Actin binding of human LIM and SH3 Protein is regulated by cGMP- and cAMP-dependent Protein Kinase phosphorylation on Serine 146

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Phosphorylation of human LASP by cGK and cAK
SUMMARY

Various drugs that elevate cGMP levels and activate cGMP-dependent protein kinase (cGK) inhibit agonist-induced platelet activation. In the present study we identified the LIM and SH3 domain protein (LASP) that was recently cloned from human breast cancer cells (Tomasetto, C. et al., 1995, Genomics 28, 267-278) as a novel substrate of cGK in human platelets. Recombinant human LASP was phosphorylated by cGMP- and cAMP-dependent protein kinase (cAK) in vitro. Cotransfection of PtK-2 cells with LASP and cGK confirmed phosphorylation of LASP in vivo. Studies with human LASP mutants identified Serine 146 as a specific phosphorylation site for cGK and cAK in vivo. LASP is an actin-binding protein, and the phospho-LASP-mimicking mutant S146D showed reduced binding affinity for F-actin in cosedimentation experiments. Immunofluorescence of transfected PtK2 cells demonstrated the localization of LASP in the tips of cell membrane extensions and at cell-cell contacts. Expression of the human LASP mutant S146D resulted in nearly complete relocalization to the cytosol and reduced migration of the cells. Taken together, these data suggest that phosphorylation of LASP by cGK and cAK may be involved in cytoskeletal organization and cell motility.
INTRODUCTION

The activation process of human platelets and vessel wall - platelet interactions are tightly regulated under physiological conditions and are often impaired in thrombosis, arteriosclerosis, hypertension, and diabetes. Platelet activation can be inhibited by a variety of agents, including aspirin and Ca²⁺ antagonists as well as cGMP- and cAMP-elevating agents such as NO and prostaglandin I₂ (for review see Ref. 1). The inhibitory effects of cGMP and cAMP are mediated by cAMP-dependent protein kinases types I and IIβ and by cGMP-dependent protein kinase Iβ (cAK I, cAK IIβ, and cGK Iβ, respectively), representing the major forms of cyclic nucleotide-dependent protein kinases in human platelets (2, 3).

The molecular mechanisms of platelet inhibition by cGMP signaling downstream of cGK activation are only partially understood. In cGK-deficient mice cGMP-mediated inhibition of platelet aggregation is impaired (4). To date, only a few substrates for cAK and cGK have been identified and characterized in human platelets. The 22-kDa small GTP-binding protein rap 1b is phosphorylated by cAK and cGK in intact platelets (5, this study). Phosphorylation of rap 1b is associated with translocation of the protein from the membrane to the cytosol (6). The vasodilator-stimulated phosphoprotein VASP is another major substrate of cAK and cGK in human platelets (7). Its three phosphorylation sites are phosphorylated with different specificities by these two kinases (8). VASP phosphorylation is thought to be involved in the negative regulation of integrin αIIbβ₃ (9). Experiments in vitro revealed reduced F-actin binding and actin polymerization of phosphorylated VASP (10). Two recent studies investigated RhoA-mediated myosin light chain (MLC) activation and its contribution to platelet aggregation and secretion, showing that cGK phosphorylates RhoA and counteracts the phosphorylation of myosin light chain (MLC) through activation of MLC phosphatase (11, 12). We previously identified heat shock protein 27 (Hsp27) as a substrate for cGK in intact platelets (13). Phosphorylation of Hsp27 by cGK reduced the stimulatory effect of MAPKAP kinase 2-phosphorylated Hsp27 on actin polymerization. There is also evidence that at least part of the inhibitory response mediated by cGK depends on phosphorylation of the thromboxane receptor (14, 15) and the IP₃-receptor (16).

Several other proteins have been reported to be phosphorylated in response to cGK activation either in vitro or in intact cells, including cGMP-specific phosphodiesterase (2), MLC kinase (17), an IP₃ receptor-associated cGMP kinase substrate (18), Na⁺/K⁺-ATPase (19), cysteine rich protein 2 (20), MEKK1 (21), and endothelial NO synthase (22). None of these proteins, however, have been established as a downstream target of cGK in platelets.
Here we report the identification of a specific substrate for cAK and cGK in intact human platelets using differential phosphoproteomic display of radiolabeled human platelets. The protein was identified as the LIM and SH3 domain protein (LASP) (27) by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). Phosphorylation of LASP at Ser-146 leads to a redistribution of the actin-bound protein from the tips of the cell membrane to the cytosol accompanied with a reduced cell migration.
EXPERIMENTAL PROCEDURES

Materials—Urea (ultrapure), [γ\(^{32}\)-P]ATP, [\(^{32}\)P]orthophoshate (HCl-free), Protein A Sepharose beads, 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), CalyculinA was from Calbiochem (Bad Soden, Germany), trypsin was from Promega (Heidelberg, Germany), 8-pCPT-cGMP was from BioLog (Bremen, Germany), IPG strips, goat anti-rabbit IgG, and nonfat dry milk were from Biorad (Munich, Germany), FuGene, the rapid ligation kit, and Complete MiniTM were from Roche (Mannheim, Germany), and nitrocellulose membrane was obtained from Schleicher und 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 was from Dianova (Hamburg, Germany), and Dulbecco’s modified Eagle’s medium was from Life Technologies (Karlsruhe, Germany), Boyden chambers were from Corning Costar (MA, USA).

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

cGK Iα and the catalytic subunit of cAK type II were purified from bovine lung and bovine heart, respectively (23). cGK Iβ and cGK II were expressed in and purified from the baculovirus-Sf9 cell system (24).

Isolation of platelets—Freshly donated blood from healthy volunteers (50 ml) was collected in acid-citrate dextrose and centrifuged for 10 min at 300 x g to yield platelet-rich plasma (PRP). PRP was centrifuged for 20 min at 500 x 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 at a density of 1 x 10^9 cells/ml. After resuspension, platelets were allowed to rest at 37°C for 15 min.

\(^{32}\)P-Labeling of platelets—Platelet preparation was carried out essentially as described above. After washing, 1 ml of platelets at a concentration of 1 x 10^9/ml was incubated with 500 µCi [\(^{32}\)P]orthophosphate (HCl-free) for 1.5 h at 37°C. Platelets were then centrifuged at 500 x g for 7 min and resuspended in 1 ml isotonic buffer. Aliquots of 100 µl (corresponding
to 200 µg protein) were treated with 500 µM 8pCPT-cGMP for 30 min at 37°C. After stimulation, platelets were briefly centrifuged (500 x g for 3 min) to yield a pellet.

2-Dimensional gel electrophoresis—Isoelectric focusing for two-dimensional gel electrophoresis was performed using the Protean IEF cell from Biorad (Munich, Germany) according to the instructions of the manufacturer. The 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), 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 7000V.

After equilibration in 50 mM Tris, pH 8.9, 6 M urea, 30% (w/v) glycerol, 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 gels of radioactively labeled platelet proteins were fixed in 30% ethanol, 10% acetic acid and exposed. Radioactive spots visualized by autoradiography were excised.

Mass-Spectrometry—Gel pieces were washed sequentially for 10 min in tryptic digestion buffer (10 mM NH₄HCO₃) and digestion buffer: acetonitrile 1:1. 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.

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). Fifteen µl of 5% (v/v) formic acid (FA) were added to the gel pieces and the peptides extracted by sonication for 15 min. The extraction step was repeated once. The supernatants were transferred to glass tubes and the peptides were automatically flushed and preconcentrated on a µ-C₁₈-precolumn (Nano-Precolumn", 0.3-mm I.D. x 1 mm, C₁₈ PepMap", LC Packings Dionex, Idstein, Germany) for 10 min with 0.1% (v/v) trifluoroacetic acid and a flow of 40 µl/min. Tryptic peptides were injected automatically on the reversed-phase C₁₈-column (75 µm I.D. x 250 mm, C₁₈ PepMap", 5 µm particle size, LC Packings Dionex) column using the Switchos"-system (LC Packings Dionex). Separation of the peptides was carried out in a gradient consisting of 0.5% (v/v) FA (solvent A) and 0.5% FA/84% acetonitrile (solvent B) with 5-15% B in 10 min, 15-20% B in 10 min, 20-50% B in 70 min and 50-100% B in 5
min. The flow rate was adjusted from 200 µl/min to 160 nl/min using a precolumn split. Eluting peptides were transferred online to a heated capillary (Pico Tip®, FS360-20-10, New Objective Incorporated, Cambridge, USA) of an ion trap mass spectrometer (LCQ® Classic, Thermo Finnigan). The following ESI parameters were used: spray voltage, 1.7 kV-2.15 kV; capillary temperature, 200 °C; capillary voltage, 42 V; tube lens offset voltage, 30 V; and the electron multiplier at -950 V. The collision energy was set automatically depending on the mass of the parent ion. Gain control was set to 10^7. The data were collected in the centroid mode using one MS experiment (Full-MS) followed by three MS/MS experiments of the three most intensive ions (intensity at least 3 × 10^5). ‘Dynamic Exclusion´ was used for data acquisition with an exclusion duration of 5 min and an exclusion mass width of ± 1.5 Da.

**Molecular cloning of LASP and LASP mutants**—Gene-specific primers used for PCR amplification of LASP and LASP mutants were designed based on the published human cDNA sequence (GenBank Access. No. X82456). Oligonucleotides used to generate wild-type LASP: AATGGATCCATGAACCCCAACTGCGCCCGGTGCGGCAAG (sense, starting at position 76) and CGGGAATTCCTAGATGGCCTCCACGTAGTTGGCGCGGCA (antisense, starting at position 862) with BamHI and EcoRI restriction sites (underlined). Full length human LASP cDNA was cloned into the BamHI/EcoRI sites of pGEX4T1 to generate a glutathione-S-transferase (GST)-LASP fusion protein. The single and double mutants were made by performing a second round of PCR using the wild type and single mutant as a template, respectively, and the appropriate pairs of oligonucleotides. For eukaryotic expression, LASP was cloned into pcDNA3. All constructs were confirmed by DNA sequence analysis.

**Expression of GST-LASP fusion proteins**—Recombinant LASP and LASP mutants S61D, S146D, and S61/146D were expressed in *E.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 was achieved by digestion with thrombin overnight at 4°C. Purity was analyzed by examination of Coomassie-stained SDS-polyacrylamide gels.

**LASP polyclonal antibody generation**—To generate a polyclonal antibody specific for LASP, recombinant human GST-LASP that had been expressed and affinity-purified from bacteria was injected into New Zealand rabbits (Immunoglobule, Himmelstadt, Germany).
Immunoreactive serum was affinity-purified against LASP protein coupled to a HiTrap-NHS-activated affinity column according to the manufacturer’s instructions.

**Western Blot Analysis of LASP**—Cell extracts were resolved by 10% SDS-PAGE. After blotting on nitrocellulose membrane and blocking with 3% nonfat dry milk in 10 mM Tris, pH 7.5, 100 mM NaCl, 0.1% (w/v) Tween 20, the membrane was first incubated with the polyclonal antibody raised against LASP (1:16,000) followed by incubation with horseradish peroxidase-coupled goat anti-rabbit IgG (1:5000) and detection by ECL.

**In vitro phosphorylation of LASP**—LASP and its mutants S61A, S146A, and S61/146A (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 MgCl₂, 1 mM EDTA, 0.2 mM dithiothreitol, and the C subunit of cAK or cGK Iα, Iβ, or II (0.05 µM each) and 5 µM cGMP. Reactions were started by the addition of 50 µM ATP containing 0.5 µCi of [γ-32P]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 32P was visualized by autoradiography.

**Eukaryotic expression and in vivo phosphorylation**—PTK2 cells were grown in DMEM medium in 6-well plates to about 70% confluency and then transiently transfected with one of the LASP constructs cloned into a pcDNA3 vector alone or together with pCMV-cGKIβ (25) using FuGene. After 48 h, cells were washed once with phosphate-free DMEM and labeled with [32P]orthophosphate for 1 h at 37°C. Then cells were exposed to buffer alone and either 10 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, then 1.5 h with pre-equilibrated protein A Sepharose. Bound immune complex was washed 3 times with ice-cold PBS. The pellets were resuspended in 30 µl Laemmli SDS stop solution. Proteins were separated by SDS-PAGE on 10% gels. Incorporation of 32P was visualized by autoradiography.

**Immunofluorescence**—For immunofluorescence microscopy, transfected PtK2 cells grown on glass chamber slides were 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 antibody.
(1:1000) followed by secondary Cy3-labeled anti-rabbit antibody.

*F-actin cosedimentation assay*—F-actin cosedimentation assays were performed essentially as described by the actin manufacturer (Cytoskeleton). Briefly, purified recombinant human 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 x 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 were analyzed by SDS-PAGE.

*Migration experiments*—PTK2 cells were grown in DMEM medium in 5 cm dishes to about 80% confluency and then transiently transfected with pcDNA3 vector containing WT-LASP or S146D-LASP using Lipofectamine. After 24 h, 1 x 10⁵ cells per 100 µl incubation medium (DMEM with 1 mM MgCl₂) were seeded in the upper chamber of BSA-coated transwells. PtK2 cells were allowed to migrate through the porous membrane for 4 h at 37°C. Cells remaining at the upper surface were completely removed using a cotton carrier. The lower surfaces of the membranes were then stained in a solution of 1 % (w/v) crystal violet in 2 % ethanol for 30 s and rinsed afterwards in distilled water. Cell-associated crystal violet was extracted by incubation in 10 % acetic acid for 20 min and measured at 595 nm.
RESULTS

Phosphorylation of LASP in intact human platelets treated with the cGK-specific stimulus 8pCPT-cGMP—To identify substrates of cGK in intact human platelets, cells were labeled with [32P]orthophosphate, stimulated with 500 µM of the specific cGMP-dependent protein kinase activator 8pCPT-cGMP, and proteins of the resulting platelet lysate were separated by two-dimensional gel electrophoresis. The 2D-phosphoproteomes resulting from this experiment (Fig. 1) demonstrate the phosphorylation/dephosphorylation of several proteins after stimulation with 8pCPT-cGMP. For identification of these proteins, the spots were excised from the gel, digested with trypsin, and the resulting peptides were analyzed by ESI-MS/MS. Spot 2a-c was identified as the vasodilator-stimulated phosphoprotein (VASP), a well known substrate of cGK in human platelets (8) with multiple phosphorylation isoforms (26). Spot 3 was characterized as rap 1b, a low molecular weight GTP-binding protein. Phosphorylation of rap 1b has been observed in nitric oxide-stimulated human platelets through stimulation of guanylyl cyclase and activation of the cGMP-dependent protein kinase (5). Spot 1 was identified as LIM and SH3 protein (LASP), a novel type of actin binding protein (27). Spots 4, 5 and 8 have not been identified yet due to their low amounts of protein in the gel.

Recombinant LASP is phosphorylated by cGK and cAK in vitro—The specificity of the phosphorylation observed in intact platelets was further investigated by studying the role of LASP as a substrate for cGK in vitro. For this purpose, purified recombinant human LASP was incubated with cGK Iβ and with the catalytic subunit of cAMP-dependent protein kinase in the presence of [γ-32P]ATP. As shown in Fig. 2, LASP is clearly a substrate of cGK Iβ and cAK, the two isoforms known to be present in human platelets.

Identification of LASP phosphorylation sites in vitro—Human LASP contains two cAK consensus motifs for serine phosphorylation: Ser-99 (KGFS) and Ser-146 (RRDS) (28). In the present study, phosphorylation of amino acids was determined by mass spectrometry. A complete trypsin digest of phosphorylated LASP was fractionated on a nano-HPLC connected online to an ion trap mass spectrometer. The resulting MS/MS-spectrum of the
phosphopeptide with the sequence QpSFTMoxVADTPENLR is presented in Fig. 3. The b-ion series shows many signals with a loss of -98 Da typical for phosphoserine- and phosphothreonine-containing peptides (29), leading to the unequivocal identification of Ser-61 as phosphorylation site for cGK. In addition, the predicted Ser-146 was identified in a second phosphopeptide (data not shown). However, no phosphorylation of Ser-99 was observed. The same results were obtained with LASP phosphorylated by cAK.

In vitro phosphorylation of LASP and LASP mutants—To study the specificity of phosphorylation, the identified serine phosphorylation sites Ser-61 and Ser-146 were mutated to alanine (S61A, S146A and S61/146A). The purified, recombinant proteins (wild-type, single mutants, and double mutant) were incubated with the cGK isoforms Iα, Iβ, and II and the catalytic subunit of cAMP-dependent protein kinase in the presence of \( \gamma^{32}\)P]ATP. Incorporation of phosphate was observed after 30 min with each of the four kinases, albeit at different levels (Fig. 4). cGK Iβ and cAK, the two isoforms present in human platelets, caused the highest phosphate incorporation. As expected, no phosphorylation was observed for the double mutant S61/146A. In a control experiment, VASP, a well known substrate for cAK and cGK (8), was equally phosphorylated by each of the four kinases (Fig. 4).

In vivo phosphorylation of LASP by cGK Iβ and cAK at Ser-146—To evaluate the role of LASP as an in vivo substrate of cGK Iβ and cAK (the two isoforms present in human platelets), LASP deficient PtK-2 cells were transfected with wild-type LASP or the mutants S61A and S146A, and cGK Iβ simultaneously or with either protein alone. The cells express cAK endogenously. Phosphorylation of the proteins by cGK Iβ or cAK was analyzed after stimulation of the cells with 8pCPT-cGMP or forskolin, respectively, followed by immunoprecipitation. Wild-type LASP and the mutant S61A showed identical in vivo phosphorylation (Fig. 5). In contrast, no phosphorylation was observed with the S146A mutant, indicating that in vivo Ser-146 is the only cGK Iβ phosphorylation site in LASP. In the absence of cGK Iβ no phosphorylation was detected. Similar results were obtained for cAK phosphorylation of LASP (data not shown).

Expression of LASP in various cell lines and tissues—The expression of human LASP in different cell types was studied using a rabbit polyclonal antibody raised against GST-tagged
human LASP. Western blot analyses of cell extracts from several human tissues and from different cell lines showed LASP expression in human platelets, brain, heart, kidney, lung, liver, fibroblasts, smooth muscle cells (SMC) and endothelial cells (HUVEC) and various cell lines (Fig. 6A and B).

**Phosphorylation of Ser-146 reduces binding of LASP to F-actin**—In earlier studies by Schreiber et al. (27) as well as in our experiments, the filamentous expression pattern of LASP suggested that the protein is colocalized with F-actin. To test whether this association might be affected through phosphorylation of LASP, we performed F-actin/LASP cosedimentation experiments with wild-type LASP and the phosphorylation-mimicking mutant S146D, because *in vitro* phosphorylation by cAK and cGK would also phosphorylate Ser-61. In the absence of F-actin, LASP was exclusively located in the soluble fraction, whereas in the presence of F-actin, about half of the LASP protein was found in the pellet (Fig. 7). However, using LASP S146D (mimicking the phosphorylation by cAK and cGK *in vivo*), two thirds of the protein remained in the supernatant (Fig. 7, left panel). In control experiments, the actin binding protein \(\alpha\)-actinin (positive control) cosedimented almost completely with F-actin, whereas >95% of BSA (negative control) remained in the soluble fraction (Fig. 7, right panel). These results suggested that upon phosphorylation of Ser-146, LASP loses its ability to bind to F-actin.

**Phosphorylation-dependent redistribution of LASP in PtK2 cells.** In view of these results, we investigated whether the intracellular localization of LASP is directly affected by phosphorylation. PtK2 cells, which express no detectable amount of endogenous LASP (Fig. 6B), were transiently transfected with expression vectors encoding either wild-type LASP, LASP mutant S146A, or LASP mutant S146D. Forty-eight hours after transfection, cells were prepared for immunofluorescence. Wild-type LASP and LASP S146A were predominantly present in the tips of cell membrane extensions and at cell-cell contacts where it co-localizes with F-actin (Fig. 8A, B, D and E) and. However, double staining analysis with the LASP antibody and Oregon green phalloidin for F-actin staining revealed no colocalization with actin stress fibers (Fig. 8D and 8E). In contrast, the phosphorylation-mimicking mutant LASP S146D was found predominantly in the cytosol (Fig. 8C). The specificity of the staining was controlled with preadsorbed LASP antibody, which showed no immunofluorescence (data not shown).
Redistribution of LASP to the cytosol reduces cell motility. Since LASP is prominently present within focal contacts and the leading edges of the cell membrane we wondered whether the protein might be involved in cell motility. Therefore we tested migration of PtK2 cells transiently expressing wild-type LASP or LASP mutant S146D in a modified Boyden Chamber system. Cells were seeded in the upper chamber of a transwell polycarbonate membrane and after 4 h cells migrated through the porous membrane were counted. Cells transfected with LASP S146D, mimicking the phosphorylation and exhibiting cytosolic localization (Fig. 8C), showed a 25% reduced motility compared to untransfected cells or WT-LASP expressing cells displaying membrane localization (Fig. 9).
DISCUSSION

As an approach to identify novel substrates of cGMP-dependent protein kinase, we analyzed cGK-mediated protein phosphorylation in intact human platelets using two-dimensional gel electrophoresis. In addition to the previously known substrates VASP and rap 1b, we identified the LIM and SH3 domain protein (LASP) as a novel substrate for cGK and cAK. LASP consists of an N-terminal zinc-binding LIM domain, followed by two actin binding sites and a Src homology region 3 (SH3) at the C-terminal end (30). The human LASP gene was previously cloned and identified from a human breast cancer cDNA library (31). It was mapped to human chromosome 17 q12-q21 and was shown to be amplified and overexpressed in breast tumors (32).

Theoretical sequence analysis of human LASP revealed two potential phosphorylation sites at Ser-99 (KGFSF) and Ser-146 (RRDSQ), with Ser-146 having an additional basic amino acid on either side of the consensus sequence. These sites agree well with the minimal motif for efficient cGK phosphorylation (RKXST) (33). The data presented here show that human LASP Ser-146 is directly phosphorylated by cAK and cGK in vivo. Using site-directed mutagenesis of all serine residues, we excluded phosphorylation at Ser-99 and at Ser-61 in vivo, although Ser-61 was phosphorylated in vitro. Interestingly, serine in position 146 is only found in human and rabbit, while the corresponding amino acid in mouse and rat is an alanine. In contrast, Ser-99 and Ser-61 are conserved in all four species. Just recently, Chew and co-workers identified Ser-146 and Ser-99 as the major in vitro and in vivo phosphorylation sites of rabbit LASP by cAK (34). Phosphorylation of rabbit LASP at Ser-146 induced a Mr band shift that is absent in human LASP phosphorylated by cAK and cGK indicating differences in the structure of the two proteins. Further studies are underway to explore phosphorylation of LASP in the different species and its possible physiological role.

LASP is expressed in all human tissues tested including platelets, brain, heart, kidney, lung, liver, endothelial cells, smooth muscle cells, and fibroblasts. Northern blot analysis of murine LASP revealed a constant expression of the protein during embryogenesis from day 7.5 to day 18.5 with various levels in all adult tissues, which is consistent with an essential role for LASP in basic cellular function (32).

Immunofluorescence analysis of LASP subcellular distribution showed that the protein colocalizes with F-actin at focal adhesion plaques and membrane edges in mouse cardiac fibroblasts and rat mesangial cells (unpublished results). These results confirmed earlier observations by Schreiber et al. who found LASP at peripheral cell extensions in individual epithelial cancer cells (27). Experiments performed using PtK2 cells transfected with wild-type LASP also demonstrated that the protein is colocalized with F-actin at membrane extensions, although not along intracellular stress fibers. In PtK2 cells, however, the LASP mutant S146D, which simulates phosphorylation at Ser-146, accumulates in the cytoplasm when transiently expressed, suggesting that phosphorylation of human LASP by cAK and cGK regulates the intracellular localization of the protein. Recently, it was shown that the cAK-dependent acid secretory agonists histamine and forskolin induce a rapid sustained rise in LASP phosphorylation in rabbit gastric parietal cells, and this increase is closely correlated with
the acid secretory response (28). In parallel, LASP redistributes from a predominantly cortical location to a region surrounding the intracellular canaliculus, which is the site of active HCl secretion (35). Mutation of the two major cAK phosphorylation sites in rabbit LASP, Ser-99 and Ser-146, to alanine appears to block this recruitment (34).

The function of LASP in living cells seems to be complex and cell type specific. The localization of LASP to the part of active membrane extension in addition to our observations of a reduced cell migration after phosphorylation and relocalization to the cytosol indicates a prominent role for LASP in cell movement – either by interacting directly with actin and promoting actin polymerization or by acting as a scaffolding molecule recruiting other motility proteins to the tips of the cells involved in the organization of the cytoskeleton. In gastric parietal cells, LASP was identified to bind to dynamin, a large GTPase involved in vesicular fission and control of membrane trafficking in the H+/K+-ATPase pathways at the apical membrane (36).

Apart from LASP, cGK phosphorylates the vasodilator-stimulated phosphoprotein VASP (8), a protein that has been implicated in the regulation of actin dynamics and associated processes such as cell adhesion and motility by its ability to associate with F-actin, profilin, zyxin, and vinculin (37). In platelets, VASP phosphorylation seems to be involved in the negative regulation of the integrin $\varepsilon_{IIb}\varepsilon_{IIIa}$ (9). Actually, in vitro phosphorylation of VASP reduces F-actin binding (10), however, in contrast to LASP, phosphorylation of VASP plays no obvious role in subcellular targeting (38).

As a newly identified signaling protein within the cGMP and cAMP pathway, the specific function of LASP is still under investigation. Future experiments will address the question of whether there are platelet-specific binding partners for LASP and determine whether LASP might be a phosphorylation-dependent molecular switch that regulates the interaction of other proteins with F-actin-rich compartments, thereby modulating platelet function.
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FOOTNOTES

1 The abbreviations used are: LASP, LIM and SH3 domain protein; C, catalytic subunit; cAK, cAMP-dependent protein kinase; cGK, cGMP-dependent protein kinase; VASP, vasodilator-stimulated phosphoprotein; 8pCPT-cGMP, 8-para-chlorophenylthio-cGMP; PBS, phosphate-buffered saline; ESI-MS, electrospray ionization mass spectrometry.
FIGURE LEGENDS

Fig. 1: Phosphorylation of LASP in intact human platelets. Human platelets were incubated in the presence of [32P]orthophosphate and treated with buffer alone (Control) or with 500 µM cGK activator 8pCPT-cGMP for 30 min (Stimulation). Platelet homogenate was separated by two-dimensional gel electrophoresis and an autoradiogram was obtained. Spot 1: LASP; spots 2a, b and c: VASP; spot 3: rap 1b; spots 4, 5 and 8 have not yet been identified. The pH gradient is indicated at the top of the gel. The gels shown are representative of three separate experiments.

Fig. 2: In vitro phosphorylation of LASP. Purified, recombinant human LASP (1 µM) was phosphorylated by cGK 1β and cAK (0.05 µM each) in a total volume of 20 µl for 30 min as described under "Experimental Procedures". In a control experiment (Co) no kinase was added to the mixture. Proteins were resolved by SDS-PAGE and the phosphorylated LASP visualized by autoradiography. Similar results were obtained in four separate experiments.

Fig. 3: Phosphoamino acid analysis of human LASP phosphorylated by cGK. MS/MS-spectrum of the phosphorylated peptide QpSFTMoxVADTPENLR. Nearly the complete b- and y-ion series are visible. The typical generation of dehydroalanine from phosphoserine resulting in the loss of -98 Da occurs from y13, indicating unequivocally Ser-61 as the phosphorylated amino acid. Similar spectra were obtained with LASP phosphorylated by cAK.

Fig. 4: In vitro phosphorylation of LASP and by cGMP- and cAMP-dependent protein kinases. Purified, recombinant human LASP or its mutants S61D, S146D, and S61/146D or VASP (each 1 µM) were phosphorylated by either cGK types 1α, 1β, and II or the C subunit of cAK (each 0.05 µM) in a total volume of 20 µl for 30 min as described under "Experimental Procedures". Proteins were resolved by SDS-PAGE and the phosphorylated proteins visualized by autoradiography. The autoradiogram shown is representative of three separate experiments.

Fig. 5: In vivo phosphorylation of LASP expressed in PtK-2 cells. LASP- deficient PtK-2 cells were transfected with wild-type LASP or the mutants S61A and S146A, and cGK 1β
simultaneously or with either protein alone. Cells were stimulated 20 min with 50 µM 8pCPT-cGMP for cGK activation or 20 min with 5 µM forskolin for cAK activation and this was followed by immunoprecipitation of LASP. Immunoprecipitates were analyzed by SDS-Page (total LASP) and then subjected to autoradiography to reveal [32P]-incorporation (upper panels). The autoradiograms shown are representative of two separate experiments.

**Fig. 6: Expression of LASP in human tissues and cell lines.** A rabbit polyclonal LASP antibody was used to detect LASP protein by Western blot analysis of crude cell lysates from various human tissues and different cell lines. **A:** Human platelets, brain, heart, kidney, lung, and liver. **B:** Human embryonic kidney (HEK293), human umbilical vein endothelial cells (HUVEC), PtK-2 cells, human fibroblasts, COS-7 cells, and human smooth muscle cells (SMC).

**Fig. 7: Binding of LASP to F-actin.** Cosedimentation analysis of 2 µM pre-polymerized actin with 1 µM wild-type LASP (WT) or the phosphorylation-mimicking mutant S146D. 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. 8: Subcellular localization of LASP.** Immunofluorescence microscopy of the subcellular distribution of wild-type LASP and the mutants S146A and S146D in PtK-2 cells. Cells were fixed with paraformaldehyde and permeabilized, and LASP was immunostained with the LASP polyclonal antibody and Cy3-labeled goat anti-rabbit secondary antibody. Actin was stained with oregon green phalloidin. Wild-type LASP (A, D) and LASP S146A (B) are predominantly present at the leading edges and focal contacts of cells (indicated by arrows) while the LASP mutant S146D is mainly localized in the cytosol (C). The co-localisation of LASP and F-actin is demonstrated by double-staining in D and E (arrows) while no LASP binding to actin stress fibers (arroheads in E) is observed.

**Fig. 9: Migration.** Relative migration of uninfected PtK2 cells (Co) and PtK2 cells expressing wild type LASP (WT) or mutant LASP S146D (S146D). Cell migration was measured over 4 h in a Transwell® cell culture chamber. At least 4 chambers from 2 separate experiments
were analyzed (* value significant different from that of Co and WT by t-test; p < 0.001). Western blot of untransfected control cells and LASP-expressing cells probed with anti-LASP polyclonal antibody are shown in the lower panel.
Fig. 2

LASP →

Co  cAK  cGK
Fig. 5
Fig. 6
Fig. 9
Actin binding of human LIM and SH3 protein (LASP) is regulated by cGMP- and cAMP-dependent protein kinase phosphorylation on Ser-146

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