LPP, a LIM protein highly expressed in smooth muscle

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Gorenne, Isabelle, Robert K. Nakamoto, Clayton P. Phelps, Mary C. Beckerle, Avril V. Somlyo, and Andrew P. Somlyo. LPP, a LIM protein highly expressed in smooth muscle. Am J Physiol Cell Physiol 285: C674–C685, 2003. First published May 21, 2003; 10.1152/ajpcell.00608.2002.—An 80-kDa protein, prominently expressed in smooth muscle, was microsequenced and identified as LPP, the product of the lipoma-preferred partner gene (Petit MMR, Mols R, Schoenmakers EFPM, Mandahl N, and Van de Ven WJM. Genomics 36: 118–129, 1996). Using a specific anti-LPP antibody, we showed, in Western blots and with immunofluorescence microscopy, the selective expression of LPP in vascular and visceral smooth muscles (~0.5–1 ng/μg total protein). In other mature (noncultured) tissues, including heart and skeletal muscle, the protein is present only in trace amounts and is closely correlated with the levels of the smooth muscle marker α-actin. In freshly isolated guinea pig bladder smooth muscle cells, immunofluorescence images showed LPP as linear arrays of punctate, longitudinally oriented staining superimposed with vinculin staining on the plasma membrane surface. A corresponding pattern of periodic labeling at the membrane in transverse sections of bladder smooth muscle suggested an association of LPP with peripheral dense bodies. In cultured rat aortic smooth muscle cells, LPP colocalized with vinculin at focal adhesions but not with p120 catenin or α-actinin. Overexpression of the protein increased EGF-stimulated migration of vascular smooth muscle cells in Transwell assays, suggesting the participation of LPP in cell motility. The Rho-kinase inhibitor Y-27632 dissociated focal adhesions and LPP staining at the cell periphery and enhanced the nuclear accumulation of LPP induced by leptomycin B, indicating that LPP has a potential for relocating to the nucleus through a shuttling mechanism that is sensitive to inhibition of Rho-kinase.

LIM protein; dense plaque; Rho-kinase; nuclear transport; cell migration

WE REPORT THE UNEXPECTED FINDING of a protein that, among adult tissues, is predominantly expressed in smooth muscles and was initially detected using an antibody generated against an unrelated epitope. The protein, identified as LPP, the product of the lipoma-preferred partner gene (21), is a member of a subfamily of LIM proteins that have in common repeats of COOH-terminally located LIM domains (5). Two related proteins, zyxin (15) and thyroid receptor-interacting protein 6 (TRIP6) (42), share the highest overall sequence homology with LPP, 41 and 53%, respectively. LIM domains, which contain two zinc finger motifs, are described as protein-protein interaction domains with broad specificity (28). The LPP sequence also contains NH2-terminal polyproline motifs that are consensus sequences for EVH1 (Ena/VASP homology) domains thought to be responsible for binding the vasodilator-stimulated phosphoprotein (VASP), a protein associated with actin filaments and focal adhesions (22). In addition, LPP possesses a nuclear export sequence (NES) that, when deleted or when the nuclear export is inhibited, is responsible for the accumulation of LPP in the nucleus (20). Until now, on the basis of RNA detection, LPP was believed to be ubiquitously expressed with no definite function attributed to it, although it was reported to have transcriptional activity in an in vitro GAL4 promoter transactivation assay (20). Our new finding that, at the protein level, LPP is selectively expressed in smooth muscle, combined with its cellular localization at the level of dense bodies in mature cells, suggests that it may have a role in forming and/or regulating the cytoskeletal architecture. In addition, we show that a Rho-kinase inhibitor enhances nuclear accumulation of LPP in aortic smooth muscle cells in which the CRM1-dependent nuclear export system is blocked. We also report that overexpression of LPP in cultured vascular smooth muscle cells increased their migration in a Transwell assay, implicating its potential for regulating cell motility. Preliminary abstracts of some of these findings have been presented (9, 10).

MATERIALS AND METHODS

Preparation of tissue extracts. Tissues were obtained from male New Zealand White rabbits and Hartley guinea pigs. The animals were euthanized by an overdose of halothane and rapid exsanguinations following protocols approved by the University of Virginia Animal Experimentation Committee. For tissue screening, pieces of different organs were removed, weighed, frozen in liquid nitrogen, and kept at −80°C until used. The tissues were homogenized in a buffer containing 1% sodium dodecyl sulfate (SDS), 20 mM Tris, pH 7.5, and 50 mM 2-β-mercaptoethanol and centrifuged at 14,000g for 15 min, and the supernatant was kept at −80°C.

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For confirmation of the LPP identity by protein sequencing, the protein was partially purified from bladder. Rabbit bladders were homogenized in a buffer containing 50 mM Tris, pH 8.0, 100 mM NaCl, 2 mM EDTA, 2 mM MgCl₂, 50 mM β-mercaptoethanol, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF; Calbiochem, La Jolla, CA), 1 mM benzamidine, and 1 μM pepstatin and then centrifuged for 30 min at 5,000 g at 4°C. The supernatant was ultracentrifuged for 45 min at 45,000 g (4°C). The p80 protein was precipitated from the supernatant by ammonium sulfate with a cut off of 15–30%. After resuspension of the pellet in a 50 mM 2-(N-morpholino)ethanesulfonic acid (MES), pH 6.8, and 1 mM dithiothreitol (DTT) buffer, the protein was enriched by passage on a SOURCE 30S cation exchange column (Pharmacia Biotech, Uppsala, Sweden). The protein, detected by Western blot analysis, was found in fractions eluted between 0.2 and 0.4 M NaCl. These fractions were pooled, and proteins were precipitated with 1% trichloroacetic acid (TCA). The precipitated proteins were resuspended in a solution containing 8 M urea, 1% NP-40, and 1 mM DTT for loading on two-dimensional (2-D) gels. The same protocol was used for extraction of proteins from cardiac tissues. The protein concentration in samples was measured using the Bradford (Bio-Rad, Hercules, CA) and bicinchoninic acid (BCA; Pierce, Rockford, IL) assays. At the dilution used, buffers did not interfere with the protein assays. In some experiments, aortic smooth muscle tissues (10-mg strips) were homogenized on ice by using glass-glass homogenizers in a buffer consisting of 10 mM Tris-Cl, pH 8.0, 5 mM EDTA, 2% glycerol, 10% β-mercaptoethanol, 10% glycerol, 1 mM AEBSF, and 1 mM benzamidine and then centrifuged at 14,000 g for 45 min. The recombinant proteins were resuspended from the inclusion bodies in the pellet in a solution containing 8 M urea and 50 mM 2-mercaptoethanol. After dilution of the sample to achieve a 2-mercaptoethanol concentration of <10 mM, the recombinant protein was purified on a nickel-NTA Superflow column (Qiagen, Valencia, CA) as described by the manufacturer for purification of proteins in denaturing conditions. The purity of the protein was calculated to be 91% of total proteins.

Plasmids expressing human LPP and transfections. The full-length LPP and enhanced green fluorescent protein (EGFP) was prepared by inserting the full sequence of LPP into the EcoRI/XhoI restriction sites of the expression vector pEGFP-C2 (BD Clontech). Human iliac vein smooth muscle cells (HIVS-125) were transfected using the optimized protocol for electroporation of human aortic smooth muscle cells with the use of the Nucleofector apparatus (Amaxa, Cologne, Germany). Seventy percent confluent cells (below 10th passage) were trypsinized, centrifuged, and resuspended in 100 μl of solution (AoSMC Nucleofector solution) and then electroporated in the presence of 5 μg of plasmid by using the program U25. The cells were seeded on gelatin-coated plastic dish and used after 24 h for migration assay.

Quantitation of LPP in smooth muscle. Rabbit portal vein, ileum longitudinal smooth muscle, and abdominal aortic segments were dissected, freed of connective tissue, weighed, and homogenized in SDS buffer (20 μl/mg tissue wet wt) as described in Preparation of tissue extracts. Serial dilutions of each homogenate were used for quantitation over the linear range of the antibody signal on Western blots, utilizing as standards known quantities of purified recombinant LPP transferred to the same PVDF membranes. The protein signals were quantitated by densitometry using an Epson Ex-
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pression 1,600 densitometer and analyzed with NIH Image 1.62 software.

**Cultured cells and dissociated cells.** Smooth muscle cells from rat thoracic aorta (gift of Dr. G. K. Owens, University of Virginia) were isolated and cultured as previously described (32). The cells, used at the 10th-26th passage, were seeded on coverslips and cultured until subconfluent in Dulbecco's modified Eagle's medium-F12 (Life Technologies) containing 10% fetal calf serum (HyClone, Logan, UT), 100 U/ml penicillin, 100 µg/ml streptomycin (Life Technologies) and supplemented with 0.68 mM L-glutamine (Sigma). HIVS-125 cells were purchased from the American Type Culture Collection (ATCC) and cultivated in Ham's F-12K medium with 10% fetal bovine serum and 2 mM L-glutamine and complemented with 50 µg/ml heparin and 0.03 mg/ml endothelial cell growth supplement (ECGS). ECGS and heparin have been shown to help maintain the proliferation of HIVS-125 cells up to 10–12 passages, a stage at which the cells' doubling time starts to increase noticeably (37).

Guinea pig bladder smooth muscle cells were isolated as previously described (31), except that papain (1 mg/ml; Sigma) and type II collagenase (1 mg/ml; Sigma) were used to disperse the cells. The cell suspensions were washed by centrifugation to remove the enzymes, and then calcium was reintroduced by the addition of CaCl2 in four incremental steps to recover a concentration of 1.2 mM CaCl2. The freshly dispersed guinea pig bladder cells were spun onto slides for 3 min at 900 g using a cytopsin centrifuge (Thermo Shandon, Pittsburgh, CA) and immediately fixed for immunofluorescence staining.

**Indirect immunofluorescence.** The guinea pig tissues were prepared for cryosectioning as previously described (13). Tissues were fixed for 1 h at room temperature or overnight at 4°C in 3% paraformaldehyde in 10 mM PBS, pH 7.4, and cryoprotected in 5% sucrose-PBS for 60 min followed by 15% sucrose-PBS for another 60 min before being frozen in liquid N2-cooled Freon-22. Sections (7–8 µm) were cut from tissues embedded in Tissue-Tek OCT compound (Miles, Elkhart, IN), permeabilized for 10 min with 0.3% Triton X-100 in PBS, rinsed, and then blocked for 1 h in 1% BSA-PBS at room temperature. Cryosections were labeled with the rabbit polyclonal LPP (318–332) antibody (dilution 1:2,000), anti-vinculin (1:500, Sigma), or anti-α-actinin (1:300; Sigma). After three washings in PBS, a rhodamine red-X-conjugated goat anti-rabbit antibody (1:1,000; Molecular Probes, Eugene, OR) was used at 1:1,000 for guinea-pig cells, anti-vinculin antibody (1:500, Sigma), anti-p120 catenin (1:200, Transduction Labs, Lexington, KY), or anti-α-actinin (1:300, Sigma) diluted in blocking solution for 2 h at room temperature or overnight at 4°C. The secondary antibodies used were a rhodamine red-X-conjugated goat anti-rabbit IgG (1:200; Molecular Probes), a rhodamine (TRITC)-F(ab’)2 goat anti-mouse IgG (1:250; Jackson ImmunoResearch, West Grove, PA), or a FITC-goat anti-rabbit IgG (1:200; Molecular Probes) diluted in blocking solution. Some cells were labeled for 1 h with rhodamine-phalloidin (1:1,000; Molecular Probes). The cells were washed four times for 5 min with PBS before being mounted with Aqua Poly/Mount.

**Cell migration assay.** The migration assay was performed using Transwell culture inserts with polyethylene terephthalate membranes (8-µm pores, Falcon; Becton Dickinson Labware, Franklin Lakes, NJ). Twenty-four hours posttransfection with LPP or control vectors, the human iliac vein smooth muscle cells were harvested with trypsin-EDTA, resuspended in serum-free medium, counted, and distributed at a density of 5 × 104 cells in the inserts or seeded in separate wells for Western blot analysis or detection of EGFP fluorescence. Each determination was performed in triplicate. The cells were allowed to settle for 1 h before the addition of EGF (10 nM) in the lower chamber and then allowed to migrate to the underside of the insert's membrane for 4 h at 37°C, 5% CO2. At the end of the experiment, the cells were fixed in methanol. Cells on the bottom of the insert were mechanically removed with a cotton swab. Cells that migrated to the lower surface of the membrane were stained with crystal violet and counted from three different fields with a ×20 objective.

**RESULTS**

**Identification of the 80-kDa protein.** In a screen for RhoA guanine nucleotide exchange factors, an antibody directed against Vav2 detected a protein in a variety of rabbit smooth muscles but not in other tissues (Fig. 1A). The migration of the protein at 80 kDa in SDS-PAGE was slightly faster than expected for Vav2 (95 kDa), and its distribution was inconsistent with the reported ubiquitous distribution of Vav2, highest in spleen and liver (29). The 80-kDa protein was concentrated from rabbit bladder homogenates and focused by 2-D gel electrophoresis. Figure 1B shows a representative Western blot of the partially purified protein detected with the Vav2 antibody (left) and the silver-stained gel obtained from the same sample (right). The protein is composed of at least three major resolved spots. The number of spots labeled with the LPP antibody could be reduced by treatment with alkaline phosphatase, suggesting that at least some of them represent phosphorylation-induced shifts (data not shown). Six peptides, generated by digestion of spot 1 and microsequenced, were 100% identical to sequences of the human LPP protein. The protein identity was also confirmed by sequencing spot 3 from rabbit ileum. LPP is a 66-kDa protein (despite its apparent migration at 80 kDa) that surprisingly shares no significant sequence similarity with Vav2. Several regions of LPP have high homology with the protein zyxin. We confirmed, using the B71 antibody specific for zyxin and the B38 antibody, which recognizes both zyxin and LPP, that the identified protein was distinct from zyxin (Fig. 1, C and D). A new LPP antibody, made against a peptide from the LPP sequence (318–332), recognized the same spots as the Vav2 antibody in 2-D gels (not shown) and did not recognize zyxin (Fig. 1C). In addition, when LPP was
immunoprecipitated using the specific LPP antibody, the 80-kDa band was also recognized by the Vav2 antibody (data not shown).

**LPP is highly expressed in smooth muscle tissues.** Western blotting with the specific LPP (318–332) antibody revealed that LPP was present at high levels in uterine, corpus cavernosum, portal vein, stomach, aorta, bladder, and ileum tissues, which are composed of a large proportion of smooth muscle cells (Fig. 2A). The same tissues also gave a strong signal when blotted for the smooth muscle-specific isoform of α-actin, whereas β-actin was found to be widely distributed. The absence of β-actin signal in skeletal muscle suggests that the antibody used did not recognize the β-actin isoform expressed in these cells, because Coomassie staining of the gels showed a similar amount of total protein, as well as strong actin and myosin staining. In given that lung, a faint band for LPP was detected but likely represents LPP from smooth muscle in blood vessels and bronchi in this tissue, because LPP was detected in pulmonary vessels and bronchi, whereas parenchyma, when dissected from the most peripheral part of the lung, did not show a strong signal (Fig. 2B). In contrast to smooth muscle, in tissues such as skeletal muscle, heart, liver, kidney, brain, and spleen, LPP was detected only with longer exposures of the PVDF membranes to film, and these low signals were also associated with stronger labeling of the smooth muscle α-actin (data not shown). Comparison of the LPP contents of bladder and heart, after concentration of the protein, showed a much higher level of LPP in bladder than heart (Fig. 2C).

The amounts of LPP in rabbit ileum longitudinal smooth muscle, abdominal aorta, and portal vein were...
determined by quantitative Western blotting, using serial dilutions of known concentrations of purified recombinant human (His)_6-LPP protein to generate a standard curve. Both the Vav2 antibody and the rabbit polyclonal anti-LPP (318–332) recognized the recombinant (His)_6-LPP. Preliminary experiments verified that LPP of human and rabbit origin were recognized with similar affinity by our LPP antibody (data not shown). The amounts of LPP in tissues, interpolated from the standard, were 0.89 ng/μg tissue proteins in ileum longitudinal muscle, 1.09 ng/μg in aortic smooth muscle, and 0.45 ng/μg in portal vein. The estimated cellular concentrations, calculated on the assumption that 80% of tissue weight is water and the recombinant (His)_6-LPP molecular weight is 66.7 kDa, were 3.4, 4.1, and 1.7 μM, for ileum, aorta, and portal vein, respectively. In addition, tissue LPP was ~8-fold greater than in the smooth muscle HIVS-125 cells and 100-fold greater than LPP content in nonmuscle cells such as NIH/3T3 cells or human umbilical vein endothelial cells (HUVEC; data not shown).

**Cellular distribution of LPP.** Aortic tissue homogenates were separated into pellet and supernatant fractions under varying NaCl concentrations (Fig. 3). At salt concentrations >150 mM, LPP moved from the 800 g pellet to the supernatant fraction. The cytosolic protein Rho-GDI was only found in the supernatant fraction, indicative of little supernatant contamination of the pellet fraction, whereas the SRF, located in nuclei, was not extracted from the pellet by 300 mM salt concentrations. Vinculin and actin were found in both fractions, and their distribution was independent of salt concentration. This experiment shows that LPP binds weakly to a structure or cytoskeletal element sedimenting in the 800 g pellet in tissue homogenate and is dissociated by salt concentrations >200 mM, presumably indicative of electrostatic binding to a pellet protein.

**Localization of LPP by immunofluorescence in tissues and isolated cells.** Immunofluorescence microscopy on guinea pig ileum, heart, kidney, and bladder sections was performed using our custom-made anti-LPP antibody (318–332), which showed monospecific reactivity in immunoblots for these tissues. Guinea pig tissues were used because the secondary antibody gave a much lower background staining than in rabbit tissues used for the biochemical experiments. As shown in Fig. 4A, LPP distribution in ileum is mainly associated with the longitudinal and circular layers of smooth muscle cells. In the mucosal layer, low-grade staining was observed, which was partly due to nonspecific staining caused by the secondary antibody. Further experiments are necessary to clarify whether the slight labeling associated with the epithelial layer lining the villi is a specific signal for LPP. In bladder sections, LPP staining was strongly associated with smooth muscle cells (Fig. 4D) and, as in heart or kidney, was present in blood vessels (Fig. 4, B–D). In transverse sections of bladder smooth muscle bundles, confocal microscopy revealed discontinuous staining localized at the periphery of the cells (Fig. 5A). The cytosolic compartments showed weak homogenous staining. Immunofluorescence patterns of vinculin and LPP in cross sections of muscle cells in the tissue showed strong overlap (data not shown). In freshly isolated bladder cells, surface scanning with confocal microscopy showed discontinuous LPP staining in a riblike pattern (Fig. 5, B and C) that resembles the localization of vinculin at dense plaques or dense bodies. Different optical sections of the cell also showed that LPP is mostly associated with the cell membrane, because intracellular space presented much weaker staining (Fig. 5D). Dual staining of LPP and vinculin also showed very strong overlap in the cellular distribution of these proteins in both tissue sections (Fig. 6, A–C) and isolated cells (Fig. 6, G–I). In these images, vinculin staining was strictly associated with the plasma membrane. The peripheral pattern of LPP staining was significantly different than that of α-actinin, which showed extensive intracellular dotlike labeling (Fig. 6, D–F and J–L).

Our anti-LPP (318–332) antibody has low affinity for cells of rat origin; therefore, we used an anti-LPP antibody directed against the NH2-terminal portion (1–109) of the protein (immunoGlobe) for labeling of the cultured rat aortic cells. Staining of LPP in cultured smooth muscle cells showed no colocalization with the cadherin-associated armadillo protein p120 catenin (Fig. 7, G–I), which is concentrated at cell-cell adhesions, or with α-actinin, known to associate with stress fibers (Fig. 7, J–L). In contrast, LPP was detected at the end of stress fibers at the level of focal adhesions in cultured smooth muscle cells (Fig. 7, F and L).
adhesions (Fig. 7, D–F), showing complete overlap with vinculin labeling (Fig. 7, A–C). 

**Rho-kinase inhibition increases leptomycin B-induced LPP nuclear localization.** To determine the effects of Rho-kinase inhibition on LPP localization by immunofluorescence, we treated the cells with 10 μM of the Rho-kinase inhibitor Y-27632 for 2 h before stimulation with leptomycin B or vehicle for an additional 3 h. Rat aortic cells treated with Y-27632 (10 μM) exhibited typical changes in cell shape, with disappearance of vinculin-stained focal adhesions and stress fibers (data not shown), as well as the appearance of cell processes (Fig. 8E). Concomitantly with the decrease in vinculin immunofluorescence signal, staining for LPP was decreased at focal adhesions and slightly increased in the cytosol (Fig. 8E). Although some nuclei were occasionally stained with anti-LPP antibody in control cells, as shown in Fig. 8, A and B, incubation of the cells for 3 h with the irreversible inhibitor of nuclear export, leptomycin B, increased the number of cells containing stained nuclei (Fig. 8F), as observed previously for zyxin (17). Nuclei of cells pretreated for 2 h with Y-27632 before the addition of leptomycin B showed much higher intensity and frequency of LPP labeling than those in cells treated with leptomycin B alone (Fig. 8G). The total cellular amount of LPP after treatment with Y-27632, leptomycin B, or both combined was not altered over the time course of the experiments (Fig. 8C).

**Effects of LPP overexpression on smooth muscle cells migration.** HIVS-125 cells are human iliac smooth muscle cells that are reported to express smooth muscle specific markers (37). We used these cells for the migration experiments because their phenotype can be modulated according to the culture conditions. The amount of LPP protein in HIVS-125 cultured cells was eightfold lower than in adult rabbit aorta when GAPDH was taken as reference (data not shown). In the presence of ECGS in the medium (still in the presence of 10% serum), the cells showed a reduction of LPP total content (~40%) as well as a reduction in cellular smooth muscle α-actin, as previously described (37). Transfection of human iliac vein smooth muscle cells with EGFP-human LPP fusion protein or EGFP...
alone was performed by electroporation using an optimized protocol for human smooth muscle aortic cells (Amaxa). The levels of transfection for HIVS-125 cells were similar for both vectors and were commonly found between 50 and 80% of total cells as assessed by counting of fluorescent cells. The overall expression of LPP was increased 10-fold compared with the initial endogenous LPP levels (Fig. 9F). In addition, the EGFP fusion protein did not interfere with LPP binding to its target, because most of the fluorescence was located on the focal adhesions (Fig. 9G). The EGFP- or EGFP-LPP-transfected cells were seeded in Transwells, and the number of cells that migrated in the lower chamber in response to EGF, used as chemoattractant, were counted. Figure 9, A–D, shows representative fields of the membrane after cell fixation and staining. Fluorescence imaging of nonstained cells on the lower surface of the membrane showed that the migrating cells expressed the EGFP constructs (data not shown). The percentage of cells stimulated with EGF that migrated to the lower surface of the membrane was significantly higher (2.5-fold) compared with unstimulated controls (vehicle). In addition, in the presence of EGF, the total number of migrated cells transfected with EGFP-LPP was 100% higher than the number of cells transfected with EGFP alone.

Fig. 6. LPP colocalizes with vinculin but not α-actinin in smooth muscle tissue sections and dissociated cells. Guinea pig bladder smooth muscle tissue cryosections (A–F) and isolated bladder smooth muscle cells (G–L) were doubly stained with antibodies against LPP (A and G) and vinculin (B and H) or antibodies against LPP (D and J) and α-actinin (E and K). Merged images in C and I show strong colocalization of LPP and vinculin at the cell periphery. Double staining with antibodies against LPP and α-actinin shows little overlap (F and L). Most α-actinin staining is found in the cytosol. Bars, 10 μm.
DISCUSSION

The main finding of this study is the recognition of LPP as a smooth muscle specific protein and its location, in tissues, at the level of peripheral dense bodies. Our results also provide the first evidence for a role of LPP in cell motility, given that the overexpression of LPP in smooth muscle cells enhanced migration in a Transwell assay. The cellular localization of LPP can be modulated by Rho-kinase, because a Rho-kinase inhibitor enhanced the nuclear accumulation of LPP induced by the nuclear export inhibitor leptomycin B. We initially detected this protein as a Western blot band recognized in rabbit tissues by an antibody for the guanine nucleotide exchange factor Vav2. However, neither the expression pattern nor the molecular weight of the protein was consistent with previously reported results for Vav2 (29). Mass spectroscopy and Edman sequencing identified this 80-kDa protein as LPP. Despite the apparent specificity of the Vav2 antibody, as indicated by the effects of the antibody-blocking peptide on Western blots, sequence analysis did not reveal any common motif between LPP and Vav2 primary protein sequences.

The LPP gene was initially discovered as a gene whose 3’ region was translocated and fused to the high mobility gene 1C (HMG1C) with a particularly high frequency in lipoma tumors (19, 21). The translocation was later found in other tumor cell types (27). Truncation of the HMG1C protein, rather than the fusion of the three LIM domains of the LPP sequence to HMG1C, is thought to be responsible for the tumor phenotype (1, 6, 27), and no definite function in tumors has yet been attributed to LPP. Of specific interest is the fact that we found the LPP protein highly expressed only in smooth muscle. LPP was detectable in the vascular system as well as other smooth muscle cell types, including intestine, stomach, uterus, bladder, esophagus, and corpus cavernosum smooth muscle. Western blots with a smooth muscle-specific α-actin antibody showed a tight correlation between the presence of LPP and this smooth muscle marker. The differences observed between the expression of the

Fig. 7. LPP is found at the end of stress fibers and colocalizes with vinculin, but not with p120 catenin or α-actinin, in cultured rat aortic cells. Rat cultured aortic cells were labeled with antibodies against LPP (A, D, G, and J) and vinculin (B), with phalloidin (E), or with antibodies against the cell-cell contact protein p120 catenin (H), or α-actinin (K). Merged image in C shows overlap of LPP and vinculin at the level of focal adhesions, as indicated by yellow staining. Merged image in F shows localization of LPP at the end of stress fibers stained with phalloidin. Merged images in I and L show no strong colocalization of LPP with p120 catenin or α-actinin, respectively.
protein reported in our study and the pattern of expression of LPP mRNA in Northern blots (21) may be explained by the presence of blood vessels in organs such as heart, lung, or kidney, as well as the possible lack of correlation between mRNA expression and protein content. Quantitation of LPP in aorta, portal vein, and ileum longitudinal muscle showed that cellular amounts of LPP are \( \sim 0.5–1 \) ng/\( \mu \)g protein, which translates into a cellular concentration of \( \sim 2–4 \) M. This concentration is consistent with the possibility of LPP having a significant role in modulating the structure of the cytoskeleton and its relation to the plasma membrane. LPP was previously found in cultured cell lines such as human foreskin fibroblasts (HFF), colon carcinoma cells (Caco2), or keratinocytes (20); therefore, LPP expression is not strictly limited to smooth muscle. However, besides its high content in smooth muscle tissue (100-fold greater than in nonmuscle cultured cells), LPP content was also eightfold higher in cultured human iliac vein smooth muscle cells (HIVS-125), than in NIH/3T3 fibroblasts or human endothelial cells (HUVECs; data not shown).

The subcellular distribution of LPP was determined in bladder tissue and freshly isolated bladder smooth muscle cells. In cross sections of bladder tissue, the labeling showed punctate staining on the periphery of the smooth muscle cells as previously reported for vinculin (2, 7). Vinculin has been shown to be a component of plasma membrane-associated dense bodies (2) and can form riblike staining on the surface of certain cells such as guinea pig taenia coli (33). Electron micrographs show dense bodies in longitudinal sections of vas deferens muscle with bundles of actin filaments of opposite polarity emerging from either side similar to the Z bands of striated bundles, forming chains of minisarcomeres throughout the cytoplasm and converging toward membrane dense bodies (3). Immunofluorescence microscopy with LPP and vinculin antibodies showed colocalization of these proteins (Fig. 5) and their exclusion from the cytosolic compartment, suggesting that, like vinculin, LPP is largely localized to membrane dense bodies, points of fixation of actin filaments to the membrane. Interestingly, the LIM protein zyxin has also been reported to be present in chicken gizzard dense plaques (4).

LPP colocalized with vinculin at focal adhesions in cultured aortic smooth muscle cells (Fig. 5) and did not colocalize with \( \alpha \)-actinin or the cadherin-binding armadillo p120 catenin (24, 25). \( \alpha \)-Actinin is associated with actin filaments in stress fibers but can also bind numerous cytoskeleton- and membrane-bound proteins, participating in the anchorage of F-actin to the membrane. Contrary to the reported interaction of LPP with \( \alpha \)-actinin (14), we found very little overlap between LPP and \( \alpha \)-actinin distribution in tissues or isolated smooth muscle cells. In smooth muscle, \( \alpha \)-actinin is associated with both peripheral and cytoplasmic dense bodies, whereas vinculin associates only with peripheral dense bodies (3). Our results indicate that LPP is also a predominantly peripheral protein. The light cytosolic immunostaining of LPP did not show a pattern significantly overlapping the \( \alpha \)-actinin
staining. In addition, the fluorescence of overexpressed EGFP-LPP in the smooth muscle cells, as well as other cell types, is not strikingly associated with actin or actin-binding proteins along the stress fibers (present study and Ref. 18). In cultured cells, focal adhesion plaques are sites of recruitment of integrins, mediating cell adhesion and supporting cell motility (12). Dissociation of focal adhesions by inhibitors of the RhoA/Rho-kinase pathway is accompanied by a decrease in cell motility (35, 36), and the dissociation of LPP from focal adhesions in the presence of a Rho-kinase inhibitor (present study) suggests its role as a regulated component of focal complexes. Whereas LPP anchors to these structures through its group of three LIM domains (18), the binding partners responsible for focal adhesion targeting are as yet unknown. Our results show that overexpression of LPP, in vascular smooth muscle cells, enhanced EGF-stimulated migration. The possible binding of LPP to VASP through its NH2-terminal proline-rich consensus sequence (20) may account for LPP effects, because VASP is known to regulate the actin polymerization processes required for cell motility (see review, Ref. 23). It will be of interest to determine the behavior and function of LPP in smooth muscle cells migrating in injured blood vessels and also to determine whether LPP has a signaling function as part of macromolecular complexes at smooth muscle dense plaques (8).

An inhibitor of CRM1-mediated nuclear export, leptomycin B, caused accumulation of LPP in the nucleus, as previously reported for LPP (20), zyxin (17), and TRIP6 (40), suggesting the existence of a normally active LPP shuttling mechanism between nucleus and cytoplasm. Our finding of nuclear LPP in untreated vascular smooth muscle cells in culture suggests that this is a normal physiological process modulated by drugs and, perhaps, other agents. The mechanism of nuclear import of LPP is unknown. The effects of leptomycin B were greatly potentiated by a specific Rho-kinase inhibitor, Y-27632 (Fig. 8). Y-27632 changed cell morphology (39), a previously described effect of RhoA inhibition (26), resulting in a decrease in cell size, the formation of long processes, and inhibition of cell migration (34). The Y-27632-induced disassembly of stress fibers and focal adhesions was accompanied by LPP staining of the cytosolic compartment and at
the termini of the few remaining stress fibers, indicating that Rho-kinase can modulate LPP localization. Leptomycin B alone did not cause dissociation of focal adhesions, suggesting that inhibition of the CRM1 nuclear exporter is sufficient to increase nuclear localization of LPP. The continuous disassembly/reassembly of adhesion proteins in migrating cells (41) suggests that these proteins are periodically disassociated and physiologically available for nuclear transport, as also indicated by the presence of LPP in the nuclei of some untreated cells (present study). Given the relatively large size of LPP, it is unlikely to be driven into the nucleus by mass action and free diffusion. Y-27632 may act by either removing a constitutive Rho-kinase-mediated brake on nuclear import or inhibiting a redundant export mechanism, parallel to CRM1, through a functional consequence of nuclear accumulation of LPP itself, or of a partner protein, by a Rho-kinase dependent mechanism. Considering the functional consequences of nuclear accumulation of LPP, we note that Y-27632 inhibits transcriptional regulation of smooth muscle-specific promoters, an effect tentatively ascribed to nuclear accumulation of G-actin (16). Our present finding of nuclear LPP localization modulated by a Rho-kinase inhibitor raises the possibility that LPP participates in modulating transcriptional regulation of cytoskeletal proteins by Rho-kinase.

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DISCLOSURES

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