The lipoma preferred partner LPP interacts with α-actinin

Bo Li[1], Lei Zhuang[1], Matthias Reinhard[2] and Beat Trueb[1,*]

[1]ITI Research Institute, University of Bern, PO Box 54, CH-3010 Bern, Switzerland
[2]Institute for Clinical Biochemistry and Pathobiochemistry, University of Würzburg, Versbacher Strasse 5, D-97078 Würzburg, Germany
*Author for correspondence (e-mail: beat.trueb@iti.unibe.ch)

Accepted 4 December 2002
Journal of Cell Science 116, 1359-1366 © 2003 The Company of Biologists Ltd
doi:10.1242/jcs.00309

Summary

The lipoma preferred partner LPP is a member of the zyxin family of proteins. In this paper, we demonstrate that the structural similarities observed between zyxin and LPP also extend to their interaction capabilities. Similar to zyxin, LPP was found to bind to α-actinin in vitro. This interaction was confirmed in yeast and mammalian cells. Studies utilizing the three-hybrid system further indicated that zyxin and LPP compete for the same binding site in α-actinin. This site was mapped to the central rod of α-actinin, which contains spectrin-like repeats 2 and 3. In the case of LPP, a conserved motif present at the N-terminus was shown to be responsible for the interaction. Constructs lacking this motif did not bind to α-actinin in the yeast two-hybrid system and were not able to recruit α-actinin to an ectopic site in mammalian cells. Quantitative data obtained with the two-hybrid and the three-hybrid system suggest that LPP has a lower affinity for α-actinin than zyxin. It is likely that this difference leads to slightly different roles played by LPP and zyxin during the assembly and disassembly of focal adhesions.

Key words: α-Actinin, Cytoskeleton, Focal adhesion, Lipoma preferred partner, LPP, Zyxin

Introduction

The controlled assembly and disassembly of actin filaments plays a critical role in a diverse palette of cellular processes, including cell adhesion, spreading, migration and signal transduction. One of the regulatory proteins involved in the fine-tuning of microfilament assembly appears to be zyxin. Zyxin was originally discovered in chicken fibroblasts as a protein associated with stress fibers, focal adhesions and cell-cell contacts (Beckerle, 1986). It represents a protein with a relative molecular mass of 82 kDa that can be phosphorylated at multiple sites (Zumbrunn and Trueb, 1996; Macalma et al., 1996). A large body of evidence suggests that zyxin plays an active role in the sequestration of components for actin assembly at specific sites within the cell and in the stimulation of spatially restricted actin polymerization (Beckerle, 1998). In fact, when artificially directed to the surface of mitochondria by incorporation of a specific signal sequence, zyxin is able to induce actin assembly at these ectopic sites (Fradelizi et al., 2001).

Zyxin has a modular structure with a proline-rich N-terminus that harbors a nuclear export signal and three C-terminal LIM domains (Fig. 1). It serves as a versatile adapter protein that brings together various regulatory molecules and cytoskeletal proteins. Although the precise function of zyxin in the organization of the actin cytoskeleton is not yet clear, the identification of its binding partners may shed some light onto its role. Zyxin has been demonstrated to interact, via its C-terminal LIM domains, with members of the cysteine-rich protein family CRP (Sadler et al., 1992; Schmeichel et al., 1998). The LIM domains have also been demonstrated to bind to LATS1, a tumor suppressor that appears to be involved in the regulation of mitosis (Hirota et al., 2000). Furthermore, zyxin binds to proteins of the Ena/VASP family, which control the organization of the actin cytoskeleton (Reinhard et al., 2001). For this interaction, the proline clusters within the N-terminal domain of zyxin are responsible (Niebuhr et al., 1997; Drees et al., 2000). The proline clusters also appear to serve as binding sites for the oncoprotein Vav, which is a guanosine exchange factor for the small GTP-binding protein Rho (Hobert et al., 1996). Moreover, zyxin interacts with α-actinin, an actin-crosslinking protein enriched at focal adhesion sites and along stress fibers (Crawford et al., 1992). The exact binding site has been mapped to the extreme N-terminus of zyxin (Reinhard et al., 1999; Drees et al., 1999). We have recently demonstrated that a linear motif of six amino acids (26-FGPVVA-31) plays a critical role in this interaction. When a single amino acid within this motif is replaced by using in vitro mutagenesis, binding of zyxin to α-actinin is abolished, and the subcellular distribution of zyxin is significantly altered (Li and Trueb, 2001).

Zyxin belongs to a small family of several related focal adhesion proteins. Another member of this family is the lipoma preferred partner LPP (Petit et al., 1996). LPP also possesses a modular structure with a proline-rich N-terminus, including a nuclear export signal and three C-terminal LIM domains (Fig. 1). Similar to zyxin, LPP has been localized to focal adhesions and to cell-cell adherence junctions (Petit et al., 2000). The gene for LPP was originally discovered during the analysis of chromosomal rearrangements in lipomas. Chromosomal translocations involving human chromosomes 3 and 12 are found with high frequency in these benign tumors of adipose tissues. The translocations often result in the fusion of the

HMGA2 gene on chromosome 12 with the *LPP* gene on chromosome 3. HMGA2 is known to code for a transcription factor of the high mobility group of proteins (Ashar et al., 1995). The generated fusion proteins contain the N-terminal sequence of HMGA2, including three DNA binding domains followed by the C-terminal sequence of LPP with two or three LIM domains. Although a direct relationship between tumorigenesis and the expression of these fusion proteins has not been demonstrated in detail, it is likely that the LIM domains of LPP contribute to the altered gene expression observed in lipomas.

A third member of the zyxin family is Trip6. This protein was originally identified in a yeast two-hybrid screen as a protein that interacted with the thyroid hormone receptor in a hormone-dependent manner (Lee et al., 1995; Yi and Beckerle, 1998). Similar to zyxin and LPP, Trip6 contains three LIM domains at the C-terminus and a proline-rich N-terminus with a nuclear export signal. Furthermore, Trip6 exhibits a subcellular distribution at focal adhesion plaques quite similar to zyxin and LPP (Wang and Gilmore, 2001). Two additional proteins, LIMD1 (Kiss et al., 1999) and Ajuba (Goyal et al., 1999), may also be regarded as members of the zyxin protein family because they possess similar domain structures. At the level of the amino-acid sequences, however, these proteins are not as closely related as zyxin, LPP and Trip6.

Trip6 and LPP exhibit the highest sequence identity (53%) among all of the zyxin family members. Their domain structures, however, are not strictly conserved, as the proline-rich N-terminus of Trip6 is considerably shorter than that of LPP (Fig. 1). On the other hand, LPP and zyxin reveal a lower sequence identity (41%), but exhibit a highly conserved domain structure. Interestingly, the sequence motif that has previously been found in zyxin to be both necessary and sufficient for α-actinin binding is conserved in LPP (Fig. 1). This motif, on the other hand, is missing in the Trip6 sequence. The aim of the present study was therefore to investigate whether LPP interacts with α-actinin in a way similar to zyxin.

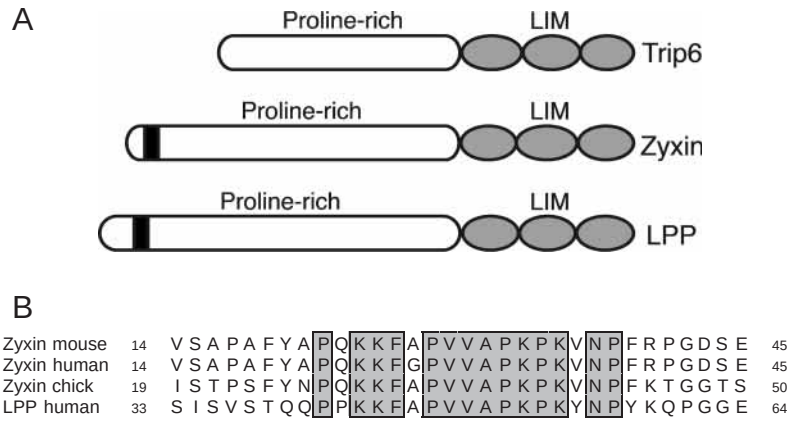

Fig. 1. Domain structures of the three proteins Trip6, zyxin and LPP. The N-terminal motif conserved in zyxin and LPP is indicated by a black bar in A. Note that this motif is not present in Trip6. The amino-acid sequences flanking the conserved motif are aligned in B. The sequences for mouse, human and chicken zyxin as well as human LPP are included. Identical residues are boxed.

Materials and Methods

cDNA cloning and plasmid construction

The cDNA sequences for human zyxin [GenBank accession number X95735; (Zumbrunn and Trueb, 1996)] and human α-actinin [accession number X15804 (Millake et al., 1989)] have previously been cloned in our laboratory (Li and Trueb, 2001). Several cDNA clones that span the entire open reading frame of human LPP [accession number U49957 (Petit et al., 1996)] were obtained from the German Resource Center for Genome Research in Berlin.

For the expression of glutathione S-transferase (GST)-tagged LPP in bacteria, the cDNA sequence for LPP was amplified from a full-length human cDNA (Marathon-Ready cDNA, Invitrogen) by the polymerase chain reaction (PCR) utilizing two synthetic primers. The resulting full-length cDNA as well as a fragment derived thereof (encoding amino acid residues 1-109) were subcloned into the expression vector pGEX-4T2 (Amersham Pharmacia Biotech) downstream of the *gst* gene.

For expression in yeast, constructs were prepared by PCR with the help of various synthetic oligonucleotide primers (Microsynth GmbH, Switzerland). Selected cDNA fragments for α-actinin were subcloned into the two-hybrid prey vector pACT2 (Li and Trueb, 2001). Two cDNA fragments for LPP that corresponded to amino-acid residues 1-40 (LPP40) and 1-61 (LPP61), respectively, were subcloned into the *Nco*I/*Pst*I restriction site of the bait vector pGBKT7. For competition experiments, a cDNA fragment coding for amino-acid residues 1-42 of zyxin was inserted into multiple cloning site I of the three-hybrid vector pBridge (Clontech Laboratories, Palo Alto, CA) downstream of the sequence for the GAL4 DNA-binding domain. As competitors, the sequences for LPP40, LPP61 or zyxin (1-42) were ligated into multiple cloning site II of the same vector downstream of the *MET25* promoter. This promoter exhibits conditional activity depending on the presence or absence of methionine in the culture medium (Tirode et al., 1997).

Fusion constructs of LPP and green fluorescent protein (GFP) were prepared by ligating the full-length LPP sequence or fragments LPP40 and LPP61, respectively, into the *Eco*RI/*Sal*I restriction site of the expression vector pEGFP-C3 (Clontech) downstream of the *GFP* reporter gene. A short deletion spanning nucleotides 367-417 (amino acids 41-57, accession number U49957) was introduced into the full-length LPP construct by the ExSite PCR-based mutagenesis method (Costa et al., 1996; Li and Trueb, 2001), resulting in the construct LPPΔ. For mitochondrial targeting experiments, a synthetic oligonucleotide coding for the membrane anchor of ActA (amino-acid residues 628-LILAMLAIGVFS LGAFIKIIQLRKNN-653) was purchased from Microsynth GmbH (Switzerland). This oligonucleotide was inserted into the *Apa*I/*Bam*HI site of the pEGFP vector, downstream of the sequences for LPP and GFP. Authenticity and reading frame of all constructs were verified by DNA sequencing.

Blot overlays

GST-tagged LPP was expressed in *E. coli* BL21 after induction with 0.1 mM isopropylthio-β-galactoside as suggested by the supplier (Amersham Pharmacia Biotech). The bacteria were collected by centrifugation and lysed by sonication. The fusion proteins were purified from the lysate by affinity chromatography on glutathione Sepharose and analyzed on SDS polyacrylamide gels. After transfer to nitrocellulose by electroblotting, the polypeptides were detected with the GST detection module (Amersham) using goat anti-GST antibodies, followed by alkaline-phosphatase-conjugated secondary antibodies (Sigma). Similar blots that had been prepared in parallel were blocked with bovine serum albumin and subsequently incubated with radiolabeled α-actinin in 10 mM NaCl, 1 mM Nonidet P-40, 0.1% 2-mercaptoethanol, 20 mM HEPES, pH 7.5 as previously

described (Crawford et al., 1992; Reinhard et al., 1999). After 4 hours at room temperature, the blots were washed twice with the same buffer and exposed to BioMax MS film (Eastman Kodak Co.).

Yeast two-hybrid and three-hybrid system

Yeast two-hybrid and three-hybrid experiments were carried out essentially as described in the manuals provided by the supplier (Clontech). Yeast reporter strain Y190 was cotransfected with the appropriate bait and prey plasmids by the lithium acetate method. Selection for *HIS3* reporter gene activation was performed on agar plates lacking histidine, tryptophan and leucine. Colonies that appeared after incubation for 5-10 days at 30°C were assayed for activation of the *lacZ* reporter gene utilizing the colony filter lift assay. For quantitative data, the colonies were grown in liquid media and analyzed for β-galactosidase activity using O-nitrophenyl β-D-galactopyranoside as a substrate.

Cell culture and GFP fusion protein expression

Cell lines were obtained from the American Type Culture Collection (Manassas, VA) and kept in the laboratory at 37°C under an atmosphere of 5% CO_2. PtK2 cells (CCL-56) were cultivated in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin. H9C2 myoblasts (CRL-1466) were grown in RPMI-1640 medium containing the same supplements. GFP plasmids (1 μg/well) were mixed with 100 μl Opti-MEM 1 (Life Technologies) containing 3 μl of FuGENE-6 reagent (Roche) and added to the cells that had grown to 60% confluence in six-well plates. Two days after transfection, the cells were fixed with formaldehyde and prepared for indirect immunofluorescence as described previously (Reinhard et al., 1999). A monoclonal antibody against human α-actinin (Sigma) was used at a dilution of 1:400. A polyclonal antiserum that had previously been prepared in our laboratory against human zyxin (amino acid residues 134-147) was used at a 1:50 dilution (Reinhard et al., 1999). After incubation with rhodamine-labeled secondary antibodies, the slides were inspected under a Zeiss Axiovert microscope equipped with epifluorescence optics. Electronic pictures were taken with filter settings optimized for green (515-565 nm) and red (>590 nm) light emission, respectively, and merged with the help of a computer software program.

Results

LPP interacts with α-actinin in vitro

The potential interaction of LPP with α-actinin was examined in vitro in a blot overlay experiment (Fig. 2). First we focused on the N-terminal sequence of LPP that harbored the motif conserved in zyxin (see Fig. 1). A GST fusion protein generated with the N-terminal amino-acid residues 1-109 of human LPP migrated on a gel with a relative mobility of 40 kDa consistent with its calculated molecular mass. The polypeptide was transferred to a nitrocellulose membrane and incubated with radioiodinated α-actinin. Our probe detected a single band, which increased in intensity with increasing amount of fusion protein loaded to the gel (Fig. 2, left). In contrast, α-actinin did not bind to GST alone or to any of the globular protein markers used.

The analogous result was obtained with a full-length GST-LPP construct, which migrated on a gel with a relative mobility of 100 kDa (Fig. 2, right). In this case, our probe reacted with the full-length polypeptide as well as with several shorter, minor polypeptides that had probably been created from the full-length construct by unspecific degradation. However, our probe did not react with GST alone or with any of the polypeptide markers included on the blot. Thus, α-actinin interacts specifically with LPP in vitro.

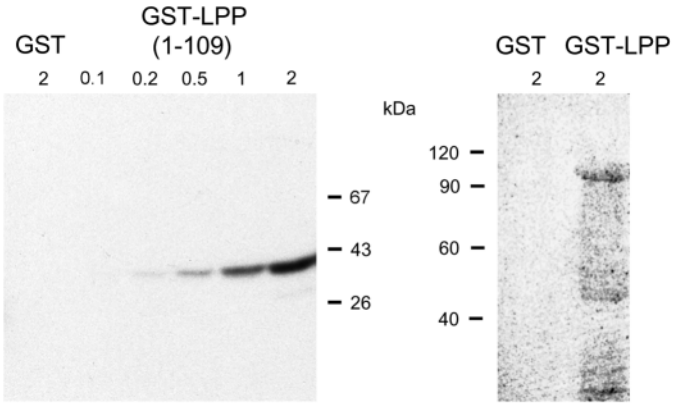

Fig. 2. Direct interaction of LPP and α-actinin in a blot overlay. Full-length LPP (right) as well as its N-terminal sequence (residues 1-109, left) were expressed in bacteria as GST-fusion proteins and resolved on polyacrylamide gels (0.1-2 μg/lane). After transfer to nitrocellulose, the proteins were probed with radiolabeled α-actinin. GST (2 μg/lane) and various protein standards were included as controls.

Mapping of binding sites in α-actinin and in LPP

To map the binding sites in detail, we employed the yeast two-hybrid system (Fig. 3). The cDNA sequence for human LPP was ligated into the bait vector pAS2-1 downstream of the sequence for the DNA-binding domain of GAL4. By contrast, various fragments derived from an α-actinin cDNA were cloned into the prey vector pACT2 downstream of the sequence for the transactivation domain of GAL4. A potential interaction of the resulting fusion proteins was analyzed by growth of transfected yeast on histidine-deficient agar plates and by transcription of the reporter gene *lacZ*. All results were compared to interactions observed between α-actinin and zyxin.

Initial studies demonstrated that the full-length LPP construct possessed autonomous transactivating properties. Yeast transfected with the bait plasmid alone grew on selective agar plates and expressed the *lacZ* reporter gene. We therefore restricted our studies to the N-terminal sequence of LPP (amino acids 1-61), which did not show autonomous transactivation as demonstrated in a control experiment. When the corresponding GAL4-LPP construct was transfected into yeast together with various fusion constructs coding for α-actinin, transcription of the reporter genes *HIS3* and *lacZ* was observed. Specific interactions were noted with those fragments that comprised the central SPEC domains of α-actinin (Fig. 3). No interaction was detected with the N-terminal calponin homology domains or with the C-terminal EF hands. The minimal fragment of α-actinin that showed a positive interaction with LPP consisted of SPEC domain 2-3. No interaction was observed with a tandem array spanning SPEC domains 1-2 or 3-4. Furthermore, no interaction was observed with SPEC domain 2 or SPEC domain 3 alone. In a parallel experiment, the tandem array of SPEC domains 2-3 was also found to be the minimal fragment interacting with the N-terminus of zyxin (residues 1-42) (Fig. 3). In this context it

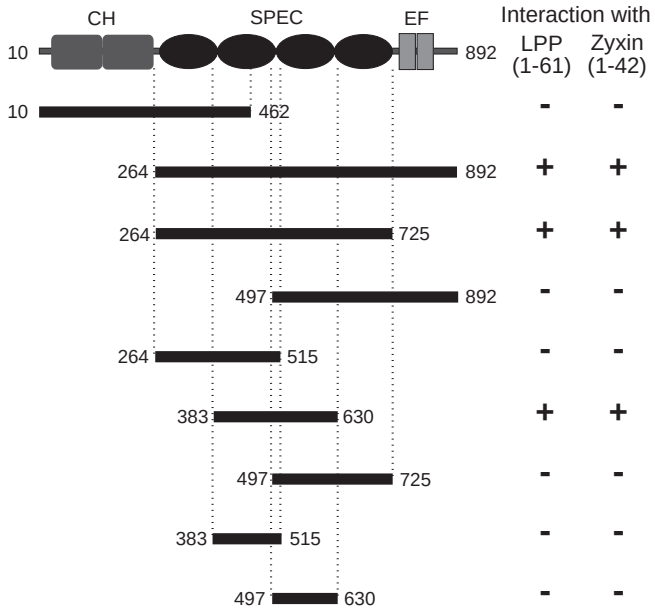

Fig. 3. Mapping of the LPP interaction site in α-actinin by the two-hybrid system. The yeast reporter strain Y190 was cotransfected with a prey vector encoding the N-terminus of LPP (residues 1-61) and different bait vectors encoding various fragments of α-actinin as indicated. Two-hybrid interactions were analyzed by growth on histidine, tryptophan and leucine-deficient plates. Positive interactions (+) were verified by the colony filter lift assay. Interactions of the same α-actinin fragments with the N-terminus of zyxin (residues 1-42) were analyzed in parallel experiments. Note that all α-actinin fragments that revealed a positive interaction are able to form dimers in vitro (Li and Trueb, 2001). CH, region containing calponin homology domains; SPEC, region containing spectrin-like repeats; EF, region containing EF hands.

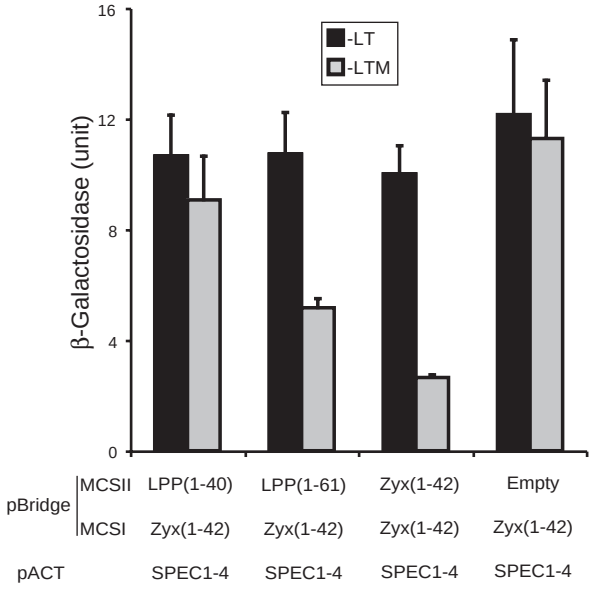

Fig. 4. Competition between LPP and zyxin for the binding site of α-actinin as revealed by the three-hybrid system. Yeast reporter strain Y190 was cotransfected with the prey vector pACT2 encoding the α-actinin rod (residues 264-725) and the bait vector pBridge. The latter vector contained the sequence for zyxin (residues 1-42) in multiple cloning site I and the sequences for zyxin (residues 1-42) or LPP (residues 1-61 or 1-40) in multiple cloning site II. Note that multiple cloning site II is under the control of the *MET25* promoter, which is active in the absence of methionine, but inactive in its presence. Colonies that grew on leucine- and tryptophan-deficient plates were inoculated into liquid medium lacking leucine and tryptophan (black bars) or, alternatively, lacking leucine, tryptophan and methionine (grey bars). Expression of the reporter gene for β-galactosidase was analyzed by a colorimetric assay. The results represent the means with standard deviation from at least three independent determinations.

should be noted that the tandem array of SPEC domains 2-3 is the shortest fragment of α-actinin that forms dimers in vitro (Djinovic-Carago et al., 1999; Li and Trueb, 2001). Taken together, our results suggest that α-actinin binds via the same site to both zyxin and LPP.

For a quantitative comparison, we utilized a colorimetric assay and determined the relative expression of the reporter gene *lacZ*. We found that the N-terminus of LPP (residues 1-61) interacted with α-actinin (residues 264-725) with a relative affinity of 1.46±0.24 (n=3), whereas the N-terminus of zyxin (residues 1-42) interacted with the same fragment with a relative affinity of 10.28±3.1. Thus, the interaction of α-actinin with zyxin is considerably stronger than that with LPP.

To map the binding site in LPP in more detail, we prepared a shorter construct corresponding to amino acids 1-40 of human LPP. We found that LPP (1-61) did interact with α-actinin, whereas LPP (1-40) did not (data not shown). The sequence deleted in the shorter LPP construct corresponded to the α-actinin-binding motif that was conserved in zyxin and LPP (see Fig. 1). Thus, the conserved motif appears to be responsible for α-actinin binding.

LPP competes with zyxin for α-actinin binding

Since both, LPP and zyxin bind to α-actinin, they may either bind simultaneously or compete for binding to the same site. To distinguish between these two possibilities, we employed the yeast three-hybrid system (Fig. 4). This system makes use of the pBridge vector, which contains two multiple cloning sites, MCSI and MCSII. Sequences cloned into the first site are expressed as fusion proteins with the DNA-binding domain of GAL4 similar to the situation in the normal two-hybrid system. Sequences cloned into the second site, however, are expressed as individual proteins (without GAL4 domain) from a conditional promoter. This promoter is active in the absence of methionine, but repressed in the presence of methionine. With the pBridge vector, it is therefore possible to investigate stimulating or inhibiting effects of a third protein onto a regular two-hybrid interaction.

The N-terminal sequences for LPP (residues 1-61 or 1-40) or zyxin (residues 1-42) were ligated into MCSII of the pBridge vector. The other cloning site harbored the N-terminal sequence of zyxin (residues 1-42). These constructs were transfected into yeast together with the bait vector pACT2 that contained the sequence for the central α-actinin rod (residues 264-725) as outlined above.

A control experiment with a pBridge construct lacking any insert in MCSII showed transcription of the reporter gene *lacZ*

in the presence as well as absence of methionine, which is indicative of a positive interaction of zyxin with α-actinin (Fig. 4). When MCSII contained the sequence for zyxin (1-42), this interaction was strongly reduced in the absence of methionine because the additionally expressed zyxin (without GAL4 DNA-binding domain) competed for α-actinin binding with the GAL4-zyxin fusion protein expressed from MCSI. In the presence of methionine, no competition was observed since transcription from MCSII was repressed. When LPP (1-61) rather than zyxin (1-42) was expressed from MCSII, the zyxin–α-actinin interaction was also substantially reduced (Fig. 4). However, the colorimetric assay showed that zyxin (1-42) inhibited the zyxin–α-actinin interaction to a larger extent than LPP (1-61). This result is consistent with the observation made above that zyxin shows stronger affinity for α-actinin than LPP. No significant inhibition was observed with LPP (1-40), which lacked the conserved α-actinin-binding site.

Our results were confirmed by the converse experiment. The sequence for LPP (1-61) was cloned into MCSI of the pBridge vector and expressed as a fusion protein with the DNA-binding domain of GAL4. When zyxin (1-42) was expressed from MCSII, it strongly inhibited the LPP–α-actinin interaction (data not shown). In this case, competition was nearly complete, suggesting once more that zyxin possessed higher affinity for α-actinin than LPP.

Taken together, our results clearly demonstrate that LPP and zyxin bind to the same site of α-actinin in a mutually exclusive manner.

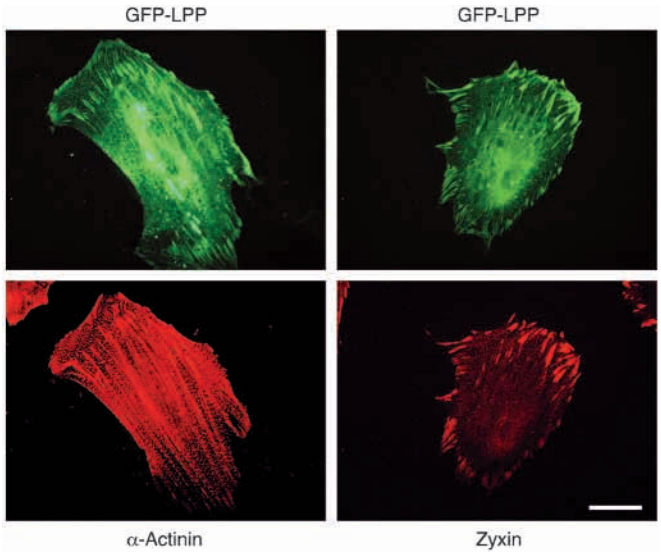

Fig. 5. Comparison of the subcellular distribution of GFP-tagged LPP and zyxin. H9C2 cells were transiently transfected with a full-length cDNA for LPP ligated into the expression vector pEGFP. Two days after transfection, the cells were fixed, permeabilized and stained with antibodies against α-actinin and zyxin, respectively. The green panel shows direct fluorescence emitted from GFP, the red panel indirect immunofluorescence from antibodies against α-actinin or zyxin. Bar, 20 μm.

Comparative subcellular distribution of LPP and zyxin

To compare the subcellular distribution of LPP and zyxin, the cDNA sequence of LPP was ligated into a GFP expression vector. H9C2 cells were transfected with this construct and inspected by epifluorescence microscopy (Fig. 5). The GFP-fusion protein was found to be distributed specifically at focal adhesion plaques as previously demonstrated (Petit et al., 2000) with specific antibodies. When the transfected cells were labeled with antibodies against α-actinin and inspected by double label fluorescence, a punctate staining was observed at focal contacts and along stress fibers. Labeling of the transfected cells with antibodies against zyxin specifically marked the focal adhesion plaques. The staining pattern was very similar to that observed above with GFP-LPP (Fig. 5). No significant differences in the distribution of zyxin were detected between GFP-LPP-transfected and non-transfected cells. Thus, LPP and zyxin exhibit a very similar subcellular distribution, but GFP-LPP is not able to displace zyxin from its normal subcellular sites, at least not to an extent detectable under the conditions used.

LPP interacts with α-actinin in vivo

The interaction of LPP with α-actinin was also verified in living cells. An experiment was designed to investigate whether LPP, which has been artificially targeted to mitochondria, recruits α-actinin to these ectopic sites (Fig. 6). As a sorting signal we used the membrane anchor of the protein ActA from *Listeria monocytogenes*, which is able to direct a fusion protein to the surface of mitochondria. Several constructs were prepared that encoded GFP fusion proteins with the membrane anchor (M) of ActA and full-length LPP or selected fragments derived thereof. PtK2 cells were transfected with these constructs and analyzed under epifluorescence. The full-length construct of LPP (GFP-LPP-M) was found to be distributed specifically at the mitochondrial surfaces (Fig. 6). Interestingly, this construct appeared to have the ability to induce aggregation of the mitochondria into a few prominent clusters. When the transfected cells were stained with antibodies against α-actinin and inspected by double label fluorescence, a strict codistribution of α-actinin and the full-length construct was observed. This phenomenon was particularly evident at larger clusters of mitochondria (Fig. 6, arrows). When the conserved α-actinin-binding motif was removed from the full-length construct by deleting residues 41-57 (construct GFP-LPPΔ-M), no codistribution of the fluorescence signal from α-actinin and GFP was observed. Analogous results were obtained with shorter fragments derived from the full-length LPP construct. A fragment spanning only the N-terminal amino acids 1-61 of LPP (GFP-LPP61-M) codistributed with α-actinin and induced clustering of the mitochondria that was very similar to the full-length construct (Fig. 6, arrows). In contrast, a shorter construct that lacked the conserved α-actinin-binding site (GFP-LPP40-M spanning residues 1-40) did not show any codistribution and did not induce mitochondrial clustering. Likewise, no codistribution of α-actinin and GFP and no clustering of mitochondria was observed in cells transfected with GFP-M alone.

These results demonstrate that LPP interacts with α-actinin in vitro as well as in vivo. Furthermore, the conserved α-actinin binding site is essential for recruitment of α-actinin to an ectopic site.

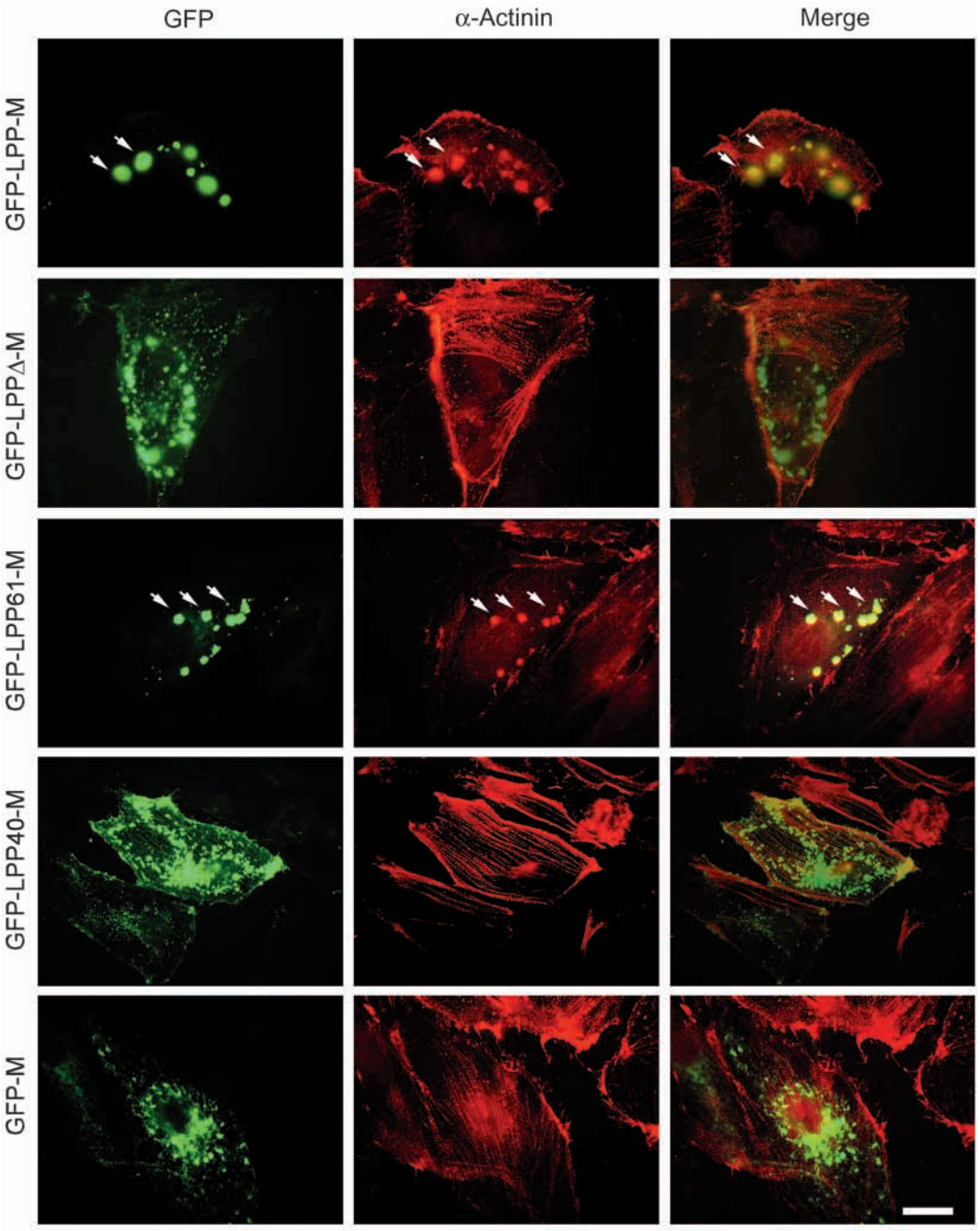

Fig. 6. Recruitment of α-actinin to LPP fusion proteins targeted to the surface of mitochondria. PtK2 cells were transiently transfected with GFP-fusion constructs encoding full-length LPP, full-length LPP lacking amino acids 41-57 (LPPΔ), the N-terminal fragment of LPP (LPP61, residues 1-61) or a shorter fragment (LPP40, residues 1-40) as well as a mitochondrial anchor (M) as indicated. Two days after transfection, the cells were fixed, permeabilized and stained with antibodies against α-actinin. The green panel shows direct fluorescence emitted from GFP, the red panel indirect immunofluorescence from antibodies against α-actinin. Arrows point to large clusters of mitochondria. Bar, 20 μm.

Discussion

LPP is a member of the zyxin family of structurally related proteins, which to date comprises zyxin, LPP, Trip6, LIMD1 and Ajuba. The present publication, as well as a recent study performed by Petit et al. (Petit et al., 2000) demonstrate that LPP does not only share structural features with zyxin, but that the two proteins also have overlapping functional properties. At the structural level, LPP and zyxin exhibit the same domain structure with a proline-rich N-terminus and three C-terminal LIM domains. At the functional level, both zyxin and LPP appear to serve as scaffold proteins in the dynamic assembly of focal adhesions and cell-cell contacts. Under certain conditions that are not yet understood in detail, both proteins can translocate to the nucleus where they may participate in the regulation of gene expression (Petit et al., 2000; Nix et al., 2001). The amount of zyxin and LPP in the nucleus is extremely small, such that it cannot be determined by direct biochemical means. A nuclear export signal found in LPP as well as zyxin appears to prevent a prolonged delay in the nucleus (Petit et al., 2000; Nix et al., 1997). Thus, the two proteins shuttle between cell adhesion sites and the nucleus and play an active role in intracellular communication. This role might also explain why both proteins possess autonomous transcriptional activation properties. When analyzed in a GAL4-based assay, both proteins are able to induce transcription of a reporter gene. The regions of the two proteins that are responsible for this autonomous transcriptional activation have broadly been mapped to the

central portion of zyxin as well as LPP (Petit et al., 2000; Li and Trueb, 2001).

The functional similarities between zyxin and LPP also extend to their binding partners. Both zyxin and LPP bind to VASP, a protein involved in the control of actin polymerization (Petit et al., 2000; Reinhard et al., 2001). In the present publication we showed that LPP, similar to zyxin, also interacts with α-actinin. This interaction could be demonstrated in vitro with the isolated proteins (blot overlay) as well as in yeast cells by the two-hybrid system. The interaction was also verified in mammalian cells in an experiment where LPP was able to recruit α-actinin to the surface of mitochondria when artificially targeted to these ectopic sites.

Truncation analyses allowed us to localize the binding site to the central rod of α-actinin, which contains the spectrin-like repeats SPEC 2 and 3. Only fragments that could dimerize in vitro were able to interact with LPP, suggesting that the dimeric conformation of α-actinin is required for binding. A similar conclusion has previously been reached with zyxin (Li and Trueb, 2001). The formation of such dimers offers a plausible explanation why the recruitment of α-actinin to LPP, which has been expressed on mitochondrial surfaces, may lead to the striking clustering of the mitochondria: α-actinin in its dimeric form might function as a divalent crosslinker and connect two LPP molecules expressed on two different mitochondria. It is possible that this crosslinking property of the α-actinin/LPP complex has functional implications in the formation of focal adhesions in living cells.

The binding site of α-actinin in LPP, by contrast, was mapped to a motif present at the extreme N-terminus of LPP that is fully conserved in zyxin. When this motif was deleted, LPP did not interact with α-actinin in the two-hybrid system and lost its ability to recruit α-actinin to an ectopic site in mammalian cells. The conserved motif contains hydrophobic and basic amino acids (KKFXPVVAPKPK) and does not occur in any other protein except zyxin and LPP.

In this publication we made extensive use of the three-hybrid system to tackle the question of whether LPP and zyxin bind to α-actinin in a mutually exclusive manner. Our results demonstrate that the two proteins compete for the same binding site in α-actinin. Since LPP and zyxin coexist in most fibroblastic and epithelial cells, the question about the biological significance for this functional redundancy arises. The answer might be found in the existence of subtle differences between the two proteins. Petit et al. described minor differences in the intracellular distribution (Petit et al., 2000). Although both proteins were found at focal adhesions and cell-cell contacts, zyxin was more prominently distributed than LPP along stress fibers. Furthermore, there was a difference in the relative abundance of the two proteins. In fibroblasts, the level of zyxin was about five times higher than that of LPP. However, no significant difference in the relative abundance was observed in epithelial cells (Petit et al., 2000). In this publication we demonstrated that there is also a manifest difference between the two proteins in their relative affinity for α-actinin. Utilizing a quantitative colorimetric assay, we found that zyxin bound to α-actinin with much higher affinity than LPP. A similar colorimetric approach was originally used to determine the dissociation constants of the retinoblastoma protein and its binding partners (Yang et al., 1995). The authors found that the binding affinities determined by surface plasmon resonance correlated well with the results obtained by the two-hybrid assay. We could confirm the difference between zyxin and LPP in the affinity for α-actinin by direct competition experiments. Zyxin completely abolished binding of LPP to α-actinin, whereas LPP just reduced binding of zyxin to α-actinin in the converse experiment.

In spite of the fact that LPP and zyxin compete for the same binding site in α-actinin, LPP was not able to displace zyxin from its normal subcellular site when overexpressed in mammalian cells. Likewise, our preliminary experiments (B. Li, unpublished) suggest that zyxin is not able to displace LPP from focal adhesions, at least not to an extent detectable under our experimental conditions. We interpret these findings as indicating the existence of a multitude of additional binding partners for zyxin and LPP. It might therefore not be possible to disturb a pre-existing focal adhesion complex by the mere overexpression of a single component.

We assume that the subtle differences between LPP and zyxin, as outlined above, play a decisive role in the differential function of the two proteins. Zyxin is actively involved in the organization of focal adhesions, complexes that are composed of more than 50 different proteins (Zamir and Geiger, 2001). When the expression of zyxin is inhibited by interfering RNAs, focal adhesions are diminished and stress fibers are greatly reduced (Harborth et al., 2001). The assembly and disassembly of these complexes is critical for cell motility and may represent the limiting step for maximal speed of migration (Palecek et al., 1998). Disassembly at the distal edge of the focal adhesions and assembly at the proximal edge is controlled by dynamic protein-protein interactions. Zyxin is one of the first molecules that dissociates from dissolving focal adhesions (Rottner et al., 2001). Our previous studies showed that the interaction with α-actinin is necessary for zyxin to localize to focal adhesions (Reinhard et al., 1999). Thus, the competition of LPP (and other competitors) with zyxin for α-actinin could provide one of the mechanisms for the dissociation of zyxin from dissolving focal adhesions. It would therefore be interesting to investigate whether zyxin and LPP show differences in their relative distribution at the distal and the proximal end of focal adhesions.

So far, all our studies have been limited to bilateral protein-protein interactions. It is obvious, however, that a comprehensive understanding of the function of focal adhesions cannot be gained without extending our analyses to multiple protein interactions as they exist under physiological conditions. It is possible that the new technology of proteomics will be instrumental in tackling this challenge.

This study was supported by the Swiss National Science Foundation (grant 31-061296) and by the Deutsche Forschungsgemeinschaft (grant Re1011/3-1).

References

Ashar, H. R., Fejzo, M. S., Tkachenko, A., Zhou, X., Fletcher, J. A., Weremowicz, S., Morton, C. C. and Chada, K. (1995). Disruption of the architectural factor HMGI-C: DNA-binding AT hook motifs fused in lipomas to distinct transcriptional regulatory domains. *Cell* **82**, 57-65.

Beckerle, M. C. (1986). Identification of a new protein localized at sites of cell-substrate adhesion. *J. Cell Biol.* **103**, 1679-1687.

Beckerle, M. C. (1998). Spatial control of actin filament assembly: lessons from *Listeria. Cell* **95**, 741-748.

Costa, G. L., Bauer, J. C., McGowan, B., Angert, M. and Weiner, M. P.

(1996). Site-directed mutagenesis using a rapid PCR-based method. *Methods Mol. Biol.* **57**, 239-248.

Crawford, A. W., Michelsen, J. W. and Beckerle, M. C. (1992). An interaction between zyxin and α-actinin. *J. Cell Biol.* **116**, 1381-1393.

Djinovic-Carugo, K., Young, P., Gautel, M. and Saraste, M. (1999). Structure of the α-actinin rod: molecular basis for cross-linking of actin filaments. *Cell* **98**, 537-546.

Drees, B. E., Andrews, K. M. and Beckerle, M. C. (1999). Molecular dissection of zyxin function reveals its involvement in cell motility. *J. Cell Biol.* **147**, 1549-1560.

Drees, B., Friederich, E., Fradelizi, J., Louvard, D., Beckerle, M. C. and Golsteyn, R. M. (2000). Characterization of the interaction between zyxin and members of the Ena/vasodilator-stimulated phosphoprotein family of proteins. *J. Biol. Chem.* **275**, 22503-22511.

Fradelizi, J., Noireaux, V., Plastino, J., Menichi, B., Louvard, D., Sykes, C., Golsteyn, R. M. and Friederich, E. (2001). ActA and human zyxin harbour Arp2/3-independent actin-polymerization activity. *Nat. Cell Biol.* **3**, 699-707.

Goyal, R. K., Lin, P., Kanungo, J., Payne, A. S., Muslin, A. J. and Longmore, G. D. (1999). Ajuba, a novel LIM protein, interacts with Grb2, augments mitogen-activated protein kinase activity in fibroblasts, and promotes meiotic maturation of *Xenopus* oocytes in a Grb2- and Ras-dependent manner. *Mol. Cell. Biol.* **19**, 4379-4389.

Harborth, J., Elbashir, S. M., Bechert, K., Tuschl, T. and Weber, K. (2001). Identification of essential genes in cultured mammalian cells using small interfering RNAs. *J. Cell Sci.* **114**, 4557-4565.

Hirota, T., Morisaki, T., Nishiyama, Y., Marumoto, T., Tada, K., Hara, T., Masuko, N., Inagaki, M., Hatakeyama, K. and Saya, H. (2000). Zyxin, a regulator of actin filament assembly, targets the mitotic apparatus by interacting with h-warts/LATS1 tumor suppressor. *J. Cell Biol.* **149**, 1073-1086.

Hobert, O., Schilling, J. W., Beckerle, M. C., Ullrich, A. and Jallal, B. (1996). SH3 domain-dependent interaction of the proto-oncogene product Vav with the focal contact protein zyxin. *Oncogene* **12**, 1577-1581.

Kiss, H., Kedra, D., Yang, Y., Kost-Alimova, M., Kiss, C., O'Brien, K. P., Fransson, I., Klein, G., Imreh, S. and Dumanski, J. P. (1999). A novel gene containing LIM domains (LIMD1) is located within the common eliminated region 1 (C3CER1) in 3p21.3. *Hum. Genet.* **105**, 552-559.

Lee, J. W., Choi, H. S., Gyuris, J., Brent, R. and Moore, D. D. (1995). Two classes of proteins dependent on either the presence or absence of thyroid hormone for interaction with the thyroid hormone receptor. *Mol. Endocrinol.* **9**, 243-254.

Li, B. and Trueb, B. (2001). Analysis of the α-actinin/zyxin interaction. *J. Biol. Chem.* **276**, 33328-33335.

Macalma, T., Otte, J., Hensler, M. E., Bockholt, S. M., Louis, H. A., Kalff-Suske, M., Grzeschik, K. H., von der Ahe, D. and Beckerle, M. C. (1996). Molecular characterization of human zyxin. *J. Biol. Chem.* **271**, 31470-31478.

Millake, D. B., Blanchard, A. D., Patel, B. and Critchley, D. R. (1989). The cDNA sequence of a human placental α-actinin. *Nucleic Acids Res.* **17**, 6725.

Niebuhr, K., Ebel, F., Frank, R., Reinhard, M., Domann, E., Carl, U. D., Walter, U., Gertler, F. B., Wehland, J. and Chakraborty, T. (1997). A novel proline-rich motif present in ActA of *Listeria monocytogenes* and cytoskeletal proteins is the ligand for the EVH1 domain, a protein module present in the Ena/VASP family. *EMBO J.* **16**, 5433-5444.

Nix, D. A. and Beckerle, M. C. (1997). Nuclear-cytoplasmic shuttling of the focal contact protein, zyxin: a potential mechanism for communication between sites of cell adhesion and the nucleus. *J. Cell Biol.* **138**, 1139-1147.

Nix, D. A., Fradelizi, J., Bockholt, S., Menichi, B., Louvard, D., Friederich, E. and Beckerle, M. C. (2001). Targeting of zyxin to sites of actin membrane interaction and to the nucleus. *J. Biol. Chem.* **276**, 34759-34767.

Palecek, S. P., Huttenlocher, A., Horwitz, A. F. and Lauffenburger, D. A. (1998). Physical and biochemical regulation of integrin release during rear detachment of migrating cells. *J. Cell Sci.* **111**, 929-940.

Petit, M. M. R., Mols, R., Schoenmakers, E. F. P. M., Mandahl, N. and van de Ven, W. J. M. (1996). LPP, the preferred fusion partner gene of HMGIC in lipomas, is a novel member of the LIM protein gene family. *Genomics* **36**, 118-129.

Petit, M. M. R., Fradelizi, J., Golsteyn, R. M., Ayoubi, T. A. Y., Menichi, B., Louvard, D., van de Ven, W. J. M. and Friederich, E. (2000). LPP, an actin cytoskeleton protein related to zyxin, harbors a nuclear export signal and transcriptional activation capacity. *Mol. Biol. Cell* **11**, 117-129.

Reinhard, M., Jarchau, T. and Walter, U. (2001). Actin-based motility: stop and go with Ena/VASP proteins. *Trends Biochem. Sci.* **26**, 243-249.

Reinhard, M., Zumbrunn, J., Jaquemar, D., Kuhn, M., Walter, U. and Trueb, B. (1999). An α-actinin binding site of zyxin is essential for subcellular zyxin localization and α-actinin recruitment. *J. Biol. Chem.* **274**, 13410-13418.

Rottner, K., Krause, M., Gimona, M., Small, J. V. and Wehland, J. (2001). Zyxin is not colocalized with vasodilator-stimulated phosphoprotein (VASP) at lamellipodial tips and exhibits different dynamics to vinculin, paxillin, and VASP in focal adhesions. *Mol. Biol. Cell* **12**, 3103-3113.

Sadler, I., Crawford, A. W., Michelsen, J. W. and Beckerle, M. C. (1992). Zyxin and cCRP: two interactive LIM domain proteins associated with the cytoskeleton. *J. Cell Biol.* **119**, 1573-1587.

Schmeichel, K. L. and Beckerle, M. C. (1998). LIM domains of cysteine-rich protein 1 (CRP1) are essential for its zyxin-binding function. *Biochem. J.* **331**, 885-892.

Tirode, F., Malaguti, C., Romero, F., Attar, R., Camonis, J. and Egly, J. M. (1997). A conditionally expressed third partner stabilizes or prevents the formation of a transcriptional activator in a three-hybrid system. *J. Biol. Chem.* **272**, 22995-22999.

Wang, Y. and Gilmore, T. D. (2001). LIM domain protein Trip6 has a conserved nuclear export signal, nuclear targeting sequences, and multiple transactivation domains. *Biochim. Biophys. Acta* **1538**, 260-272.

Yang, M., Wu, Z. and Fields, S. (1995). Protein-peptide interactions analyzed with the yeast two-hybrid system. *Nucleic Acids Res.* **23**, 1152-1156.

Yi, J. and Beckerle, M. C. (1998). The human TRIP6 gene encodes a LIM domain protein and maps to chromosome 7q22, a region associated with tumorigenesis. *Genomics* **49**, 314-316.

Zamir, E. and Geiger, B. (2001). Molecular complexity and dynamics of cell-matrix adhesions. *J. Cell Sci.* **114**, 3583-3590.

Zumbrunn, J. and Trueb, B. (1996). A zyxin-related protein whose synthesis is reduced in virally transformed fibroblasts. *Eur. J. Biochem.* **241**, 657-663.