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Phosphorylation of mouse LASP-1 on threonine 156 by cAMP- and cGMP-dependent protein kinase

Christian Keicher[a], Stepan Gambaryan[a], Elfriede Schulze[a], Katrin Marcus[b], Helmut E. Meyer[b], Elke Butt[a,*]

[a] *Institute of Clinical Biochemistry and Pathobiochemistry, University of Würzburg, Josef-Schneider-Str. 2, D-97080 Würzburg, Germany*
[b] *Medical Proteome Center, Ruhr University of Bochum, Universitätsstr. 150, D-44780 Bochum, Germany*

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Abstract

LIM and SH3 domain protein (LASP-1) is a specific focal adhesion protein involved in cell migration. Overlay studies demonstrate that LASP-1 directly binds to the proline-rich domains of zyxin, lipoma preferred partner (LPP), and vasodilator-stimulated phosphoprotein (VASP), with zyxin being the most prominent interacting partner. Despite the LIM/zinc-finger domain, hypothesized to be involved in homodimerization, LASP-1 exists as a monomer. In vitro phosphorylation of recombinant mouse LASP-1 by cAMP- and cGMP-dependent protein kinase (PKA and PKG, respectively) occurs at serine 61, serine 99, and threonine 156 whereas in intact cells mouse LASP-1 is phosphorylated only at threonine 156. This site is different from the known in vivo phosphorylation sites in human (serine 146) and rabbit (serine 99 and serine 146). Nevertheless, immunofluorescence of LASP-1 in human and mouse mesangial cells revealed no difference in subcellular distribution. Exposure of the cells to forskolin induced a translocation of both, human and mouse LASP-1, from the focal contacts to the cell interior without affecting F-actin structure. Immunoblotting of LASP-1 in various mouse and human tissues detected a similar prominent expression in non-muscle tissue. Altogether, our data suggest so far no functional differences between human and mouse LASP-1.

Keywords: LASP; cAMP-dependent protein kinase; cGMP-dependent protein kinase; Threonine; Phosphorylation; Mouse; F-actin; Mass spectrometry; Zyxin; LPP; SH3 domain; Proline rich domain; Platelets; Dimerization; 2-Dimensional gel electrophoresis; Mesangial cells

Lim and SH3 domain protein (LASP-1) was previously identified in a cDNA library of breast cancer metastasis and the gene was mapped to the q21 region of the long arm of chromosome 17 [1,2]. Human LASP-1 encodes a protein of 261 amino acids containing an N-terminal LIM domain, followed by two actin binding sites and a C-terminal src homology SH3 domain. In general, LIM domains are a specialized double zinc-finger motif interacting with many different proteins in association with the cytoskeleton and even form homeodomains to become nuclear [3]. The actin binding domain in the core of the LASP-1 protein is in favor of an interaction between LASP-1 and the actin cytoskeleton at the site of cell membrane extensions, but not along the actin stress fibers [4]. The SH3 domain at the C-terminus is involved in protein–protein interactions through binding to proline-rich sequences. Although the specific cellular functions of LASP-1 have not been defined, the protein-protein interactions mediated by the LIM and SH3 domains can be regarded as scaffolds for the formation of higher order complexes. In this regard, we will show here that LASP-1 binds to zyxin, a proline-rich protein detected primarily in the focal adhesion plaques at steady state [5]. In parietal cells, LASP-1 interacts with the proline–arginine rich domain of dynamin, a large GTPase that regulates the fission of vesicles from the plasma membrane [6]. In addition,

* Corresponding author. Fax: +49 931 201 45153.
E-mail address: butt@klin-biochem.uni-wuerzburg.de (E. Butt).

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LASP-1 is identified as a cAMP-depending signaling protein. In rabbit parietal cells, elevation of intracellular cAMP induces a partial translocation of LASP-1 to the apically directed F-actin rich intracellular canaliculus, which is the site of active HCl secretion [7,8]. Recently, the phosphorylation sites of human and rabbit LASP-1 were characterized. While in rabbit the major PKA phosphorylation sites are Ser-99 and Ser-146 [10] in human LASP-1 only Ser-146 was identified as a specific phosphorylation site for PKA and PKG [9]. Phosphorylation of LASP-1 increased the K_d and decreased the B_{max} for LASP-1 binding to F-actin [9,10]. Moreover, phosphorylation resulted in a translocation of the protein from the membrane to the cytosol and is associated with a reduced migration of the cells [9]. Theoretical sequence analysis of human, rabbit, rat, and mouse LASP-1 revealed different potential phosphorylation sites. Ser-146 is only found in human and rabbit, whereas the corresponding amino acid in mouse and rat is an alanine. In contrast, Ser-99 and Ser-61 are conserved in all four species. Phosphorylation of rabbit LASP-1 at Ser-146 induced a M_r band shift that is absent in human LASP-1 phosphorylated by PKA and PKG, indicating differences in the structure of the two proteins [9,10].

Concerning the mouse protein not very much is known. Murine LASP-1 was mapped to the C–D region of chromosome 11. The gene was found to be expressed during mouse embryonic development from 7.5 to 17.5 dpc and was detected in all mice adult tissues tested [11].

Since genetically engineered mice are used increasingly to investigate the function of target proteins, we extended our studies on LASP-1 including the mouse protein. In the course of these experiments our results suggest that human and mouse LASP-1 behave similar despite their differences in the sequence and phosphorylation pattern.

Materials and methods

Materials. Urea (ultrapure), [γ-^{32}P]ATP, [^{32}P]orthophoshate (HCl-free), protein A–Sepharose beads, Sephadex G-25, thrombin, IPTG, glutathione, HiTrap-NHS-activated HP column, pGEX4T1, and the ECL detection kit were purchased from Amersham Biosciences (Freiburg, Germany).

Actin was obtained from Cytoskeleton (Denver, USA), ethylene glycol bis(succinimidylsuccinate) was from Molecular Probes (Leiden, The Netherlands), and VASP antibody 16C2 was purchased from Nanotools (Freiburg, Germany). Actin was obtained from Cytoskeleton (Denver, USA), Calyculin A was from Calbiochem (Bad Soden, Germany), trypsin was from Promega (Heidelberg, Germany), 8pCPT-cGMP was from BioLog (Bremen, Germany), IPG strips, goat anti-rabbit IgG, and non-fat dried milk were from Bio-Rad (Munich, Germany), Complete Mini and the rapid ligation kit were from Roche (Mannheim, Germany), and nitrocellulose membrane was obtained from Schleicher and Schuell (Kassel, Germany). Primers were ordered from MWG Biotech (Ebersberg, Germany), restriction enzymes were from New England Biolabs (Frankfurt, Germany), the first strand cDNA synthesis kit was from MBI Fermentas (St. Leon-Rot, Germany), Lipofectamine, TA-vector, and pcDNA3 were from Invitrogen (Groningen, The Netherlands), xL1-blue competent cells were from Stratagene (Amsterdam, The Netherlands), Cy3 and Cy2 were from Dianova (Hamburg, Germany), and Dulbecco's modified Eagle's medium was from Life Technologies (Karlsruhe, Germany).

All other chemicals, reagents, and solvents of the highest purity available and anti-vinculin were purchased from Sigma (Deisenhofen, Germany).

Catalytic subunit of PKA type II was purified from bovine heart [12]. PKG Iβ was expressed in and purified from the baculovirus-Sf9 cell system [13].

LPP, VASP, and zyxin were a kind gift from Dr. Reinhard, Immunoglobe (Himmelstadt, Germany).

Molecular cloning of mouse LASP-1 and mouse LASP-1 mutants. Gene-specific primers used for PCR amplification of mouse LASP-1 and mouse LASP-1 mutants were designed based on the published mouse cDNA sequence (GenBank Accession No. X96973). Oligonucleotides used to generate wild-type LASP-1 include: AATGGATCC ATGAACCCTAACTGTGCCCGGTG (sense, starting at position 65) and CGGGAATTCTCAGATGGCCTCCACGTAGTTG (antisense, starting at position 835) with *Bam*HI and *Eco*RI restriction sites (underlined). Full length mouse LASP-1 cDNA was cloned into the *Bam*HI/*Eco*RI sites of pGEX4T1 to generate a glutathione *S*-transferase mouse (GST)-LASP-1 fusion protein. The single, double, and triple mutants were made by performing a second round of PCR using the wild-type and single or double mutant as a template, respectively, and the appropriate pairs of oligonucleotides. For eukaryotic expression, LASP-1 was cloned into pcDNA3. All constructs were confirmed by DNA sequence analysis.

Expression of GST-LASP-1 fusion proteins. Recombinant mouse LASP-1 and mouse LASP-1 mutants S61A, S99A, T156A, T156E, S61/99A, and S61/99A-T156E were expressed in *Escherichia coli* as GST fusion proteins using pGEX-4T1. Expression and purification of the GST fusion proteins were performed according to the manufacturer's protocol. Removal of GST from LASP-1 was achieved by digestion with immobilized thrombin for 2 h at RT. Purity was analyzed by examination of Coomassie-stained SDS–polyacrylamide gels. Human LASP-1 was expressed and purified as previously described [9].

In vitro phosphorylation of mouse and human LASP-1. Mouse LASP-1 and its mutants S61A, S99A, S61/99A, and S61/99A-T156E (0.5 μM each) were incubated at 30 °C in a total volume of 20 μl with 10 mM Hepes, pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA, 0.2 mM dithiothreitol, and the C subunit of PKA or PKG Iβ (0.05 μM each) and 5 μM cGMP. Reactions were started by the addition of 50 μM ATP containing 0.5 μCi [γ-^{32}P]ATP and terminated after 30 min or at the times indicated in the figures by the addition of 10 μl Laemmli SDS stop solution. Proteins were separated by SDS–PAGE on 10% gels. Incorporation of ^{32}P was visualized by autoradiography.

For overlay experiments radioactive ATP was removed by spinning the human LASP-1 preparation through a Sephadex G-25 column.

Eukaryotic expression and in vivo phosphorylation of mouse LASP-1. PTK2 cells were grown in DMEM in 6-well plates to about 70% confluency and then transiently transfected with the mouse LASP-1 constructs cloned into a pcDNA3 vector alone or together with pCMV-PKGIβ [14] using metafectene. After 48 h, cells were washed once with phosphate-free DMEM and labeled with [^{32}P]orthophosphate for 1 h at 37 °C. Then cells were exposed to buffer alone and either 5 μM forskolin or 50 μM 8pCPT-cGMP for 20 min. After washing with ice-cold PBS, the cells were scraped into RIPA buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 10% sodium pyrophosphate (all w/v %), 10 mM EDTA, 10 mM NaF, 100 U/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM benzamidine, 10 nM calyculin A, and 0.5 mM PMSF). Immunoprecipitation was performed with 0.3 mg of proteins. Lysates were incubated with 1 μl antibody (1.6 μg) for 2 h at 4 °C and then 1.5 h with pre-equilibrated protein A–Sepharose. Bound immune complex was washed three times with ice-cold PBS. The pel-

lets were resuspended in 30 μl Laemmli SDS stop solution. Proteins were separated by SDS–PAGE on 10% gels. Incorporation of ^{32}P was visualized by autoradiography.

Isolation of human platelets. Freshly donated blood from healthy volunteers (50 ml) was collected in acid-citrate dextrose and centrifuged for 10 min at 300*g* to yield platelet-rich plasma (PRP). PRP was centrifuged for 20 min at 500*g* and the platelet pellet was resuspended and washed once in an isotonic buffer containing 10 mM Hepes (pH 7.4), 137 mM NaCl, 2.7 mM KCl, 5.5 mM glucose, and 1 mM EDTA.

Two-dimensional gel electrophoresis. Isoelectric focusing for two-dimensional gel electrophoresis was performed using the Protean IEF cell from Bio-Rad (Munich, Germany) according to the instructions of the manufacturer. The human platelet pellet (about 200 μg protein) was solubilized for 15 min by sonication in 320 μl lysis buffer containing 7 M urea, 2 M thiourea, 4% (w/v) Chaps, 15 mM DTT (electrophoresis grade), and 0.5% carrier ampholytes, pH 3–10. Pellet homogenate was loaded on a 17-cm immobilized IPG strip, pH 3–10, and reswollen overnight at 50 V. Focussing was carried out for 1 h at 250 V, 1 h at 500 V, and 15 h at 7000 V. After equilibration in 50 mM Tris, pH 8.9, 6 M urea, 30% (w/v) glycerol, and 2% (w/v) SDS, gels were immediately applied to a vertical 10% SDS gel without a stacking gel. Electrophoresis was carried out at 8 °C with a constant current of 40 mA per gel. The proteins were transferred to nitrocellulose for overlay experiments.

Mass spectrometry. Gel pieces from one-dimensional- or two-dimensional-gels, respectively, were washed sequentially for 10 min in tryptic digestion buffer (10 mM NH_4HCO_3) and digestion buffer:acetonitrile 1:1 as described in Marcus et al. [15]. These steps were repeated three times and led to a shrinking of the gel. It was reswollen with 2 μl protease solution (trypsin at 0.05 μg/μl) in digestion buffer and incubated overnight at 37 °C. Alternatively Glu-C digests were performed (overnight at 25 °C) in digestion buffer (25 mM ammonium carbonate, pH 7.8) using 2 μl of protease solution (Glu-C at 0.05 μg/μl).

Analysis of the resulting peptides was carried out using a nano-HPLC system coupled directly to an ESI-iontrap mass spectrometer equipped with a custom-built nano-electrospray ion source (LCQ Classic, Thermo Finnigan, San Jose, USA) as described earlier [9].

F-actin co-sedimentation assay. F-actin co-sedimentation assays were performed essentially as described by the actin manufacturer (Cytoskeleton). Briefly, purified recombinant mouse LASP (40 μg/ml) was incubated with 400 μg/ml freshly polymerized actin (F-actin) for 30 min at room temperature. Following incubation, the LASP/F-actin solution was subjected to centrifugation at 160,000*g* to pellet F-actin and LASP bound to F-actin. After solubilization of the pellet fraction in a volume equal to the initial incubation volume, 20 μl of the pellet and supernatant fractions was analyzed by SDS–PAGE.

Overlay experiments. Nitrocellulose sheets were incubated with either radioactive labeled or purified LASP-1 (0.01 μM) for 1 h in PBS/1% hemoglobin before autoradiography or immunodetection by LASP-1 Western blot, respectively. LPP and Zyxin were used as GST-fusion proteins.

Cross-linking of LASP-1. Human and mouse LASP-1 were cross-linked in PBS at final concentrations of 25 and 5 μM. The reaction was started by the addition of 500 μM ethylene glycol bis(succinimidyl-succinate) (EGS). After 1-h incubation at RT samples were directly stopped by the addition of Laemmli-buffer, heated for 2 h at 37 °C, and analyzed by SDS–gel electrophoresis followed by Coomassie staining. For control experiments glutathione *S*-transferase, known to be a dimer, was used.

Mesangial cell culture. Mesangial cells were isolated and cultured using a sieving technique. Mouse kidneys were minced with razor blade and this suspension was sequentially passed through metal sieves with pore sizes of 125 and 75 μm, and then collected on a 22.5 μm sieve. Cells were cultured on plastic at 37 °C in RPMI 1640 containing 20% fetal calf serum, 2 mM L-glutamine, 0.1 mM sodium pyruvate, 5 mM Hepes, pH 7.2, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.1% non-essential amino acids, and 0.1% growth supplements. Cells from passages 4–9 were used. Normal human mesangial cells (NHMC) were purchased from Cell Systems (St. Katharinen, Germany).

Immunofluorescence. For immunofluorescence microscopy, mouse mesangial cells and normal human mesangial cells were grown on glass chamber slides, fixed in 4% (w/v) paraformaldehyde in PBS, permeabilized with 0.1% (w/v) Triton X-100 in PBS, and then stained with affinity-purified LASP-1 antibody (1:2000) followed by secondary Cy3-labeled anti-rabbit antibody.

Western blot analysis. Tissues used for Western blots were dissected from mouse anesthetized with Nembutal and homogenized in PBS containing protease inhibitors (Complete mini). Cell extracts were resolved by 10% SDS–PAGE. After blotting on nitrocellulose membrane and blocking with 3% non-fat dried milk in 10 mM Tris, pH 7.5, 100 mM NaCl, and 0.1% (w/v) Tween 20, the membrane was first incubated with the polyclonal antibody raised against LASP-1 (1:20,000) [9] followed by incubation with horseradish peroxidase-coupled goat anti-rabbit IgG (1:5000) and detection by ECL.

Results

In vitro mouse LASP-1 is phosphorylated at Ser-61, Ser-99, and Thr-156

It was reported previously that in intact cells human LASP-1 is phosphorylated at Ser-146 by PKA and

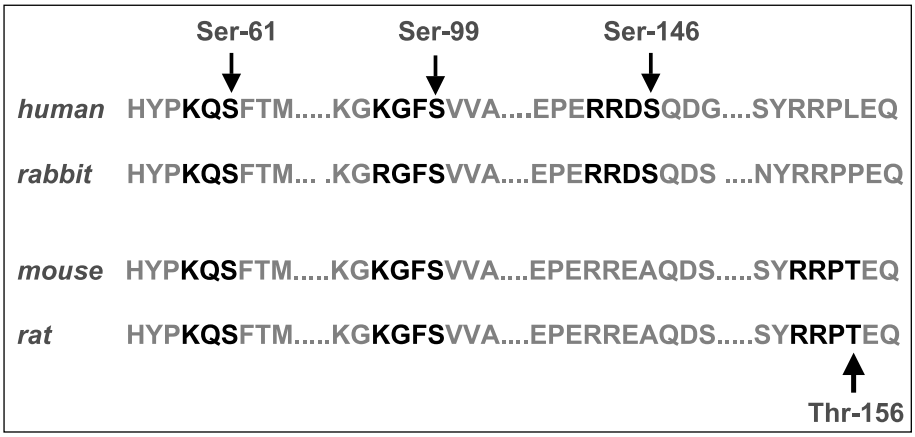

Fig. 1. Partial amino acid sequence alignment of human, rabbit, mouse, and rat LASP-1. The potential phosphorylation sites for cAMP- and cGMP-dependent protein kinase are indicated by arrows.

PKG. Since a corresponding phosphorylation site is not present in rat and mouse (Fig. 1) initial phosphorylation experiments were performed with mouse LASP-1, which was expressed as GST-fusion protein in bacteria and subsequently purified on glutathione–Sepharose. Due to a thrombin cleavage site in the LASP-1 protein, the GST was removed specifically by running the protein through an immobilized thrombin column. Coomassie staining of the purified protein fraction demonstrated the purity of the recombinant LASP-1. The phosphorylation by PKG and catalytic subunit of PKA revealed specific phosphorylation of mouse LASP-1. As shown in Fig. 2A, phosphorylation induced a molecular M_r-band shift from 36 to 38 kDa that is absent in human LASP-1.

Three putative phosphorylation consensus sites for cyclic nucleotide-regulated kinases are present in mouse LASP-1: Ser-61, Ser-99, and Thr-156 (Fig. 1). To examine whether phosphorylation of LASP-1 occurs at these sites, the two radioactive bands were excised from the gel, digested with trypsin, and fractionated on a nano-HPLC connected online to an ion trap mass spectrometer. The resulting MS/MS-spectra led to the unequivocal identification of Ser-61 and Ser-99 as in vitro phosphorylation sites for PKA and PKG (Fig. 3). Based on the length of the predicted proteolytic fragments the third site at Thr-156 was not accessible.

Therefore, additional analyses were performed with mutated mouse LASP-1 in which one, two or all three putative phosphorylation sites were mutated to alanine or glutamic acid. As shown in Fig. 2B, PKG catalyzes the phosphorylation of WT-LASP-1, S61A-LASP-1, S99A-LASP-1, and S61/99A-LASP-1. No [^{32}P] incorporation was observed with the triple mutant, suggesting that all three sites are phosphorylated in vitro—with Ser-61 and Thr-156 the most prominent phosphorylation sites. Mutation of Ser-61 and Ser-99 to alanine did not block the phosphorylation-induced M_r-shift, concluding that the shift results from phosphorylation of Thr-156. Similar results were obtained with PKA (data not shown).

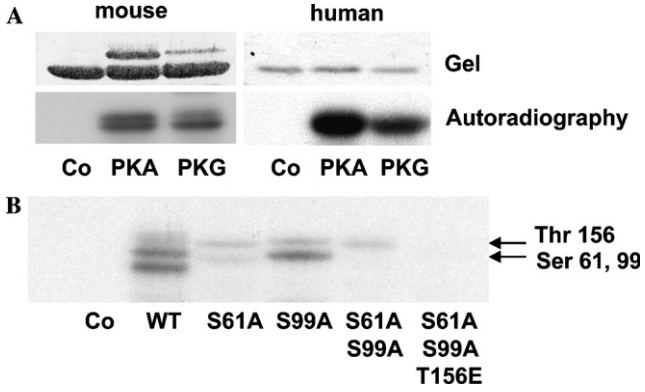

Fig. 2. In vitro phosphorylation of mouse and human LASP-1. (A) Purified, recombinant mouse and human wild-type LASP-1 (1 μM each) were phosphorylated by PKG Iβ and PKA (0.05 μM each) in a total volume of 20 μl for 30 min. In a control experiment (Co) no kinase was added to the mixture. Proteins were resolved by SDS–PAGE and the phosphorylated LASP-1 was visualized by autoradiography. Similar results were obtained in four separate experiments. (B) Purified, recombinant mouse LASP-1 or its mutants S61A, S99A, S61/99A, and S61/99A,T156E (1 μM each) were phosphorylated by PKG type Iβ (0.05 μM) in a total volume of 20 μl for 30 min. Proteins were resolved by SDS–PAGE and the phosphorylated proteins were visualized by autoradiography. The autoradiogram shown is representative of three separate experiments. Similar results were obtained with the C subunit of PKA. Be aware that all mutants run at higher molecular weight than that of the wild-type. There is no M_r after phosphorylation of Ser-61 or Ser-99.

In vivo mouse LASP-1 is phosphorylated at Thr-156

To examine the significance of the in vitro phosphorylation sites in vivo, LASP-1 deficient PTK2 cells were transiently transfected with wild-type LASP-1 and PKG simultaneously or with either protein alone. The cells express PKA endogenously. Phosphorylation of LASP-1 by PKG or PKA was analyzed after incubation of the cells with *ortho*-phosphate and stimulation with 8-pCPT-cGMP or forskolin, respectively, followed by immunoprecipitation. Phosphorylation of WT-LASP by PKG resulted in a double band with a non-phosphorylated 36 kDa band and a radioactive phosphorylated shifted 38 kDa band (Fig. 4), indicating that the in vivo phosphorylation occurs at least at Thr-156. Similar results were obtained for PKA (data not shown). To further analyze masked phosphorylation at Ser-61 and/or Ser-99 both LASP-1 bands (control and potentially phosphorylated protein band) were cut out of the gel, digested with the protease Glu-C, and analyzed for phosphopeptides by nanoLC-ESI-MS/MS. In two replicate analyses only Ser-61 and Ser-99 were found to be non-phosphorylated. No unequivocal localization of Thr-156 was possible.

Finally, the most likely phosphorylation site in LASP-1 (Thr-156) was mutated to alanine (LASP-T156A). Immunoprecipitation of LASP-T156A from ^{32}P-labeled PTK2 cells treated with forskolin or 8-pCPT-cGMP did not demonstrate any phosphorylation (Fig. 4), thus proving Thr-156 the only in vivo phosphorylation site of mouse LASP-1 for PKG and PKA.

Human and mouse LASP-1 are monomers

The LIM domain is a crucial protein structure for assembling multiple protein interactions. In addition, LIM domain proteins can form homodimers that act as bridging factors for protein complexes [16]. In this respect we analyzed the oligomeric state of recombinant human and mouse LASP-1 by chemical cross-linking with ethylene glycol bis(succinimidylsuccinate). However, addition of the crosslinker did not reveal any higher molecular weight band, indicating that LASP-1 exists as a monomer in solution (Fig. 5).

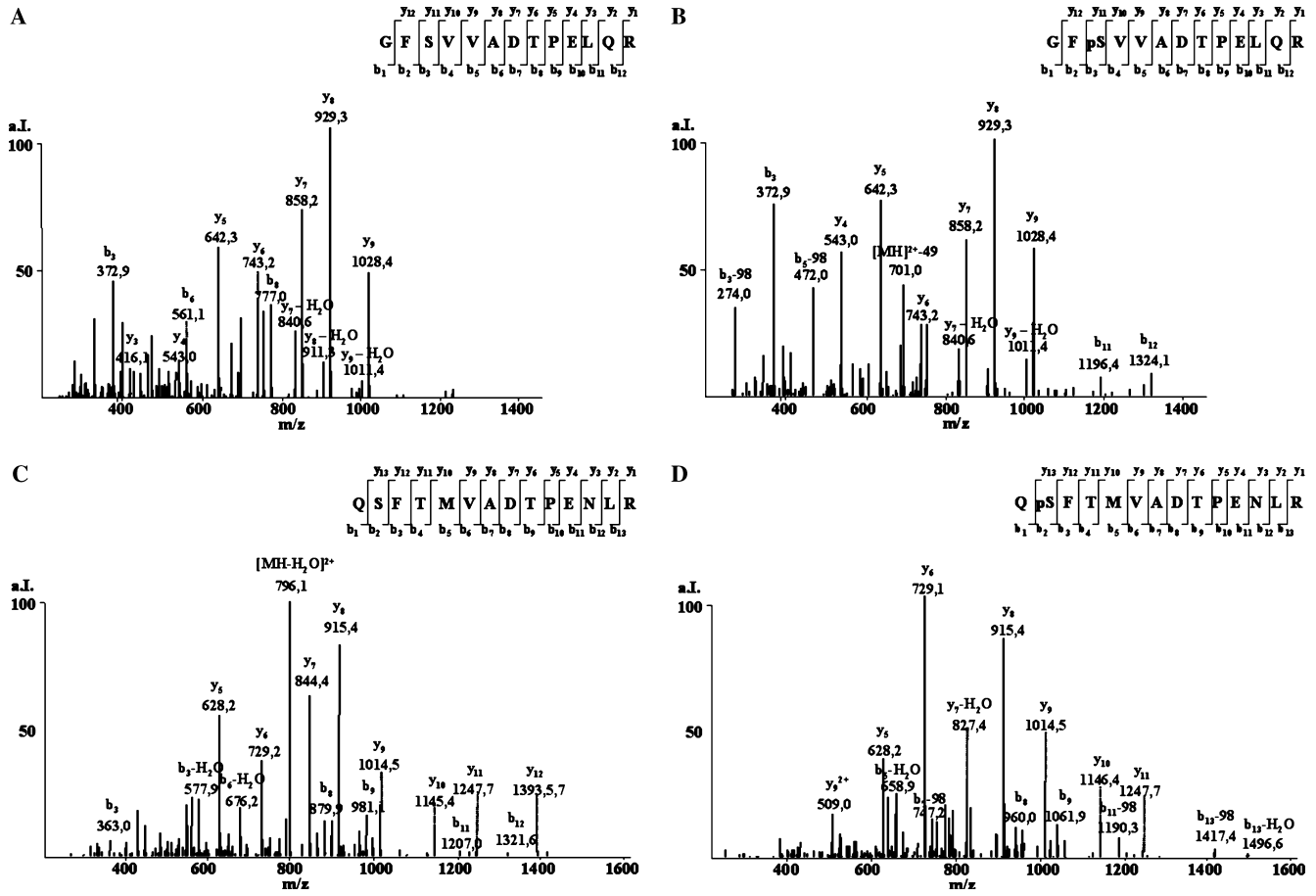

Fig. 3. Localization of the in vitro phosphorylation site of mouse LASP-1. (A) MS/MS spectrum of the non-phosphorylated peptide GFSVVADTPELQR. (B) MS/MS spectrum of the phosphorylated peptide GFpSVVADTPELQR. The typical generation of dehydroalanine from phosphoserine resulting in the loss of −98 Da occurs from b_3 indicating unequivocally Ser-99 as the phosphorylated amino acid. (C) MS/MS-spectrum of the non-phosphorylated peptide QSFTMVADTPENLR. (D) MS/MS-spectrum of the phosphorylated peptide QpSFTMVADTPENLR. Nearly the complete b- and y-ion series are visible. Ser-61 was found to be phosphorylated. In both cases, a direct comparison of the spectra of the non-phosphorylated and phosphorylated peptide facilitated the localization of the phosphorylation site.

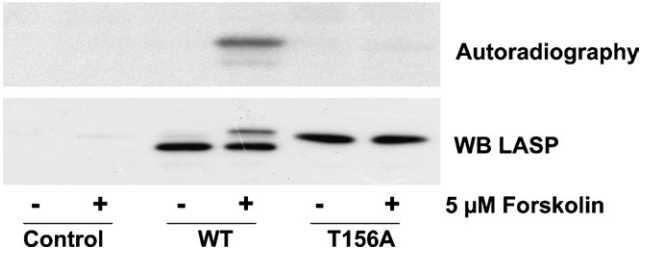

Fig. 4. In vivo phosphorylation of LASP-1 expressed in PTK2 cells. LASP-deficient PTK2 cells were transfected with wild-type LASP or the mutant T156A and cGK Iβ simultaneously or with either protein alone. Cells were stimulated for 20 min with 50 µM 8pCPT-cGMP for PKG activation or 20 min with 5 µM forskolin for PKA activation followed by immunoprecipitation of LASP-1. Immunoprecipitates were analyzed by SDS–PAGE (total LASP, lower panel) and subjected to autoradiography to reveal [^{32}P]-incorporation (upper panel). The autoradiogram shown is representative of two separate experiments.

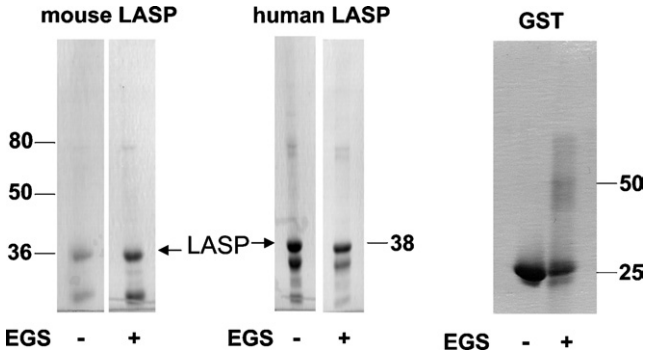

Fig. 5. Analysis of cross-linked LASP-1. Mouse LASP-1 and human LASP-1 (25 µM each) were cross-linked with or without 500 µM EGS and analyzed by SDS–PAGE and Coomassie staining yielding no dimer. For positive control the known dimer GST (25 µM) was used showing partial cross-linking at 50 kDa.

Phosphorylation of mouse LASP at threonine 156 reduces binding to F-actin

In earlier studies by Chew et al. [10] with rabbit LASP-1 as well as in our experiments with human LASP-1 [9] phosphorylation of LASP-1 inhibited co-sedimentation with F-actin in vitro. To test whether this association is similar in mouse LASP-1, we performed F-actin/LASP co-sedimentation experiments with wild-type LASP-1 and the phosphorylation-mimicking mutant T156E. In the absence of F-actin, mouse LASP-1 was exclusively located in the soluble fraction (not shown), whereas in the presence of F-actin, 60% of the LASP-1 protein was found in the pellet (Fig. 6). However, using LASP T156E (mimicking the phosphorylation by PKA and PKG in vivo), 70% of the protein remained in the supernatant (Fig. 6). In control experiments, the actin binding protein α-actinin (positive control) co-sedimented almost completely with F-actin, whereas >95% of BSA (negative control) remained in the soluble fraction (Fig. 6, right panel). These results suggested that phosphorylation at Thr-156 reduces the ability of mouse LASP-1 to bind to F-actin.

LASP-1 binds to proline-rich domain proteins

Because binding between SH3 domains and their ligands occurs through proline-rich sites, we tested the possible interaction between LASP-1 and several proline-rich proteins which had been separated by SDS–PAGE and blotted onto nitrocellulose. In detail, overlay experiments with PKG-phosphorylated radioactive LASP-1 revealed binding to full length vasodilator stimulated phosphoprotein (VASP) and lipoma preferred partner (LPP) as well as to the partial expressed forms of zyxin and LPP presenting only the proline-rich sequence elements, suggesting that the XPPPP motif is involved in LASP-1 binding (Fig. 7A). No unspecific

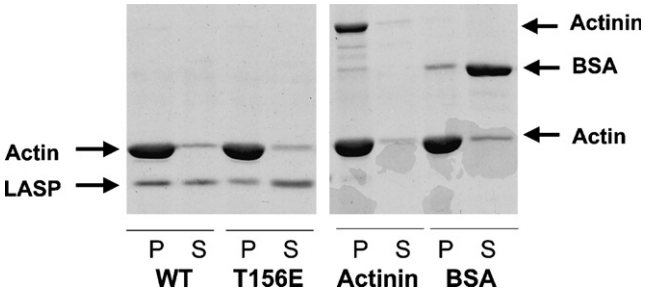

Fig. 6. Binding of LASP-1 to F-actin. Co-sedimentation analysis of 2 μM pre-polymerized actin with 1 μM mouse wild-type LASP (WT) or the phosphorylation-mimicking mutant T156E. Densitometric analysis of two separate experiments determined the amount of LASP remaining in the supernatant (S) versus the amount of LASP in the pellet (P). The positive control with actinin and the negative control with BSA are shown in the right panel.

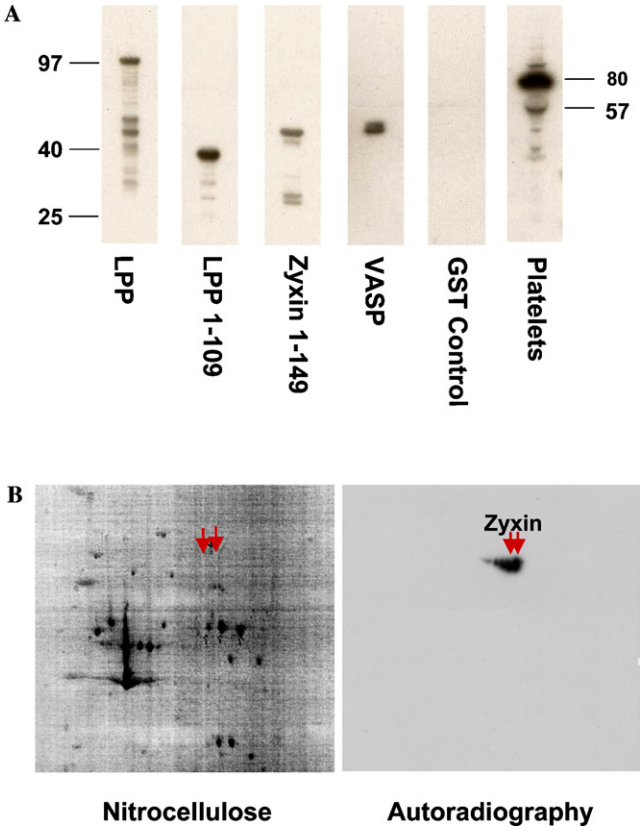

Fig. 7. Binding of LASP-1 to zyxin, VASP, and LPP in [^{32}P]LASP-1 overlays. (A) Total protein from human platelets (20 μg), full length (98 kDa), and partial GST-LPP 1–109 (42 kDa), partial GST-zyxin 1–149 (48 kDa), GST alone (25 kDa), and full length VASP (46 kDa) were separated by SDS–PAGE and blotted onto nitrocellulose. The membrane was blocked and overlaid with radioactive LASP-1. (B) Total protein from human platelets (200 μg) was separated by two-dimensional-SDS–PAGE, blotted onto nitrocellulose, and overlaid with radioactive LASP-1 (autoradiography). The marked radioactive signal (arrow) was cut out from the corresponding gel and identified as zyxin by mass spectrometry.

binding to the GST-tag alone was observed. Similar results were obtained in the reciprocal experiment with non-phosphorylated LASP-1, detected by polyclonal antibody (data not shown), indicating that the binding of LASP-1 to proline-rich domains is independent of the phosphorylation state of LASP-1.

In addition, we analyzed platelet homogenate for new LASP-1 binding partner. Although the one-dimensional SDS–PAGE overlay showed several positive interactions of LASP-1 (Fig. 7A) in the two-dimensional gel of human platelets only strong binding to zyxin (80 kDa) could be confirmed (Fig. 7B).

Expression of LASP-1 in various tissues of human and mouse origin

The expression of human and mouse LASP-1 in different tissues was studied using a rabbit polyclonal antibody raised against GST-tagged human LASP-1.

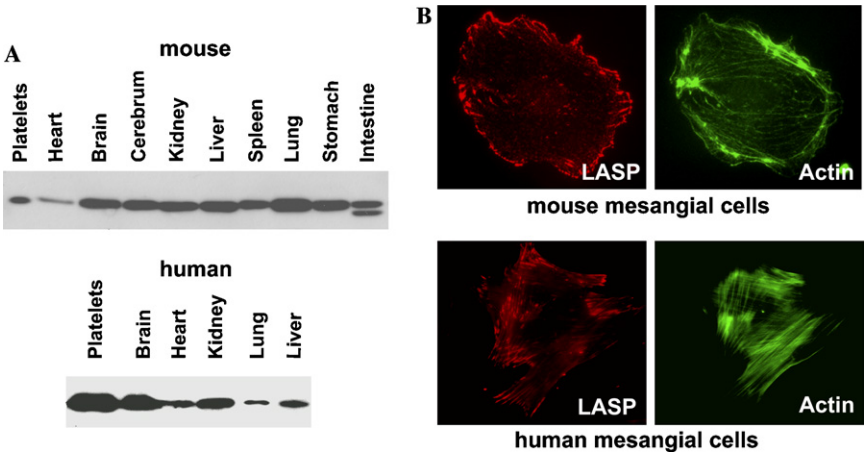

Fig. 8. Analysis of LASP-1 protein expression in human and mouse tissue. (A) Tissue extracts were separated by SDS–PAGE and analyzed by Western blot technique. (B) Immunological co-staining of LASP-1 (visualized with Cy3-tagged sheep-anti rabbit; red) and actin (visualized with Oregon green phalloidin; green) in mouse and human mesangial cells. In both species LASP-1 is present in the focal adhesion plaques and at the ends of actin fibers but not along the actin stress fibers.

Western blot analyses showed LASP-1 expression in platelets, brain, heart, kidney, lung, and liver as well as in mouse cerebrum, spleen, stomach, and intestine (Fig. 8A). For these mouse probes no human tissue was available.

Localization of human and mouse LASP-1 in mesangial cells

A prerequisite for the interaction of LASP-1 with the identified binding partner is the co-localization of the proteins in specific cell compartments. LASP-1 was detected by immunostaining in human, mouse, and rat mesangial cells in the focal adhesion plaques and at the ends of actin fibers (Fig. 8B, data for rat not shown), structures known to be associated with proteins like zyxin, paxillin, LPP, and vinculin [17]. The focal contact origin was confirmed by double staining with anti-vinculin–antibody, a marker of these plaques (Figs. 9A and B). No co-localization of LASP-1 with F-actin along the stress fibers was observed (Fig. 8B).

Relocalization of mouse LASP-1 after forskolin stimulation

Recently, we showed that phosphorylation of human LASP-1 resulted in a cytosolic relocalization of the protein accompanied with a decrease of cell migration [9]. Since cell motility has been correlated with small transient focal adhesions, mouse mesangial cells were allowed to attach and spread on glass slides, and double labeled for LASP-1 and actin, or LASP-1 and vinculin, a marker for focal adhesions. In control cells, LASP-1 and vinculin are co-localized at the cell periphery and to the adhesion plaques (Figs. 9A and B). Stimulating the cells with forskolin (increasing the level of cAMP

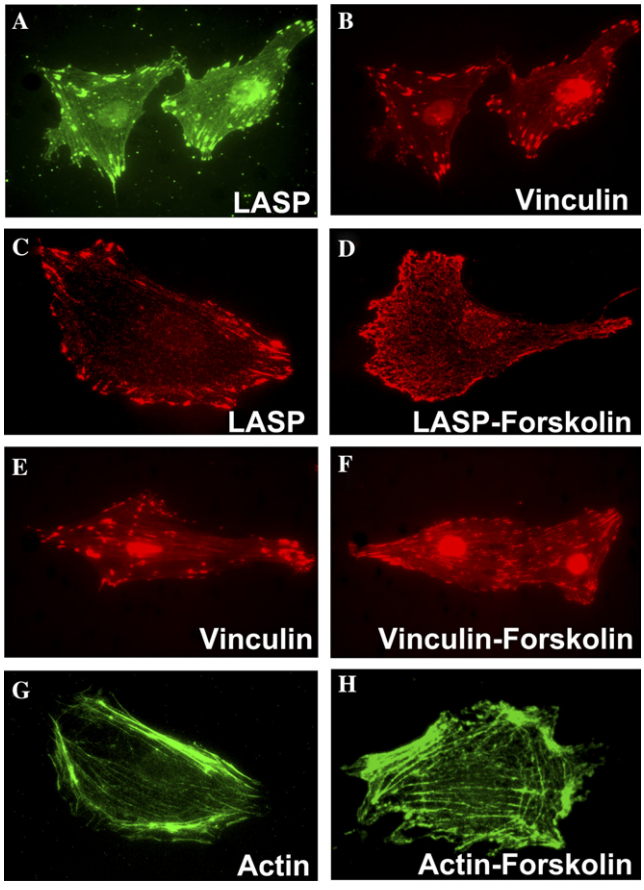

Fig. 9. Expression and subcellular distribution of LASP-1 in mouse mesangial cells. Co-localization of LASP-1 (A) and vinculin (B) at the focal adhesion sites in mouse mesangial cells was revealed by double staining. The binding of LASP-1 was visualized with Cy3-goat anti rabbit secondary antibody. Stimulation of the cells with forskolin leads to a redistribution of LASP-1 from the leading edges and focal contacts (C) to the cytosol (D). This relocalization does not involve changes in the vinculin distribution (E,F) nor actin cytoskeleton organization (G,H).

and activating PKA) resulted in a LASP-1 rearrangement from the focal contacts to a more diffuse cytosolic localization (Figs. 9C and D) Under these conditions no changes either in the actin stress fiber bundles (Figs. 9G and H) or in the vinculin distribution are observed (Figs. 9E and F), indicating that the LASP-1 relocalization is not due to changes in the actin or focal contact structure.

Discussion

In the present study, the phosphorylation specific differences but also functional similarities between human and mouse LASP-1 were studied. The human LASP-1 gene was previously identified on the q12-q21 region of chromosome 17 while the mouse gene was mapped to the 11C–11D region of chromosome 11 [11]. Both proteins have N-terminal LIM domains, C-terminal SH3 domains, and internal nebulin repeats. Recently, a novel actin-binding protein (LASP-2) was identified from chicken brain [18]. LASP-2 has the same domain structure as LASP-1, but the sequence between the second nebulin repeat and the SH3 domain is LASP-2 specific and conserved in chicken, mouse, and human. In contrast to LASP-1, which is widely distributed in non-muscle tissue, LASP-2 is highly expressed in brain.

The amino acid sequences of human, rabbit, mouse, and rat LASP-1 are highly similar to each other. Between mouse and rat 99.6% of the aligned positions are identical (only one amino acid exchange from serine to proline at position 183). Human and mouse share 97.3% identical amino acids while human and rabbit show 95.8% similarity. Despite this high analogy, sequence analysis of the four species revealed different phosphorylation sites for PKA and PKG. The known in vivo phosphorylation site in human is Ser-146 [9] while in rabbit Ser-99 and Ser-146 are phosphorylated in intact cells [10]. Interestingly, Ser-146 is only found in human and rabbit, whereas the corresponding amino acid in mouse and rat is an alanine (Fig. 1). On the other hand, in mouse and rat a new potential phosphorylation site at Thr-156 with a typical consensus motif for PKA/PKG can be identified (RRPT) that is absent in human and rabbit. Actually, our data confirm this site to be phosphorylated in vivo by PKA and PKG.

Despite these differences, phosphorylation of human and mouse LASP-1 resulted in an apparent translocation of both proteins from the membrane to the cytosol (Fig. 9). This relocalization does not reflect changes in the focal contacts since immunohistochemical staining of vinculin, a marker for focal contacts, was unaffected. Indeed, phosphorylation reduces the binding of human and mouse LASP to F-actin in vitro.

The regulation of actin dynamics, cell movement, and signal transduction is mediated by proteins like zyxin and paxillin by their association with a variety of cytoskeletal and signaling proteins [5]. Based on our overlay experiments, there is strong evidence that human and mouse LASP-1 associate with its SH3 domain to the proline-rich domains of the tested proteins VASP, zyxin, and LPP, all connected to focal contacts. Just recently, the interaction of zyxin to the C-terminal SH3 domain of LASP-1 has been confirmed using the two-hybrid system [19]. Truncation of the C-terminus disrupted translocation of LASP-1 to focal adhesions [20]. However, the binding of LASP-1 to zyxin is not disconnected after LASP-1 phosphorylation whereas with F-actin a phosphorylation dependent disruption of the binding between LASP-1 and F-actin can be observed, resulting in a relocalization of the protein from the cell membrane to the cytosol. A recently observed c-Abl-dependent tyrosine phosphorylation of LASP-1 at Tyr-171 is a distinct signaling event that specifically blocks LASP-1 transiting to focal complexes in apoptotic but not migratory cells [20].

The LIM domain is a specialized double zinc-finger motif found in a variety of proteins and is thought to function as protein interaction modules. Several important LIM proteins like zyxin and paxillin are associated with the cytoskeleton and can act with other LIM proteins forming heterodimers for the coordinated, localized assembly of multimeric complexes involved in cell adhesion, spreading, and motility [3]. In this respect, LIM domains have also been implicated in conferring specific protein–protein interactions by homodimerization [21]. However, in our experiments LASP-1 was shown to act as a monomer although heterodimerization with other LIM proteins to serve as an adapter molecule or scaffolding protein cannot be excluded and is still under investigation.

Taken together, our findings indicate that human and mouse LASP-1 behave similar by, despite their differences in sequence and phosphorylation sites. This now establishes the possibility to generate transgenic mice for the functional study of LASP in vivo.

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