75.2 (a’–d’). Primary antibodies were detected with Oregon Green-labeled anti-rabbit and Cy3-labeled anti-mouse antibodies, respectively. The mitochondrial targeting vector contained either wild type zyxin coding sequence (a and a’), zyxin \( \Delta 19–41 \) (b and b’), zyxin \( \Delta 261–283 \) (c and c’), or zyxin \( \Delta 19–41/\Delta 261–283 \) (d and d’). Sites of prominent \( \alpha \)-actinin recruitment to mitochondrial clusters are marked with open arrows (a’ and c’). Bar in d (valid for all panels), 20 \( \mu \)m.
with this latter human zyxin peptide are in contrast, however, with overlay experiments performed with synthetic peptides (not shown). 9–21-mer peptides were synthesized in parallel arrays on a solid paper support using the SPOT™ technology (43). When these peptides were incubated with α-actinin, we observed spurious binding to peptides corresponding either to the actual binding site as characterized here or to the nonfunctional downstream element and even to a reverse sequence. This indicates that—in the case of α-actinin—binding data based primarily on this type of assay should be interpreted with caution unless confirmed by independent approaches. It will be interesting to investigate whether or not this downstream motif is related to a putative second α-actinin binding site that is responsible for α-actinin binding in the reciprocal overlay assay. However, the demonstration that chicken zyxin interacts with α-actinin in 125I-labeled zyxin overlays (18), although chicken zyxin lacks an element related in sequence to residues 261–283 of human zyxin, argues against such a relationship.

Requirement of the α-Actinin-Zyxin Interaction for Proper Subcellular Targeting of Zyxin—As judged from transfection experiments with GFP-zyxin fusion constructs, the zyxin α-actinin interaction appears to be essential for zyxin to adopt the typical dotted pattern of stress fiber localization, because GFP-zyxinA19–41 failed to locate at stress fibers. Nevertheless, interactions of zyxin with proteins other than α-actinin appear to recruit the same fusion protein to focal adhesions, although not very efficiently. However, we cannot distinguish whether these differences in subcellular targeting to focal adhesions versus stress fibers reflect true qualitative or only quantitative differences. Zyxin is predominantly concentrated at focal adhesions and the terminal portions of stress fibers, whereas its association with stress fibers proper is characterized by a much finer and weaker staining pattern in indirect immunofluorescence analysis (Fig. 7) (14, 18, 19, 21, 23). Thus, if the deletion of the α-actinin binding site would equally impair association with both types of structures, the weaker staining of the fusion protein at stress fibers might be lost, whereas some residual staining of focal adhesions could persist.

GFP-zyxin A19–41 failed to associate with stress fibers. Obviously, the known zyxin interactions with CRPs (14–17) and VASP (23) cannot compensate for direct α-actinin binding, although both proteins localize with zyxin along stress fibers and their terminal portions (14, 17, 23, 37). This may be surprising in light of the observation that VASP and zyxin display a virtually indistinguishable subcellular distribution, including a dot-like stress fiber association of identical spacing and periodicity (23) that (at stress fibers) also coincides with α-actinin staining (37). In contrast, the indirect immunofluorescence patterns of zyxin and α-actinin vastly overlap but are also distinctly different. Specifically, α-actinin staining is more pronounced along stress fibers, whereas zyxin is concentrated in the focal adhesions proper (18). However, it has been pointed out that possible differences in antigen accessibility may contribute to these immunofluorescence patterns (3, 18).

Zyxin-dependent α-Actinin Recruitment in Living Cells—Chicken gizzard vinculin, the vinculin head, and the vinculin hinge plus tail domains were very inefficient in recruiting α-actinin when tested in the same mitochondrial targeting system as used in this report (34). This is despite the fact that an interaction has been shown in vitro that involves an α-actinin binding site in the vinculin head domain (13). These results obtained with mitochondrial targeting of vinculin and vinculin fragments are in sharp contrast to the strong α-actinin recruitment observed with a hybrid construct replacing most of the vinculin head by the ActA N-terminal part (umino acids 31–236) (34). Similar α-actinin recruitment has been reported for wild type ActA (33, 44). It is still an unresolved issue whether α-actinin recruitment by ActA or the ActA-vinculin chimera is due to direct binding or whether α-actinin is recruited by the considerable amounts of F-actin that accumulate at the mitochondrial surfaces of cells transfected with these constructs. Like ActA, zyxin is able to recruit F-actin to the mitochondria of transfected cells. Nevertheless, in the case of zyxin, we can rule out the possibility that α-actinin recruitment is primarily due to an indirect association mediated by F-actin (18) or some other protein(s); a deletion, which is confined to a short zyxin peptide motif that has been positively identified as an α-actinin binding site in a parallel in vitro assay, virtually abolishes α-actinin recruitment in the targeting assay and α-actinin binding to GST-zyxin fusion proteins in blot overlays in vitro.

Regardless of whether the α-actinin recruitment by ActA (33, 44) or ActA chimeras (34) turns out to be direct or indirect, the efficient α-actinin binding to zyxin and zyxin-dependent F-actin recruitment in living cells lend further support to the view that ActA and zyxin share common functional features.

Similar to the failure of α-actinin binding site deletion mutants to localize to stress fibers, the equivalent fusion proteins assayed in the mitochondrial targeting system demonstrated that there was no efficient backup system for recruitment of α-actinin to zyxin. In particular, at least in these cells, cytoene-rich protein family members, which bind both zyxin and α-actinin (17, 32), do not functionally substitute for the direct α-actinin-zyxin interaction.

In conclusion, we have identified and evaluated an α-actinin binding site in human zyxin that maps close to the zyxin N terminus and is required for zyxin-dependent α-actinin recruitment and proper zyxin localization in living cells. The results presented here have set the stage for investigating the contribution of the α-actinin-zyxin interaction in microfilament architecture and regulation.

Acknowledgments—M. R. is grateful to Jürgen Otte and Dietmar von der Ahe (Bad Nauheim, Germany) for sharing human zyxin cDNA prior to publication. We thank Susanne Pistor and Jürgen Wehland (Braunschweig, Germany) for the kind gift of the mitochondrial targeting vector pSPL61 and our colleagues for valuable criticism and comments on the manuscript.

REFERENCES
1. Yamada, K. M., and Geiger, B. (1997) Curr. Opin. Cell Biol. 9, 76–85
2. Schulze, H., Huckriede, A., Noegel, A. A., Schleicher, M., and Jockusch, B. M. (1989) EMBO J. 8, 3587–3593
3. Pavalko, F. M., Schneider, G., Burridge, K., and Lim, S. S. (1995) Exp. Cell Res. 217, 534–540
4. Puiu, Y. A., Mahoney, N. M., and Almo, S. C. (1998) Curr. Opin. Cell Biol. 10, 25–34
5. Critchley, D. R., and Flood, G. (1999) in Guidbook to the Cytoskeletal and Motor Proteins, (Kreis, T., and Vale, R., eds) 2nd Ed., Oxford University Press, San Francisco, in press
6. Otey, C. A., Pavalko, F. M., and Burridge, K. (1990) J. Cell Biol. 111, 721–729
7. Pavalko, F. M., and LaRoche, S. M. (1993) J. Immunol. 151, 3798–3807
8. Carpen, O., Pallai, P., Staunton, D. E., and Springer, T. A. (1992) J. Cell Biol. 118, 1223–1234
9. Heisaka, L., Kantor, C., Parr, T., Critchley, D. R., Vilja, P., Gahmberg, C. G., and Carpen, O. (1996) J. Biol. Chem. 271, 20214–20219
10. Pavalko, F. M., Walker, D. M., Graham, L., Goheen, M., Doerschuk, C. M., and Kansas, G. S. (1995) J. Cell Biol. 129, 1155–1164
11. Wysznyski, M., Jin, L., Rao, A., Nigh, E., Beggs, A. H., Craig, A. M., and Sheng, M. (1997) Nature 385, 439–442
12. Mukai, H., Toshimori, M., Shibata, H., Takanaga, H., Kitagawa, M., Miyashara, M., Shimakawa, M., and Ono, Y. (1997) J. Biol. Chem. 272, 4740–4746
13. Kroecker, M., Rudiger, A.-H., Jockusch, B. M., and Rudiger, M. (1994) FEBS Lett. 355, 259–262
14. Sadler, I., Crawford, A. W., Michelsen, J. W., and Beckerle, M. C. (1992) J. Cell Biol. 119, 1573–1587
15. Schmeichel, K. L., and Beckerle, M. C. (1994) Cell 79, 211–219
16. Schmeichel, K. L., and Beckerle, M. C. (1998) Biochem. J. 331, 885–892

3 M. Reinhard and U. Walter, unpublished observations.
17. Louis, H. A., Pino, J. D., Schmeichel, K. L., Pomiès, P., and Beckerle, M. C. (1997) J. Biol. Chem. 272, 27484–27491
18. Crawford, A. W., Michelsen, J. W., and Beckerle, M. C. (1992) J. Cell Biol. 116, 1381–1393
19. Crawford, A. W., and Beckerle, M. C. (1991) J. Cell Biol. 116, 1381–1393
20. Macalma, T., Otte, J., Hensler, M. E., Bockholt, S. M., Louis, H. A., Kalf-Suske, M., Grzeschik, K.-H., von der Ahe, D., and Beckerle, M. C. (1996) J. Biol. Chem. 271, 31470–31478
21. Macalma, T., Otte, J., Hensler, M. E., Bockholt, S. M., Louis, H. A., Kalf-Suske, M., Grzeschik, K.-H., von der Ahe, D., and Beckerle, M. C. (1996) J. Biol. Chem. 271, 31470–31478
22. Petit, M. M. R., Mols, R., Schoenmakers, E. F. P. M., Mandahl, N., and Van De Ven, W. J. M. (1996) Genomics 36, 118–129
23. Reinhard, M., Jouvenal, K., Triperi, D., and Walter, U. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 7906–7910
24. Gertler, F. B., Niebuhr, K., Reinhard, M., Wehland, J., and Soriano, P. (1996) Cell 87, 227–239
25. Niebuhr, K., Ebel, F., Frank, R., Reinhard, M., Domann, E., Carl, U. D., Walter, U., Gertler, F., Wehland, J., and Chakraborty, T. (1997) EMBO J. 16, 5433–5444
26. Hobert, O., Schilling, J. W., Beckerle, M. C., Ullrich, A., and Jallal, B. (1996) Oncogene 12, 1577–1581
27. Chakraborty, T., Ebel, F., Domann, E., Niebuhr, K., Gerstel, B., Pistor, S., Temm-Grove, C. J., Jockusch, B. M., Reinhard, M., Walter, U., and Wehland, J. (1996) EMBO J. 14, 1314–1321
28. Golstein, R. M., Beckerle, M. C., Koay, T., and Friederich, E. (1997) J. Cell Sci. 110, 1893–1906
29. Dramsi, S., and Cossart, P. (1998) Annu. Rev. Cell Dev. Biol. 14, 137–166
30. Zigmond, S. H. (1998) Curr. Biol. 8, R654–R657
31. Reinhard, M., Giehl, K., Abel, K., Haffner, C., Jarchau, T., Hoppe, V., Jockusch, B. M., and Walter, U. (1995) EMBO J. 14, 1583–1589
32. Pomiès, P., Louis, H. A., and Beckerle, M. C. (1997) J. Cell Biol. 139, 157–166
33. Peti, M. M. R., Mols, R., Schoenmakers, E. F. P. M., Mandahl, N., and Van De Ven, W. J. M. (1996) Genomics 36, 118–129
34. Reinhard, M., Jouvenal, K., Triperi, D., and Walter, U. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 7906–7910
35. Frank, R. (1992) Tetrahedron 48, 9217–9232
36. Bubeck, P., Pistor, S., Wehland, J., and Jockusch, B. M. (1997) J. Cell Sci. 110, 1361–1371
37. Koller, E., Hayman, A. R., and Trueb, B. (1991) Nucleic Acids Res. 19, 485–491
38. Reinhard, M., Halbrügge, M., Scheer, U., Wiegand, C., Jockusch, B. M., and Walter, U. (1992) EMBO J. 11, 2063–2070
39. Otey, C. A., Vasquez, G. B., Burridge, K., and Erickson, B. W. (1993) J. Biol. Chem. 268, 21193–21197
40. Balzar, M., Bakker, H. A. M., Briaire-De-Bruijn, I. H., Fleuren, G. J., Warraan, S. O., and Litvinov, S. V. (1996) Mol. Cell. Biol. 16, 4833–4843
41. Xia, H., Winokur, S. T., Kuo, W.-L., Altherr, M. R., and Bredt, D. S. (1997) J. Cell Biol. 139, 507–515
42. Young, P., Ferguson, C., Bañuelos, S., and Gautel, M. (1998) EMBO J. 17, 1614–1624
43. Frank, R. (1992) Tetrahedron 48, 9217–9232
44. Pistor, S., Chakraborty, T., Walter, U., and Wehland, J. (1995) Curr. Biol. 5, 517–525