The Focal Adhesion and Nuclear Targeting Capacity of the LIM-containing Lipoma-preferred Partner (LPP) Protein*

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Targeting of proteins to a particular cellular compartment is a critical determinant for proper functioning. LPP (LIM-containing lipoma-preferred partner) is a LIM domain protein that is localized at sites of cell adhesion and transiently in the nucleus. In various benign and malignant tumors, LPP is present in a mutant form, which permanently localizes the LIM domains in the nucleus. Here, we have investigated which regions in LPP target the protein to its subcellular locations. We found that the LIM domains are the main focal adhesion targeting elements and that the proline-rich region of LPP, which harbors binding sites for α-actinin and vasodilator-stimulated phosphoprotein (VASP), has a weak targeting capacity. All of the LIM domains of LPP cooperate in order to provide robust targeting to focal adhesions, and the linker between LIM domains 1 and 2 plays a pivotal role in this targeting. When overexpressed in the cytoplasm of cells, the LIM domains of LPP can deplete endogenous LPP and vinculin from focal adhesions. The proline-rich region of LPP contains targeting sites for focal adhesions and stress fibers that are distinct from the α-actinin and VASP binding sites, and the LPP LIM domains are dispensable for targeting LPP to the nucleus. Our studies have defined novel functional domains in the LPP protein.

In recent years, it has become clear that compartmentalization within mammalian cells is a key factor for the correct functioning of the complex network of signaling pathways in these cells. Trafficking of signaling molecules between the cytoplasmic and nuclear compartments, for instance, has important implications for the magnitude and specificity of gene expression. An interesting recent development is the realization that adhesion receptors and their cytoskeletal partners can regulate this nucleocytoplasmic trafficking of signaling proteins. Specialized cell adhesion sites not only play an architectural role in organizing cell structure and polarity but also are dynamic units directly involved in communication via the nuclear trafficking capability of several adhesion site-associated proteins. One such protein that may play a role in this process is the LIM-containing lipoma-preferred partner (LPP).1

LPP is a protein that is composed of an extensive proline-rich N-terminal region and three C-terminal LIM domains (Fig. 1A) (2). LIM domains are cysteine- and histidine-rich double zinc finger protein motifs that comprise ~55 residues, with the primary sequence CXCX2CX16–23HX3CX2CX16–23CX3C (where X is any amino acid) (3, 4) (Fig. 1C). The LPP protein localizes in focal adhesions, which are membrane attachment sites of cells to the extracellular matrix (5). In addition, LPP can be transiently translocated to the nucleus (5). The nucleocytoplasmic distribution of this protein involves a nuclear export signal (NES) that resides in the proline-rich region (Fig. 1A). At cell adhesions, LPP interacts with VASP (vasodilator-stimulated phosphoprotein) via its proline-rich region that contains two VASP-binding (FPζ) motifs (Fig. 1A) (5). In addition, LPP also interacts with α-actinin at these sites via its α-actinin binding site located near its N terminus in the proline-rich region (Fig. 1A).2

Although the molecular function of LPP is not yet known in detail, several characteristics of this protein suggest it has multiple functions in different compartments of the cell. LPP binding to VASP and α-actinin suggests that it has a role in certain aspects of cell motility and actin dynamics. VASP appears to have a universal role in the control of these processes (6, 7). α-Actinin is a cross-linker of filamentous actin and a dynamic constituent of focal adhesions (8, 9). In this regard, the cytoskeletal role of LPP may be quite similar to that of zyxin, which is a family member of LPP that also localizes to focal adhesions and binds to VASP and α-actinin (10–13). Several lines of evidence implicate zyxin in actin assembly and organization, and in cell movements that are known to depend on actin (14). In the nucleus, LPP harbors a significant transcriptional activation capacity residing in the proline-rich region as well as in the LIM domains suggesting that LPP is directly involved in the regulation of gene transcription (5).

In addition, LPP may play a role in the development of some benign and malignant tumors. In a subgroup of lipomas, which are benign tumors of adipose tissue, the LPP gene acts as the preferred translocation partner of HMG2 in these tumors, HMG2/LPP fusion transcripts are expressed (2, 15, 16). Identical fusion transcripts have also been found in a subgroup of pulmonary chondroid hamartomas (17) as well as in a parosteal lipoma (18). In a case of acute monoclonal leukemia, the LPP gene acts as translocation partner of the MLL gene, and the tumor expresses MLL/LPP fusion transcripts (19). All tu-

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1 The abbreviations used are: LPP, LIM-containing lipoma-preferred partner; FA, focal adhesion; NES, nuclear export signal; VASP, vasodilator-stimulated phosphoprotein; HMG2, high mobility group A-2; MLL, myeloid/lymphoid leukemia or mixed lineage leukemia; GFP, green fluorescent protein; TRIP6, thyroid hormone receptor-interacting protein 6; βGAL, β-galactosidase; PBS, phosphate-buffered saline; LMB, leptomycin B; CRM1, chromosomal region maintenance 1.


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mor-specific fusion transcripts that are expressed in the above mentioned tumors encode similar LPP fusion proteins containing AT-hooks (DNA binding domains) of the HMGA2 or MLL proteins followed by LIM domains of LPP. As we have shown before, these fusion proteins are mainly expressed in the nucleus (5).

As LPP appears to execute different functions depending on its intracellular localization, in focal adhesions or in the nucleus, targeting of LPP to these intracellular compartments is expected to be crucial for the differential functioning of this protein. It is possible that LPP may contribute to the tumorigenic process when its targeting is deregulated. To date however, little is known about the parts of LPP that target the protein to focal adhesions or to the nucleus. To obtain more insight into this matter, we made a variety of GFP-LPP fusion constructs containing either full-length LPP molecules or a number of mutated forms there off. We investigated the intracellular distribution of these chimeras. In this way, we were able to identify distinct regions in the LPP protein as key regulators of the subcellular distribution of LPP.

EXPERIMENTAL PROCEDURES

Plasmids—Plasmids for the expression of GFP (green fluorescent protein)-tagged parts of the human LPP protein were made by subcloning the appropriate PCR products in the EcoRI and PvuI sites of the pEGFP-C2 vector (Clontech). The plasmid for the expression of GFP-tagged full-length LPP was described before (5). Plasmids for the expression of GFP-tagged human zyxin, GFP-tagged human TRIP6, and GFP-tagged mouse ajuba were made by cloning the appropriate cDNAs into the EcoRI and BamHI sites of the pEGFP-C1 vector (for zyxin), in the SmaI site of the pEGFP-C2 vector (for TRIP6), or in the BglII and EcoRI sites of the pEGFP-C1 vector (for ajuba). Plasmids for the expression of N-terminal GFP-tagged and C-terminal β-galactosidase (β-gal)tagged parts of LPP were made by subcloning the appropriate PCR products in the NheI and XhoI (or SacII) sites of the pHM830 vector (20). The pHM840 construct has been described before (20). LPP constructs containing mutations or small internal deletions were made by site-directed mutagenesis using the QuikChange™ site-directed mutagenesis kit (Stratagene) according to the supplier’s instructions. All PCR amplifications were done with the Pico DNA Polymerase (Roche Molecular Biochemicals). All synthetic mutants and PCR-amplified regions were verified by sequencing.

Cell Culture and Transfections—Cell lines used in this work included CV-1 (African green monkey kidney fibroblast cells; ATCC CCL-70), NIH/3T3 (mouse embryonal fibroblast cells; ATCC CRL-1658), and 293T (human embryonal kidney epithelial cells containing SV40 T-antigen). Cell lines were grown in Dulbecco’s modified Eagle’s medium/ F12 (1:1) (Invitrogen) supplemented with 10% fetal bovine serum (HyClone). Cell lines were cultured at 37 °C in a humidified CO2 incubator.

Transient transfections were performed using FuGENETM 6 transfection reagent (Roche Molecular Biochemicals). Cells were grown on glass coverslides (coated with fibronectin in case of NIH/3T3 cells) to 50–60% confluency in 24-well plates. For each transfection 1.5 μl of FuGENETM 6 transfection reagent in 50 μl of serum-free Dulbecco’s modified Eagle’s medium (Invitrogen) was added to 0.5 μg of DNA and incubated at room temperature for 20 min after which the mixture was applied directly to the growth medium of the cells. Cells were incubated further at 37 °C for 18–24 h before analysis.

SDS-PAGE and Western Blotting—Expression of GFP-tagged and GFP-β-galactosidase-tagged proteins was verified by Western blotting using a polyclonal rabbit anti-GFP antibody, dilution 1:5000 (Santa Cruz Biotechnology). Cell extracts from transfected cells in 24-well plates were prepared by washing the cells three times in phosphate-buffered saline (PBS) followed by direct lysis in 100 μl of SDS-PAGE sample buffer (60 mM Tris-HCl, pH 6.8, 12% glycerol, 4% SDS, 5% β-mercaptoethanol). 25 μl of each cell extract were heated at 95 °C for 5 min and were loaded onto a 7.5% SDS-polyacrylamide gel. After size-separation, proteins were electrophoretically transferred to PROTRAN membrane (Schleicher and Schuell). ECL Western blotting was performed using Renaissance Western blotting detection reagents (PerkinElmer Life Sciences) according to the supplier’s instructions. In Fig. 7A, a rabbit polyclonal anti-LPP antibody MP2 was used for ECL Western blotting at a dilution of 1:3000.

Immunofluorescence—Cells were fixed in 4% formaldehyde for 20 min followed by three washes in PBS containing 0.9 mM CaCl2 and 0.5 mM MgCl2 (PBS−). Quenching was performed by incubating the cells for 10 min at room temperature in PBS− containing 50 mM NH4Cl. Cells were then permeabilized with 0.4% Triton X-100 for 10 min at room temperature. Subsequently, the slides were incubated with primary antibodies for 30 min at room temperature. After washing the cells three times in PBS−, bound primary antibodies were detected with fluorescently labeled secondary antibodies (Molecular Probes) for 30 min at room temperature. Following three washes in PBS−, slides were mounted in Vectashield mounting medium (Vector Laboratories). For detection of GFP fluorescence, cells were fixed and thereafter directly mounted. Slides were analyzed on a Zeiss Axioshot fluorescence microscope equipped with a cooled digital CCD camera system (PhotoMetrix using SmartCapture™ software). Primary antibodies used included rabbit polyclonal anti-LPP antibody MP2, dilution 1:200 (5) and a mouse monoclonal anti-vinculin antibody hVIN-1, dilution 1:400 (Sigma).

RESULTS

The LPP LIM Domains Play an Important Role in Targeting the Protein to Focal Adhesions—As outlined above, certain benign tumors express HMGA2/LPP fusion transcripts encoding HMGA2/LPP fusion proteins. These proteins are mainly localized in the nucleus (5). In lipomas, two different fusion transcripts are found encoding HMGA2/LPP fusion proteins composed of the three DNA binding domains of HMGA2 followed by either the two most C-terminal LIM domains of LPP (HMGA2/LPP-short) or a portion of the proline-rich region (amino acids 372–413) and all three LIM domains of LPP (HMGA2/LPP-long) (2). Our previous observations show that when GFP-tagged forms of these HMGA2/LPP fusion proteins are overexpressed in cells, both forms are expressed only in the nucleus (5). However, in cells expressing very high levels of HMGA2/LPP-long, this protein is also present in the cytoplasm and in focal adhesions while in cells expressing similar levels of HMGA2/LPP-short, staining in focal adhesions is not observed (5). These observations were the first indication that the LIM domains of LPP could play a role in targeting the LPP protein to focal adhesions.

To investigate the role of the LIM domains in targeting the LPP protein to focal adhesions, we made a number of GFP fusion proteins containing full-length LPP molecules carrying mutations in one or two of its LIM domains (Fig. 1B). The mutations in the LIM domains were made in such a way that these domains were completely destroyed: four of eight conserved zinc-binding cysteine and histidine residues were mutated to alanine (Fig. 1C). We compared the intracellular distribution of these mutant LPP molecules to that of the wild-type protein also expressed as a GFP fusion protein (Fig. 1B). The distribution of the GFP-tagged wild-type LPP protein is indistinguishable from that of the endogenous protein: GFP-LPP is highly concentrated in focal adhesions and, at steady state, only very low levels of the protein can be detected in the nucleus (Fig. 1, D and D′, and our previous observations, Ref. 5).

Mutations in any of the LPP LIM domains resulted in a reduction of the focal adhesion targeting capacity of the LPP protein (Fig. 1, E–G and E′–G′). The amount of reduction was different depending on which of the LIM domains was targeted by mutations. While mutations in the third LIM domain caused a minor reduction in focal adhesion targeting capacity (Fig. 1, G and G′), mutations in the first LIM domain caused a more severe reduction (Fig. 1, E and E′), and mutations in the second LIM domain caused the most severe reduction (Fig. 1, F and F′). When two of the LPP LIM domains were targeted by mutations at the same time, a severe reduction in focal adhesion targeting capacity was observed in all possible cases (Fig. 1, H–J and H′–J′). Mutations in the second and third LIM domain (Fig. 1, I and I′), or in the first and the third LIM domain (Fig. 1, D–D′ and D–D′), caused a similar severe reduction in the targeting capacity of the fusion proteins.
domain (Fig. 1, J and J') reduced the level of LPP in focal adhesions in a similar way as when the second LIM domain was mutated. The most severe phenotype was observed when the first and second LIM domains were mutated at the same time. In this case, focal adhesion targeting of the LPP protein was almost completely abolished (Fig. 1, H and H'). In conclusion, our results suggest that the LIM domains of LPP play an important role in targeting the LPP protein to focal adhesions.

Role of the Zyxin, TRIP6, and Zyxin/TRIP6/LIMD1 Similar Regions in Targeting LPP to Focal Adhesions—LPP is a member of a family of proteins, which are all proline-rich in their N-terminal region and have three LIM domains in their C-terminal region. LPP family members include zyxin (10), TRIP6 (21), ajuba (22), and LIMD1 (23). While all family members are quite similar in their C-terminal LIM domains, there is only limited similarity in their proline-rich regions. The LPP protein contains regions similar to zyxin near the N terminus (the α-actinin binding site) (Fig. 2, A and B), TRIP6 in the center of the proline-rich region (Fig. 2, A and C), and a region similar to zyxin, TRIP6, and LIMD1 at the C terminus of the proline-rich region (Fig. 2, A and D).

Recently, it was shown that when human or chicken zyxin lacking their α-actinin binding site are expressed as fusion proteins with GFP, targeting to focal adhesions is grossly impaired (14, 24, 25). We investigated whether this region has the same function in targeting LPP to focal adhesions as it has in zyxin. For this purpose, we made a construct expressing a GFP-LPP protein containing a deletion of its α-actinin binding site (GFP-LPPΔA41–57) (Fig. 2, A and B). However, no difference in focal adhesion targeting could be detected between the mutant LPP protein and the wild-type protein (Fig. 2, E and E').

We also investigated whether deletion of the TRIP6 similar region or the zyxin/TRIP6/LIMD1 similar region in LPP has an influence on the focal adhesion targeting capacity of the LPP protein. The function of the TRIP6 similar region is not known, either in LPP or in TRIP6. The zyxin/TRIP6/LIMD1 similar region contains a nuclear export signal (NES) in zyxin (26). In LPP, the function of this region is not known. According to our previous results (5) and Fig. 2, G and G', it does not function as a NES in LPP. No difference in focal adhesion targeting could be detected between the wild-type protein and mutant GFP-LPP proteins containing a deletion of the TRIP6 similar region (GFP-LPPΔ205–230) (Fig. 2, A, C, F, and F') or a deletion of the zyxin/TRIP6/LIMD1 similar region (GFP-LPPΔ387–408) (Fig. 2, A, D, G, and G'). In conclusion, none of the similar regions in the proline-rich region of LPP are important for the focal adhesion targeting of LPP.

The Three LIM Domains of LPP Cooperate for Robust Targeting to Focal Adhesions—To obtain more insight into how the LIM domains of LPP function to target LPP to focal adhesions, we deleted the entire proline-rich region of the protein. In this case, the targeting capacity of LPP was almost completely abolished (Fig. 1, H and H'). In conclusion, the LIM domains of LPP cooperate for robust targeting to focal adhesions.
way, we were able to investigate the focal adhesion targeting capacity of the LIM domains as a separate entity. We made a construct expressing a GFP fusion protein containing all three LIM domains of LPP (Fig. 3A). In CV-1 cells, this GFP-LPP-(412–612) protein displayed robust targeting to focal adhesions (Fig. 3, B and B'). However, staining in focal adhesions was not as strong as the staining obtained with the full-length wild-type LPP protein. These observations indicate that targeting of the LIM domains to focal adhesions is not as powerful as for the full-length protein. This suggests that also the proline-rich region of LPP might have a function in targeting the protein to focal adhesions.

To further analyze the focal adhesion targeting capacity of the LPP LIM domains, we investigated the targeting capacity of paired LIM domains and individual LIM domains of LPP. To do so, we made a number of constructs expressing GFP fusions containing LIM domains 1 and 2 (GFP-LPP-(412–531)), LIM domains 2 and 3 (GFP-LPP-(471–612)), LIM domain 1 (GFP-LPP-(412–473)), LIM domain 2 (GFP-LPP-(471–531)), or LIM domain 3 (GFP-LPP-(531–612)) (Fig. 3A). Upon expression in CV-1 cells of these GFP-LPP fusion proteins, important observations could be made (Fig. 3, C–G and C'–G'). In contrast to the GFP-LPP protein containing all three LIM domains of LPP, which had strong focal adhesion targeting, paired LIM domains, either LIM 1 and 2, or LIM 2 and 3, as well as each individual LIM domain showed a drastic reduction in their focal adhesion targeting capacity. These results suggest that the three LIM domains of LPP cooperate to provide robust targeting of LPP to focal adhesions.

The LPP Proline-rich Region Harbors Targeting Capacity for Focal Adhesions and Stress Fibers—The above-mentioned results on the focal adhesion targeting capacity of the LIM domains of LPP, as compared with the complete LPP protein, suggested that the proline-rich region of LPP also has a function in targeting the protein to focal adhesions. To confirm these results, we made a construct expressing a GFP-LPP protein containing only the proline-rich region, lacking all three LIM domains (GFP-LPP-(2–412)) (Fig. 4). CV-1 cells expressing this protein presented staining in focal adhesions indicating that the proline-rich region of LPP has focal adhesion targeting capacity (Fig. 5, A and A'). However, whereas the strength of focal adhesion targeting capacity of the LIM domains was comparable to that of the full-length protein, the targeting capacity of the proline-rich region, although clearly detectable, was found to be much weaker.

To narrow down the area in the proline-rich region responsible for its focal adhesion targeting capacity, we made several deletion constructs of the proline-rich region of LPP as depicted in Fig. 4. In this way, we were able to study the effect of deletions in the proline-rich region as a separate entity, isolated from the strong focal adhesion targeting effect of the LIM domains. We first deleted the actin-binding site in LPP (GFP-LPP-(62–412)), because α-actinin is known to be a component of focal adhesion sites and as such could provide a
docking site for the proline-rich region of the LPP protein. However, deletion of the α-actinin binding site did not alter the targeting of the proline-rich region to focal adhesions (Fig. 5, B and B'). Deletion of the VASP binding sites in addition to the α-actinin binding site (GFP-LPP-(94–415)) had no effect on targeting to focal adhesions (Fig. 5, C and C'). Further deletion of the TRIP6 similar region in addition to the α-actinin and VASP binding sites (GFP-LPP-(94–415)Δ(205–230)) also had no effect (results not shown). These results indicate that the focal adhesion targeting capacity of the proline-rich region of LPP is located either between the VASP binding sites and the TRIP6 similar region or between the TRIP6 similar region and

**Fig. 3.** The three LIM domains of LPP cooperate for robust targeting to focal adhesions. A, schematic representation of GFP molecules containing LIM domains of LPP in different combinations, and their focal adhesion targeting capacity as compared with the wild-type protein. B–G, CV-1 cells transiently transfected with constructs expressing GFP-LPP-(412–612) (B, B'), GFP-LPP-(412–531) (C, C'), GFP-LPP-(451–612) (D, D'), GFP-LPP-(412–473) (E, E'), GFP-LPP-(471–531) (F, F'), GFP-LPP-(531–612) (G, G'). Cells were fixed and labeled for vinculin. Cells were visualized either for GFP (B–G) or vinculin (B'–G').

**Fig. 4.** The focal adhesion targeting capacity of the LPP proline-rich region. Schematic representation of GFP molecules containing the proline-rich region of LPP or portions of it, and their focal adhesion targeting capacity as compared with the wild-type protein.
the C-terminal end of the proline-rich region.

In order to discriminate between these two possibilities, we made constructs expressing GFP-LPP proteins containing either of these segments of the proline-rich region with the TRIP6 similar region (GFP-LPP-(94–258), GFP-LPP-(179–415)) or without the TRIP6 similar region (GFP-LPP-(94–258Δ(205–230)), GFP-LPP-(179–415Δ(205–230)) (Fig. 4). GFP-LPP-(94–258) and GFP-LPP-(94–258Δ(205–230)) almost entirely lost the ability to target to focal adhesions (Fig. 5, D, D’, E, E’, and F’). However, occasionally, targeting to stress fibers was observed (Fig. 5, E, E’, G, and G’). In this regard, we noticed that wild-type LPP protein is also occasionally observed along stress fibers.3 On the other hand, GFP-LPP-(179–415) and GFP-LPP-(179–415Δ(205–230)) did show targeting to focal adhesions in a way that was indistinguishable from the targeting capacity of the entire proline-rich region (Fig. 5, H, H’, I, and I’). These results indicate that the LPP proline-rich region harbors targeting capacity for focal adhesions and stress fibers and that these capacities are located between the TRIP6 similar region and the C-terminal end of the proline-rich region, and between the VASP binding sites and the TRIP6 similar region, respectively.

The LPP LIM Domains Can Target the β-Galactosidase Protein to Focal Adhesions—Our results suggest that the LIM domains are the main focal adhesion targeting elements of the LPP protein. To further investigate these results, we wanted to determine which parts of the LPP protein were able to transport a non-related molecule, such as β-galactosidase to focal adhesions. For this purpose, we made a number of constructs expressing GFP-LPP-βGAL proteins containing the full-length or parts of the LPP protein fused to GFP and βGAL at the N terminus and the C terminus, respectively (Fig. 6A). The GFP-βGAL protein itself is distributed throughout the cytoplasm in CV-1 cells, and no staining in focal adhesions can be detected (results not shown). We first investigated whether the full-length LPP protein (GFP-LPP-(2–612)–βGAL) could target βGAL to focal adhesions. As shown in Fig. 6, B and B’, this was indeed the case. Focal adhesion staining of the GFP-LPP-(2–612)–βGAL protein was in fact as strong as the staining obtained with the GFP-LPP protein. We next investigated whether the proline-rich region of LPP (GFP-LPP-(2–415)–βGAL) or the LIM domains of LPP (GFP-LPP-(412–612)–βGAL) were able to target β-galactosidase to focal adhesions.

While focal adhesion staining could hardly be detected when the GFP-LPP-(2–415)–βGAL protein was expressed in CV-1 cells (Fig. 6, C and C’), robust targeting to focal adhesions was observed when the GFP-LPP-(412–612)–βGAL protein was expressed in these cells (Fig. 6, D and D’). Staining in focal adhesions of the GFP-LPP-(412–612)–βGAL protein was slightly diminished as compared with the GFP-LPP-(2–612)–βGAL protein, and a higher level of diffuse cytoplasmic staining was observed. We next investigated the ability of paired and individual LIM domains of LPP to target the β-galactosidase protein to focal adhesions. GFP-LPP-βGAL proteins containing LIM domains 1 and 2 (GFP-LPP-(412–531)–βGAL), LIM domains 2 and 3 (GFP-LPP-(471–612)–βGAL), LIM domain 1 (GFP-LPP-(412–473)–βGAL), LIM domain 2 (GFP-LPP-(471–531)–βGAL), or LIM domain 3 (GFP-LPP-(531–612)–βGAL) were expressed in CV-1 cells. However, careful examination of these cells did not reveal any focal adhesion staining (Fig. 6, E–I and E’–I’). In conclusion, under these experimental conditions, only the full-length protein or the three LIM domains of LPP were able to target the β-galactosidase protein to focal adhesions.

The LIM Domains of LPP Can Deplete Endogenous LPP and Vinculin from Focal Adhesions—As our results indicated that the LIM domains of LPP showed robust targeting to focal adhesions, we investigated whether these domains could compete for the subcellular localization sites of endogenous LPP and as such could interfere with the function of this protein. For this purpose, we expressed the GFP-LPP-(412–612) molecule containing all three LIM domains of LPP in CV-1 cells and examined the intracellular distribution of the endogenous LPP protein with our MP2 antibody. This antibody recognizes an epitope in the proline-rich region of LPP (5). As a consequence of this, the antibody can discriminate between the endogenous LPP protein and the ectopically expressed GFP-LPP-(412–612) protein that lacks the proline-rich region. The MP2 antibody also does not cross-react with LPP family members zyxin, TRIP6, and ajuba (Fig. 7A). Our results showed that high levels of the GFP-LPP-(412–612) protein could displace the endogenous LPP protein from focal adhesions and could induce an accumulation of endogenous LPP in the cytoplasm of cells (Fig. 7, B and B’). This result suggests that the LIM region of LPP can indeed compete for the subcellular localization sites of endogenous LPP, interfering with the normal function of the LPP protein.

The GFP-LPP-(412–612) molecule is not only targeted to focal adhesions but was also found to accumulate in the nucleus.

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3 M. Petit, unpublished results.
of cells, most likely via passive diffusion (Fig. 7B). In this context, we wondered whether the nuclear accumulation of the LPP LIM domains could play a role in the observed reorganization of LPP’s intracellular distribution. To investigate this, instead of GFP-LPP-(412–612), we expressed the GFP-LPP-(412–612)-βGAL protein in cells. Like GFP-LPP-(412–612), GFP-LPP-(412–612)-βGAL localizes to focal adhesions but by fusing the LIM domains of LPP to βGAL, nuclear accumulation of these LIM domains is prevented (Fig. 7C). Our results showed that high levels of GFP-LPP-(412–612)-βGAL could deplete endogenous LPP from focal adhesions and induce cytoplasmic accumulation of LPP in a way that was indistinguishable from GFP-LPP-(412–612) (Fig. 7, C and C’). These results indicate that nuclear accumulation of the LPP LIM domains is not necessary to deplete LPP from focal adhesions and cause its cytoplasmic accumulation.

To further investigate the consequences of overexpression of the LIM domains of LPP, we looked into the distribution of vinculin in CV-1 cells that overexpressed the GFP-LPP-(412–612) protein. As shown in Fig. 7, D and D’, also vinculin was depleted from focal adhesions in cells expressing high levels of the GFP-LPP-(412–612) protein.

The Linker between LIM Domains 1 and 2 in LPP Is Important for Its Focal Adhesion Targeting—If LPP family members are compared with respect to their focal adhesion targeting capacity, a striking difference arises between LPP, TRIP6, and zyxin on the one hand, and ajuba on the other hand (at this moment no data are available regarding the focal adhesion targeting capacity of LIMD1). While LPP, TRIP6, and zyxin were all reported to localize in focal adhesions (5, 10, 14, 27), ajuba was reported not to be targeted to focal adhesions in NIH/3T3 cells (22). We used this information to investigate further the focal adhesion targeting capacity of the LPP protein. As our results suggest that the LIM domains of LPP are the main focal adhesion targeting determinants of this protein and as the zyxin LIM domains were reported to have a similar function (14), we compared the ajuba LIM domains to the LIM domains of the other LPP family members on the amino acid level. This shows a significant difference: in ajuba, the linker between LIM domain 1 and LIM domain 2 is 5 amino acids longer as compared with the linker in LPP, TRIP6, and zyxin (Fig. 8A).

To investigate whether in LPP the linker between the first and the second LIM domain played a role in the focal adhesion targeting capacity of this protein, we replaced this linker in LPP with the linker of ajuba as depicted in Fig. 8A. A GFP-LPP protein containing this mutation (GFP-LPPmut_ajuba) was introduced in NIH/3T3 cells plated on fibronectin and its focal adhesion targeting capacity was compared with the targeting capacity of the wild-type GFP-LPP protein. Our results showed that, while GFP-LPP was prominent in focal adhesions (Fig. 8B), focal adhesion staining of GFP-LPPmut_ajuba was significantly reduced (Fig. 8C). Similar findings were obtained in CV-1 cells (results not shown). These results indicate that the

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**Fig. 6.** The LPP LIM domains target the β-galactosidase protein to focal adhesions. A, schematic representation of GFP-LPP-βGAL molecules containing full-length or different portions of LPP and their focal adhesion targeting capacity. B–I, CV-1 cells transiently transfected with constructs expressing GFP-LPP-(2–612)-βGAL (B, B’), GFP-LPP-(2–415)-βGAL (C, C’), GFP-LPP-(412–612)-βGAL (D, D’), GFP-LPP-(412–531)-βGAL (E, E’), GFP-LPP-(471–612)-βGAL (F, F’), GFP-LPP-(412–473)-βGAL (G, G’), GFP-LPP-(471–531)-βGAL (H, H’), GFP-LPP-(531–612)-βGAL (I, I’). Cells were fixed and labeled for vinculin. Cells were visualized either for GFP (B–I) or vinculin (B’–I’).
The LIM domains of LPP can deplete endogenous LPP and vinculin from focal adhesions. A, anti-LPP MP2 antibody does not cross-react with zyxin, TRIP6, or ajuba. Cell extracts of 293T cells transiently transfected with constructs expressing GFP-LPP (lanes 1 and 5), GFP-zyxin (lanes 2 and 6), GFP-TRIP6 (lanes 3 and 7), or GFP-ajuba (lanes 4 and 8) were analyzed by SDS-PAGE and Western blotting with an anti-GFP antibody (lanes 1–4) or with the MP2 antibody (lanes 5–8). B–D, CV-1 cells were transiently transfected with constructs expressing GFP-LPP-(412–612) (B, B’, D’), or GFP-LPP-(412–612)-βGAL (C, C’) and stained with the MP2 antibody (B’, C’), or an anti-vinculin antibody (D’).

The linker between LIM domains 1 and 2 in LPP plays a role in focal adhesion targeting of this protein.

We next investigated whether reciprocal substitution in ajuba (Fig. 8A) could generate focal adhesion targeting capacity for ajuba in NIH/3T3 cells plated on fibronectin. A GFP-ajuba protein containing this mutation (GFP-ajubamut_LPP) was expressed in these cells; however, no focal adhesion staining could be observed (results not shown).

Interestingly, while no focal adhesion staining could be detected in NIH/3T3 cells when introducing a wild-type GFP-ajuba protein in cells that were already spread for 18–24 h on fibronectin (Fig. 8D), we did detect GFP-ajuba in focal adhesions in these cells when the transfected cells were trypsinized and allowed to spread for only 1 h on fibronectin (Fig. 8E). However, focal adhesion staining of GFP-ajuba in these cells was not as strong as the staining observed with GFP-LPP expressing NIH/3T3 cells that were treated in the same way (Fig. 8F).

The Nuclear Targeting Capacity of the LPP Protein—We have shown before that LPP has the ability to shuttle to the nucleus and that its nucleocytoplasmic distribution is regulated by an NES (5). This NES prevents accumulation of LPP in the nucleus in steady state cells. However, a very low percentage of cells show accumulation of LPP in the nucleus, suggesting that LPP shuttling to the nucleus is tightly regulated (5). To further study the nuclear targeting capacity of the LPP protein, we investigated the influence of mutations in the LPP protein on its capacity to shuttle to the nucleus. Previously (5), we have shown that the LPP nuclear export can be blocked by leptomycin B (LMB), a drug that blocks nuclear export by preventing the formation of the NES/CRM1/Ran-GTP complex (28–31). Therefore, we incubated cells expressing mutant LPP molecules with LMB and determined whether these molecules could still accumulate in the nucleus as an indication that these molecules could still shuttle to the nucleus.

In this way, we investigated the nuclear targeting capacity of LPP molecules containing mutations in the first, second, or third LIM domain (GFP-LPPmutLIM1, GFP-LPPmutLIM2, GFP-LPPmutLIM3), or in every combination of two LIM domains (GFP-LPPmutLIM1/2, GFP-LPPmutLIM2/3, GFP-LPPmutLIM1/3) (Fig. 1, B and C). All of these molecules accumulated in the nucleus of cells upon incubation with LMB, indicating that these mutations had no influence on the ability of LPP to shuttle to the nucleus (Fig. 9, A–F). These results suggest that any LIM domain of the LPP protein is dispensable for nuclear targeting. We investigated further this result by incubating cells expressing the GFP-LPP-(2–415) molecule with LMB. We noticed that this molecule, which contains only the proline-rich region of LPP lacking all three LIM domains (Fig. 4), also accumulated in the nucleus under these conditions (Fig. 9G). These results suggest that the LIM domains of LPP are dispensable for its nuclear targeting.

To get more insight into the LPP nuclear targeting capacity, we investigated whether small regions of the protein could transport the GFP-βGAL protein into the nucleus. We used the β-galactosidase protein to increase the molecular weight of these short LPP peptides. In this way, we were able to discriminate between active targeting of short fragments of the LPP protein to the nucleus and their accumulation in the nucleus caused by passive diffusion. When dividing the LPP molecule...
Elements in the LPP Protein

FIG. 9. The LPP LIM domains are dispensable for targeting LPP to the nucleus. A–G, CV-1 cells were transiently transfected with constructs expressing, GFP-LPPmutLIM1 (A), GFP-LPPmutLIM2 (B), GFP-LPPmutLIM3 (C), GFP-LPPmutLIM1/2 (D), GFP-LPPmutLIM2/3 (E), GFP-LPPmutLIM1/3 (F), or GFP-LPP-(2–415) (G). Cells were incubated with leptomycin B for 2 h. Detection of GFP fluorescence was done as described under “Experimental Procedures.”

into smaller parts, we took into consideration the domain structure of LPP as presently known, to avoid affecting functional domains in the protein.

As far as the proline-rich region is concerned, we made six constructs expressing small parts of LPP as GFP-LPP-βGAL fusions (Fig. 10A). These proteins contained the N terminus of LPP (GFP-LPP-(2–43)-βGAL), the α-actinin binding site (GFP-LPP-(36–65)-βGAL), the VASP-binding sites (GFP-LPP-(62–101)-βGAL), the region between the VASP binding sites and the TRIP6 similar region with a deletion of the NES (GFP-LPP-(94–208)-βGAL), the TRIP6 similar region (GFP-LPP-(201–234)-βGAL), or the remaining C-terminal part of the proline-rich region (GFP-LPP-(227–413)-βGAL).

As a positive control for nuclear accumulation, we used the plasmid pHM840, which expresses a GFP-βGAL fusion protein containing the NLS of Simian Virus 40. This NLS directs the GFP-βGAL fusion protein to the nucleus (20). We expressed these proteins in CV-1 cells but none of the GFP-LPP-βGAL fusions accumulated in the nucleus (Fig. 10, C–H) in contrast to our positive control pHM840 (Fig. 10B). We also investigated the nuclear targeting properties of the GFP-LPP-(2–413)ΔNES-βGAL protein, which contains the entire proline-rich region with a deletion of the NES; however, we found that it was not accumulating in the nucleus (Fig. 10D).

Concerning the LIM domains of LPP, we investigated the capability of each of its LIM domains individually, paired LIM domains (LIM 1 and 2 or LIM 2 and 3), or all of its LIM domains to target βGAL to the nucleus by expressing GFP-LPP-βGAL proteins in CV-1 cells (Fig. 6A). However, these fusion proteins did not exhibit any accumulation in the nucleus (Fig. 6, D–I). Finally, we made a construct expressing full-length LPP containing a deletion of the NES as a GFP-LPP-βGAL fusion protein and investigated its intracellular distribution. Whereas the protein was expressed in focal adhesions, it did not exhibit any nuclear staining (results not shown).

DISCUSSION

From our previous observations (5), it is suggested that the LPP protein has different functions depending on its localization in focal adhesions or in the nucleus. Therefore, it is expected that the intracellular localization of LPP is crucial for its differential functioning. We have used several strategies in order to map specific regions in the LPP protein that are responsible for targeting to focal adhesions and to the nucleus.

The LIM Domains Are the Main Focal Adhesion Targeting Elements in the LPP Protein—Our results showed that each of the LPP LIM domains had the capacity to be targeted to focal adhesions. However, while GFP fusion proteins containing individual or paired LIM domains only possessed a rudimentary capacity to localize to these sites, fusion proteins containing all of the LIM domains of LPP showed robust targeting to focal adhesions. These results indicate that the LIM domains of LPP cooperate to target the protein to these sites of close cellular contact with the extracellular matrix. These findings are an example of the fact that, although individual LIM domains can operate as protein binding units as demonstrated by structural analysis, protein binding is often enhanced or dependent on the presence of more than one LIM domain (32).

Our findings on the focal adhesion targeting of LPP are somewhat different to the recently published findings on the focal adhesion targeting of its family member zyxin (14). Like LPP, the three LIM domains of zyxin show robust targeting and paired LIM domains show very weak targeting to focal adhesions. However, in contrast to LPP, individual LIM domains of zyxin do not target to focal adhesions (14).

The finding that the LIM domains in LPP and zyxin cooperate to target the proteins to focal adhesions is quite different from what has been found for the protein paxillin. Paxillin is a focal adhesion-associated adapter protein that contains four LIM domains in its C-terminal region (33). Brown et al. (34) showed that the third LIM domain plays a major role in targeting paxillin to focal adhesions, whereas the second LIM domain plays a minor role.

Our analysis of the focal adhesion targeting capacity of the LPP LIM domains opens a number of possibilities on the mechanism by which these LIM domains incorporate into focal adhesions. As LIM domains are known to function as protein–protein interaction units and as each individual LIM domain still has the capacity to localize in focal adhesions, it can be suggested that each LIM domain binds to a protein that resides in focal adhesions. In this way, each LIM domain can interact with a different protein, meaning that there could be more than one protein in focal adhesions that binds to the LIM domains of LPP. However, it could also be that only one protein contains three docking sites for each of the LIM domains of the LPP protein. Alternatively, it is also possible that the three LIM domains of LPP form one large binding interface. It would be very interesting to further investigate these possibilities. To date, no binding partners of the LPP LIM domains have been identified that have a robust localization to focal adhesions and therefore could be focal adhesion targeting modules for LPP.

By replacing the linker between LIM domains 1 and 2 in LPP by the one from ajuba, we showed that the level of this mutant LPP protein in focal adhesions was lower as compared with the wild-type protein. These results indicate that not only the LIM domains per se but also the linker between the LIM domains, at least the one between LIM 1 and 2, has a role in the focal adhesion targeting of LPP. Our results suggest that the three LIM domains of LPP form a specific structure, and that it is this structure that is important for robust focal adhesion targeting of LPP rather than the presence of the three individual LIM domains as separate entities.
Fig. 10. GFP-LPP-βGAL molecules are not targeted to the nucleus. A, schematic representation of GFP-LPP-βGAL molecules containing different portions of the proline-rich region of LPP. B–I, CV-1 cells transiently transfected with constructs expressing GFP-SV40NLS-βGAL (pHM840), GFP-LPP-(2–43)-βGAL (C), GFP-LPP-(36–65)-βGAL (D), GFP-LPP-(62–101)-βGAL (E), GFP-LPP-(94–208)ΔNES-βGAL (F), GFP-LPP-(201–224)-ΔGAL (G), GFP-LPP-(227–415)-βGAL (H), GFP-LPP-(2–415)ΔNES-βGAL (I). Detection of GFP fluorescence was done as described under “Experimental Procedures.”

With regard to the functional relevance of focal adhesion targeting of LPP through its LIM domains, we have identified in yeast two-hybrid experiments proteins that interact with the LIM domains of LPP and have preliminary confirmed this interaction by alternative approaches. One of these proteins is Raly, an RNA-binding protein, pointing toward the possible involvement of LPP, as a component of focal adhesion complexes, in rapid post-transcriptional changes in gene expression mediated by repositioning of translational components to sites of signal reception, as has been suggested for zyxin, a family member of LPP (38). In that report, the suggestion was based on the potential nucleic acid binding capacity of zyxin. We cannot exclude the possibility that LPP also possesses direct nucleic acid binding capacity. However, our data indicate that, in case LPP is mechanistically involved in such a post-transcriptional change in gene expression at focal adhesion complexes, mRNA binding to LPP is probably not direct, as suggested for zyxin, but is mediated via a protein-protein complex involving LPP. Such an indirect mechanism has recently been described for another LIM domain protein, i.e. paxillin (36). The results of these studies suggest a new mechanism whereby a paxillin-poly(A) binding protein complex facilitates transport of mRNA from the nucleus to sites of protein synthesis at the endoplasmic reticulum and the leading lamella during cell migration.

The LPP Proline-rich Region Harbors Targeting Capacity for Focal Adhesions and Stress Fibers—In addition to the LIM domains, the proline-rich region of LPP also contains focal adhesion targeting capacity. However, while the LIM domains showed robust targeting to focal adhesions, the targeting capacity of the proline-rich region was much weaker. These results are similar to those reported for zyxin (14) and paxillin (37). In both of these proteins, the LIM domains are the main targeting units for focal adhesions; however, the non-LIM region also has some weak targeting activity.

Near its N terminus, the LPP proline-rich region contains binding sites for two proteins, VASP (5) and α-actinin, which are both localized in focal adhesions in addition to other sites in the cell (38, 39). However, deletion of these binding sites from the proline-rich region did not cause a reduction in the focal adhesion targeting capacity of this region. These results suggest that VASP and α-actinin most likely do not play a role in the focal adhesion targeting of LPP.

Additional evidence for the fact that α-actinin binding probably does not play a significant role in targeting LPP to focal adhesions was obtained from the fact that the focal adhesion targeting of an LPP protein containing an internal deletion of the α-actinin binding site was indistinguishable from that of the wild-type protein. These results are quite different from those observed for zyxin. Recently, it was shown that the α-actinin binding site in zyxin is essential for its subcellular localization (24). It was shown that point mutations of specific amino acids in the α-actinin binding site or deletion of the entire α-actinin binding site grossly impaired zyxin targeting to focal adhesions (24, 25). Our different results for LPP are probably due to the fact that LPP has a lower affinity for α-actinin as compared with zyxin.2

By deletion analysis, we mapped the focal adhesion targeting unit of the proline-rich region of LPP to the C-terminal half of this region. This result suggested that this part of the proline-rich region contains an interaction site for a protein that is localized in focal adhesions. Further studies will be needed to test this hypothesis. Furthermore, our studies indicate that the functionality of this focal adhesion-targeting unit is influenced by the presence of mutated LIM domains. Indeed, the focal adhesion targeting capacity of the proline-rich region as a

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Targeting of LPP to Focal Adhesions and to the Nucleus

The amino acid sequence of the LPP protein does not contain any consensus nuclear localization signals suggesting that it may be imported into the nucleus via an interaction with a nuclear localization signal containing transport protein (5). Efforts to induce nuclear accumulation in fibroblasts of zyxin (14) or TRIP6 (40) by manipulation of tissue culture conditions have been unsuccessful, however. Similar experiments, performed by us, with LPP also gave no results. In our studies, we showed that the LIM domains of LPP are dispensable for its nuclear targeting. These results are similar to those found for zyxin (14) and TRIP6 (40) but are unlike those observed for ajuba for which it was shown that the LIM domains are necessary for nuclear accumulation (41).

The LPP Nuclear Targeting Capacity—LPP shuttles between the cytoplasm and the nucleus and its nucleocytoplasmic distribution is in part regulated by a nuclear export signal (5). According to recent literature, the regulation of the nucleocytoplasmic distribution of LPP appears to be different from that of zyxin. During treatment of HeLa cells with the nuclear export inhibitor leptomycin B, accumulation of GFP-zyxin within cell nuclei occurs with kinetics that vary from cell to cell (14) while the kinetics of the nuclear accumulation of GFP-LPP in these cells is similar in every cell (5). Accumulation of TRIP6 in the nucleus of chicken embryo fibroblasts upon treatment with leptomycin B also occurs with similar kinetics in most cells (40).

Since LPP and its family members shuttle between the cytoplasm and the nucleus, the question arises as to how these molecules are imported into the nucleus. For ajuba, it was shown that induction of endodermal differentiation of P19 embryonal carcinoma cells by means of all-trans retinoic acid resulted in nuclear accumulation of ajuba in these cells (41). Efforts to induce nuclear accumulation in fibroblasts of zyxin (14) or TRIP6 (40) by manipulation of tissue culture conditions have been unsuccessful, however. Similar experiments, performed by us, with LPP also gave no results. In our studies, we showed that the LIM domains of LPP are dispensable for its nuclear targeting. These results are similar to those found for zyxin (14) and TRIP6 (40) but are unlike those observed for ajuba for which it was shown that the LIM domains are necessary for nuclear accumulation (41).

The amino acid sequence of the LPP protein does not contain any consensus nuclear localization signals suggesting that it may be imported into the nucleus via an interaction with a nuclear localization signal containing transport protein (5). In an effort to map the interaction site with this transport protein or to map any unconventional nuclear localization sequences in the LPP protein, the protein sequence was divided into shorter sequences, attempting to conserve known functional domains, and investigated the nuclear targeting capacity of these portions by expressing these as GFP-LPP-β-gal fusion proteins. However, this approach did not give any positive results. Our β-galactosidase fusion proteins were not translocated into the nucleus, either by the LPP LIM domains or by its proline-rich region containing a deletion of the NES. These findings differ from those obtained for TRIP6 whose LIM domains and proline-rich region are able to target β-galactosidase to the nucleus in chicken embryo fibroblasts (40). It is possible that the nuclear targeting in LPP is indeed different from that of TRIP6. However, another explanation for these differences might be that in our case the GFP-β-gal fusion proteins were made in such a way that GFP was fused to the N terminus and β-galactosidase to the C terminus of the LPP-portion (or vice versa). In this way, both LPP termini are blocked by external sequences and this may affect functioning due to steric interference or incorrect folding. This might also be the reason why our GFP-LPP-β-gal fusion protein containing full-length LPP is able to localize at focal adhesions, indicating that the LPP entity is functioning, but cannot be translocated to the nucleus anymore. Our studies have provided us with interesting tools for future experiments to obtain more insight in the physiological role of LPP, e.g., we can express an LPP molecule in cells that is still able to localize in focal adhesions (the full-length and the LIM domain versions) but has lost its ability to be translocated into the nucleus.

The LIM Domains of LPP Can Deplete Endogenous LPP and Vinculin from Focal Adhesions—We have shown that the LIM domains of LPP were able to compete for the subcellular localization sites of LPP thereby interfering with the subcellular distribution of endogenous LPP. We also showed that overexpression of these LIM domains could deplete endogenous vinculin from focal adhesions. Our results are similar but not identical to those obtained for zyxin (14). High level expression of the zyxin LIM domains was shown to deplete endogenous zyxin from focal adhesions; however, vinculin retained a normal focal adhesion distribution in the majority of cells (14).

There are several possibilities why the LIM domains of LPP are able to deplete the endogenous protein from focal adhesions. From our results we know that the LPP LIM domains have a strong focal adhesion targeting capacity that is comparable to that of the wild-type protein. This suggests that the endogenous LPP protein and the ectopic LIM domains compete for the same binding site(s) in the focal adhesions. However, because of their overexpression, the LIM domains apparently win this competition thereby depleting the endogenous LPP from these sites and as such populating the focal adhesions with LPP LIM domains uncoupled from the proline-rich region. In this regard, it is noteworthy that only in cells expressing high levels of the LPP LIM domains an obvious redistribution of endogenous LPP molecules was observed whereas in cells expressing lower levels of the LIM domains such a redistribution of LPP molecules was not detectable. On the other hand, it is possible that the LIM domains of LPP, when highly overexpressed in cells, recruit binding partners of these LIM domains, and as such the LPP, to the cytoplasm. In this way, focal adhesions would be depleted from the LPP LIM domain binding partner repertoire, and, because these LIM domains are LPP’s main focal adhesion targeting units, this would mean that LPP’s focal adhesion targeting is disturbed. In this regard, it is interesting to note that in cells that express high levels of the LIM domains of LPP, focal adhesion targeting of these LIM domains is diminished as compared with cells with lower expression levels.

In conclusion, by using different strategies, we have identified several regions in the LPP protein that have a function in...
Targeting of LPP to Focal Adhesions and to the Nucleus

targeting this protein to focal adhesions, stress fibers, and the nucleus. In addition, we show that the LIM domains of LPP can compete for the subcellular localization sites of LPP interfering with the molecular composition of focal adhesions.

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The Focal Adhesion and Nuclear Targeting Capacity of the LIM-containing Lipoma-preferred Partner (LPP) Protein
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