Phosphatidylinositol Phosphate Kinase Type Iγ Directly Associates with and Regulates Shp-1 Tyrosine Phosphatase*

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Tyrosine phosphorylation plays a critical role in many regulatory aspects of cellular signaling, and dephosphorylation of phosphotyrosine residues is crucial for termination of signals initiated by tyrosine kinases. Previous work has shown that the tyrosine kinase Src phosphorylates Tyr644 on phosphatidylinositol phosphate kinase type I (PIPKI)γ661 in a focal adhesion kinase-dependent manner. Phosphorylation of this residue is essential for high affinity binding of PIPKIγ661 to the focal adhesion protein talin and for targeting of PIPKIγ661 to focal adhesions. A yeast two-hybrid screen performed with the C-terminal 178-amino acid tail of PIPKIγ661 identified an interaction with the phosphatase domain of the tyrosine phosphatase Shp-1. The interaction between PIPKIγ661 and Shp-1 was confirmed via co-immunoprecipitation from HEK293 cell lysates. In addition, Src-phosphorylated PIPKIγ661 is a substrate for Shp-1, and Shp-1 modulates both the association between PIPKIγ661 and talin and the targeting of PIPKIγ661 to focal adhesions in mammalian cells. Finally, we showed that Shp-1 phosphatase activity is inhibited by the product of PIPKIγ661, phosphatidylinositol 4,5-bisphosphate, in vitro. These combined results suggest a model in which the reciprocal actions of Src tyrosine kinase and Shp-1 tyrosine phosphatase dynamically regulate the association between PIPKIγ661 and talin.

Phosphorylation of tyrosine residues is an important regulatory mechanism for numerous signaling pathways in cells (1, 2). These pathways are initiated by the catalytic action of tyrosine kinases. One of the many roles for phosphotyrosine residues generated by these kinases is to serve as docking sites for phosphotyrosine binding domain- and Src homology 2 (SH2) domain-containing proteins. Not only does this target SH2 domain-containing proteins to these sites but, in many cases, the association modulates the activity of the assembled protein. Termination of these signaling pathways then occurs via dephosphorylation by tyrosine phosphatases.

At least 107 genes have been identified as coding for protein tyrosine phosphatases, and they have been subdivided into four distinct classes (3). The SH2 domain-containing phosphatase Shp-1 and its functionally distinct homologue, Shp-2, are members of the class 1 nonreceptor protein tyrosine phosphatases. These phosphatases have two tandem SH2 domains at their N termini that serve as regulators of catalytic activity (4, 5). Shp-1 has typically been regarded as a negative regulator of cellular functions, whereas Shp-2 has been found to serve a more positive role in signaling (6).

Study of Shp-1 function has been greatly aided by the discovery of the motheaten (me) and motheaten viable (mev) allele mutant phenotypes observed in mice (7, 8). The name is derived from the phenotypic patchy hair loss resulting from the formation of dermal abscesses. These mice exhibit severe defects in control of hematopoietic cell development and proliferation (8, 9). Increased extracellular matrix adherence and significant deficiencies in cell motility have also been observed in macrophages and neutrophils derived from these mice (10, 11). Numerous other functions have been identified for Shp-1, including roles in receptor tyrosine kinase signaling, regulation of the Janus kinase/signal transducers and activators of transcription pathway, apoptosis, and inhibitory immunoreceptor signaling (6, 12–14). Recently, Shp-1 has been linked to regulation of actin dynamics via dephosphorylation of both actin filaments and the actin cross-linker α-actinin (15, 16).

The type I phosphatidylinositol phosphate kinases (PIPKIs) are also key modulators of the actin cytoskeleton. These enzymes generate the lipid second messenger phosphatidylinositol 4,5-bisphosphate (PI4,5P2), which serves as an important regulator of the actin cytoskeleton by directly stimulating proteins involved in actin polymerization, such as N-WASP and the Arp2/3 complex, while inhibiting actin-severing proteins, such as gelsolin (17).

Structures important for cellular adhesion and migration known as focal adhesions (FAs) are also highly linked to the actin cytoskeleton. Similarly, PI4,5P2 is also a major regulator in several aspects of FA signaling (18, 19). Previously, PIPKIγ661 was shown to be specifically targeted to FAs via a direct association with the FA scaffolding protein talin (20, 21). This is thought to result in generation of a highly localized pool of PI4,5P2 at FAs. PI4,5P2 may then facilitate integrin activation via direct modulation of the talin band 4.1, ezrin, radixin, moesin homology domain, resulting in enhanced binding of talin to β1 integrin (22).

Elucidating the underlying mechanism for targeting of PIPKIγ661 to talin is key for understanding how FAs may be activated by PI4,5P2. PIPKIγ661 is tyrosine-phosphorylated by Src on Tyr644 of its C terminus (23). This phosphorylation event
PIPKI-661 Associates with Shp-1 Phosphatase

was made by mutation of residue Tyr278 into a stop codon using the restriction site (5′-GCCATCCTAGG-GAGAGAATATGAGTGGACGTGG-3′) following mutagenic primers and their complements: 5′-ATAGTTCTAATCTGTGACCAACTGC-3′ and 5′-GGCATCCTAGGTAGACGGAGG-3′. The resulting PCR fragment was then subcloned into pcDNA3.1 (Expressing PIPKI-661) using primers that incorporated a 5′ EcoRI restriction site (5′-ATTAGATCTAGCTGAGCCTGTTG-3′) and a 3′ XhoI restriction site (5′-GGGCGATTCGTAATCACCCTCCTCTTTGAGG-3′). The resulting PCR fragment was then subcloned into pcDNA3.1 (Invitrogen) and subcloned by serial dilution. using FuGENE 6. Stably transfected cells were selected via neomycin sulfate (Invitrogen) and subcloned by serial dilution.

Expression Constructs—Murine PIPKI-661, PIPKI-635, and kinase-dead PIPKI-661 (PIPKI-661kd) mammalian and bacterial expression vectors were described previously (21, 23). The 483x C-terminal truncation of PIPKI-661 was generated by introducing a stop codon in place of amino acid Glu683 using the QuikChange mutagenesis kit (Stratagene) according to the manufacturer’s protocol using the following mutagenic primer and its complement: 5′-GCCATCCCGAC-GAGAGAATATGAGTGGACGTGG-3′ and 5′-GCCATCCTAGGTAGACGGAGG-3′. The resulting PCR fragment was then subcloned into pcDNA3.1(Invitrogen), pET28, and pET42 (Novagen) expression vectors. The Shp-1 C545S mutant was made using QuikChange using the following mutagenic primer and its complement: 5′-CCATCATGCTCCTACGGCAGCCGCGCATCG. The Shp-1 SH2 domain truncation was made by mutation of residue Tyr277 into a stop codon using the QuikChange method and the following mutagenic primer and its complement: 5′-GGCGAAGACCCGTAAAGACACCTTCCC-3′. The following Shp-1 truncations were made in the same fashion with the following mutagenic primers and their complements: 5′-GCCCTCGCCCTACGAGGGCGCGAGG-3′ and 5′-CCCTCGGCTCTAGTGCAGAAGGGCC-3′; 5′-GGGCATCCTAGGTAGACGGAGG-3′ and 5′-GCGCATCCTAGGTAGACGGAGG-3′; 5′-CGCGCATCCTAGGTAGACGGAGG-3′ and 5′-GGGGCTACCATTAGCTGGGCT-3′; and 5′-CGCTGTATTTGGGCCTAAATTGCTGAGCAGAAGG-3′.

The Shp-1 SH2 domain truncation was generated by digestion of the Shp-1 open reading frame with Smal and XhoI. The resulting fragment was subcloned into pET42b using EcoRI and XhoI restriction sites. Shp-1T22 was cloned by reverse transcription-PCR from the mammalian expression vector. The c-Src and HA-tagged FAK mammalian expression constructs were gifts from Dr. Patricia J. Keely (University of Wisconsin, Madison, WI). The c-Src open reading frame was cloned by PCR from the mammalian expression vector using primers that incorporated a 5′ BamHI restriction site (5′-CCGGATCCGCCAACATCATGAGCAGACGAGGAGG-3′) and a 3′ XhoI restriction site (5′-GCTGCTGCTACATGGATCTGAACTTCTTCTCTGTG-3′). The resulting fragment was subcloned into a pCMV-Tag3B (Stratagene) expression vector. The c-Src mammalian expression constructs were gifts from Dr. Patricia J. Keely (University of Wisconsin, Madison, WI). The c-Src open reading frame was cloned by PCR from the mammalian expression vector using primers that incorporated a 5′ Ncol restriction site (5′-ATCATGGATCCCTATCAGGCGGACGACGAGAAGGAGGAGG-3′) and a 3′ BamHI restriction site (5′-ATGGATCCCTATCAGGCGGACGACGAGAAGGAGGAGG-3′). The resulting fragment was subcloned into the pALTER-Ex2 (Promega) bacterial expression vector.

Cell Cultures and Transfection—HEK293 cells and NRK cells were cultured using Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. NRK cells were plated on glass coverslips and transfected using FuGENE 6 (Roche Applied Science) following the manufacturer’s instructions. The cells were then used for immunofluorescence 16 h after transfection. HEK293 cells were transfected via calcium phosphate with 2 μg of each expression vector. The cells were used for immunoprecipitation 48 h after transfection. Stable NRK cells expressing PIPKI-661 were generated by transfection with PIPKI-661 using FuGENE 6. Stably transfected cells were selected via neomycin resistance by supplementing the media with 1 mg/ml Geneticin G418 sulfate (Invitrogen) and subcloned by serial dilution.

Antibodies—An anti-phosphotyrosine (4G10) and anti-Src (EC10) were obtained from Upstate Biotechnology. Monoclonal mouse anti-phosphotyrosine (4G10) and anti-Src (EC10) were obtained from Upstate Biotechnology. Monoclonal mouse anti-HA (ab1612) was obtained from Covance. Monoclonal mouse anti-talin (8d4) was obtained from Sigma-Aldrich. Horseradish peroxidase-conjugated anti-OST antibody was purchased from Amersham Biosciences, and horseradish peroxidase-conjugated anti-T7 antibody was obtained from Novagen. Polyclonal PIPKIγ rabbit anti-serum was generated and purified as described previously (21). Secondary antibodies were obtained from Jackson ImmunoResearch Laboratories

Immunoprecipitation and Immunoblotting—Cells were washed twice with ice-cold PBS and subsequently resuspended and lysed in immunoprecipitation buffer (50 mm Tris-HCl, pH 7.4, 150 mm NaCl, 0.2% Nonidet P-40, 2 mm NaN3, 1 mm EDTA, 1 mm EGTA, and 1 mm MgCl2) supplemented with Complete Protease Inhibitor Mixture (Roche Applied Science). Cell lysates were incubated with 50 μl of 1:1 diluted protein A-Sepharose and 2 μg of the specified antibody as indicated at 4 °C overnight. The immunocomplexes were extensively washed, separated by 7.5% or 10% SDS-PAGE, and transferred to polyvinylidene difluoride (Millipore Corp.). Chemiluminescent substrate (Pierce) was used for visualization on X-ray film (RPI Corp.).

Protein Expression and Purification in Escherichia coli—Constructs in pET-28b were transformed into BL21(DE3) competent cells (Novagen). Proteins were expressed and purified using His-Bind Resin following the manufacturer’s instructions (Novagen) or using glutathione-Sepharose 4B Fast Flow as per the manufacturer’s instructions (Amersham Biosciences).

GST Pull-down Assays—Recombinant T7-tagged PIPKIγ was incubated with GST-Shp-1 together with glutathione-Sepharose 4 Fast Flow beads (21) in 500 μl of binding buffer (50 mm HEPES, pH 7.5, 150 mm NaCl, 2 mm dithiothreitol) for 4 h or overnight at 4 °C. The beads were washed with 1 ml of buffer B four times, resolved by SDS-PAGE, and analyzed via Western blot. GST was used as a control for nonspecific binding. All other GST pull-down assays were performed with the proteins indicated in the same manner.

Expression of c-Src-phosphorylated PIPKIγ—Bacterial expression constructs of Hi-tagged PIPKIγ (DE3) were transformed into BL21(DE3) competent cells (Novagen). The phosphorylated PIPKIγ proteins were expressed and then purified with His-Bind Resin following the manufacturer’s instructions (Novagen).

In Vivo Dephosphorylation of PIPKIγ—PIPKIγ-661 was immunoprecipitated from HEK293 cells co-transfected with PIPKIγ-661 and c-Src. Shp-1 was linked to a Hi-tag by PCR from HEK293 cells and the immunoprecipitate was then purified twice in phosphate buffer and twice in phosphate buffer (100 mm HEPES, pH 7.4, 150 mm NaCl, 1 mm EDTA, and 5 mm diethiothreitol). The immunoprecipitates were then split equally into two tubes for treatment with Shp-1 or mock treatment. Treatment consisted of addition of 2 μg of Shp-1 to the immunoprecipitate and incubation at 37 °C for 1 h. The reactions were then quenched with SDS sample buffer, boiled, and resolved by SDS-PAGE. Bacterially expressed, c-Src-phosphorylated PIPKIγ was dephosphorylated in phosphate buffer in the same manner.

Immunofluorescence and Microscopy—NRK cells were washed with PBS at room temperature 16 h after transfection, fixed by 4% paraformaldehyde in PBS at room temperature for 15 min, and permeabilized with 0.2% Triton X-100 in PBS at room temperature for 10 min. The cells were blocked with 3% bovine serum albumin in PBS at room temperature for 1 h, incubated with the primary antibody for 1 h at 37 °C, and washed with 0.1% Triton X-100 in PBS. The cells were then incubated with fluorophore-labeled secondary antibody and secondary antibody at room temperature for 45 min and washed with 0.1% Triton X-100 in PBS. The coverslips were mounted to glass slides in Vectashield (Vector Laboratories) mounting medium. Fluorescent images were captured using a Zeiss Axiosvert 135 microscope with a CoolSNAP charge-coupled device camera (BS Photographics) or using a ×60 Plan oil immersion lens on a confocal laser scanning microscope (model MR-1000; Bio-Rad Laboratories) mounted transversely to an inverted microscope (Diaphot 200, Nikon; W. M. Keck Laboratory for Biological Imaging, Madison, WI). Images were processed as described previously using Photoshop 8.0 (21).

Phosphotyrosine “Prophosphophosphatase” Assays—Phosphotyrosine phosphatase assays were performed with 2 μg of Shp-1 dissolved in phosphate buffer with or without lipid at a final volume of 100 μl. The reactions were preincubated at 37 °C for 10 min prior to addition of p-NPP. After 20 min, the reactions were quenched with 1 μl NaOH. The amount of p-nitrophenol ion produced was assessed by absorbance at 405 nm. All phosphoinositides used in these experiments were purchased from Echelon Biosciences.
which includes nearly half of the phosphatase domain. Shp-1 is expressed as three distinct splice variants: a 595-residue hematopoietic variant, a 597-residue epithelial variant, and a recently identified 624-residue “long” variant termed Shp-1L (24). The epithelial and hematopoietic variants differ only in the first 5 residues of their N termini. Shp-1L, however, contains an alternatively spliced exon, resulting in a distinct C terminus. The yeast two-hybrid fragment did not contain this alternative C terminus. Therefore, we focused on the epithelial (595-residue) variant of human Shp-1 for characterizing the interaction with PIPKIγ661.

The direct interaction between Shp-1 and PIPKIγ661 was first confirmed via GST pull-down assays. Both proteins were expressed in E. coli and purified as either His-tagged or GST-tagged fusion proteins. His-tagged Shp-1 and GST-tagged PIPKIγ661 were incubated in the presence of glutathione-conjugated Sepharose beads. After the beads were extensively washed, His-tagged Shp-1 remained bound to the GST-tagged PIPKIγ661 but did not bind to the GST control (Fig. 1A). This result was consistent in the reciprocal experiment because His-tagged PIPKIγ661 also bound to GST-tagged Shp-1 but not to GST. Interestingly, the interaction observed in the GST pull-down assays is not dependent on tyrosine phosphorylation of PIPKIγ661 because the recombinant proteins used in this experiment were not phosphorylated.

We next assessed the interaction in vivo by immunoprecipitating endogenous Shp-1 from HEK293 cells and blotting for associated PIPKIγ. As seen in Fig. 1B, endogenous PIPKIγ was associated with Shp-1 immunoprecipitates but was not significantly detected in the normal mouse IgG control. We further confirmed these results by co-expressing Shp-1 and PIPKIγ661 in HEK293 cells. Shp-1 was immunoprecipitated with PIPKIγ661 using a polyclonal antibody specific for the C terminus of PIPKIγ661 (Fig. 1C). The reciprocal experiment yielded similar results because PIPKIγ661 was immunoprecipitated with a monoclonal antibody specific for Shp-1. Neither protein was immunoprecipitated with normal mouse or rabbit IgG. These results and those observed in the GST pull-down experiments independently confirm a direct interaction between Shp-1 and PIPKIγ661. However, nonspecific binding of phosphatases is often problematic in immunoprecipitation experiments. Therefore, as an additional control, we co-expressed the highly homologous tyrosine phosphatase, Shp-2, with PIPKIγ661 in HEK293 cells. Under identical conditions, Shp-2 was not co-immunoprecipitated with PIPKIγ661 (Fig. 2A). This result indicates that the interaction between Shp-1 and PIPKIγ661 is quite specific.

The Phosphatase Domain of Shp-1 Binds to the C Terminus of PIPKIγ—These experiments, however, did not address the specific regions involved in the interaction between Shp-1 and PIPKIγ661. Because PIPKIγ is also expressed as a shorter splice variant, PIPKIγ635, we performed co-immunoprecipitations using both splice variants. Our results indicate that PIPKIγ635 is also capable of binding to Shp-1 (Fig. 2A) because both splice variants were immunoprecipitated with Shp-1. Additionally, phosphatidylinositol phosphate kinase activity does not appear to be required for this interaction because the kinase-dead PIPKIγ661 (Fig. 2A, Iγ661 KD) was immunoprecipitated with Shp-1 at levels similar to the wild-type enzyme.

Because the yeast two-hybrid screen indicated that the last 178 amino acids of PIPKIγ661 were sufficient for binding Shp-1, we focused on this region of PIPKIγ661 by utilizing C-terminal truncations. Using the co-immunoprecipitation approach, we co-expressed Shp-1 with PIPKIγ661, PIPKIγ635, or a C-terminal truncation of the last 178 amino acids generated by mutation of Glu183 into a stop codon (Fig. 2B, Iγ483x). As shown in Fig. 2B, the results for the two splice variants of PIPKIγ were consistent with those shown in Fig. 2A. However, truncation of the last 178 amino acids of PIPKIγ661 resulted in a significant reduction of immunoprecipitated Shp-1, indicating that this region of PIPKIγ661 is necessary for efficient binding to Shp-1.

A GST pull-down approach was then used to determine the specific binding site for PIPKIγ661 on Shp-1. GST fusions of full-length Shp-1, the N-terminal SH2 domains (residues 1–277), and the C-terminal phosphatase domain (residues 300–597) were first used to identify the PIPKIγ661 binding site. As seen in Fig. 2C, PIPKIγ661 preferentially binds to the C-terminal phosphatase domain of Shp-1. To refine the putative binding site, we generated a series of C-terminal Shp-1 truncations by mutating the codons encoding amino acids 560, 529, 502, 427, and 379 into stop codons. These residues were selected from regions of random coil within the Shp-1 crystal structure (25). Unfortunately, the truncations of Shp-1 beyond amino acid 529 were insoluble when expressed in E. coli. However, the two soluble truncations, Shp-1 560x and Shp-1 529x, bound to GST-tagged PIPKIγ661 but did not bind to GST alone (Fig. 2D). These results suggest that PIPKIγ661 binds to Shp-1 via the N-terminal portion of the phosphatase domain, which would include the active site of the phosphatase. As seen in the immunoprecipitation experiments, Shp-2 was not capable of binding to GST-tagged PIPKIγ661, further demonstrating that the interaction between Shp-1 and PIPKIγ661 is specific.
Phosphorylated PIPKIγ661 Is a Substrate for Shp-1 Both in Vivo and in Vitro—PIPKIγ661 becomes strongly tyrosine-phosphorylated upon adhesion to extracellular matrix ligands such as type I collagen, and we have previously shown that Src phosphorylates Tyr^{644} of PIPKIγ661 in a FAK-dependent manner (21, 23). Therefore, we sought to determine whether Shp-1 is capable of dephosphorylating PIPKIγ661. Co-expression of c-Src with PIPKIγ661 in HEK293 cells results in a strong tyrosine phosphorylation of PIPKIγ661, as demonstrated by Western blot using a phosphotyrosine-specific antibody (Fig. 3A). However, co-expression of Shp-1 with c-Src and PIPKIγ661 significantly attenuated the level of PIPKIγ661 tyrosine phosphorylation. The catalytically inactive mutant (C455S) of Shp-1, when co-expressed in a similar manner, has no effect on the phosphorylation level of PIPKIγ661 (data not shown). These combined results suggest that Shp-1 dephosphorylates PIPKIγ661 when expressed alone in HEK293 cells, although to a much lower extent than when it is co-expressed with c-Src, and co-expression with Shp-1 also dephosphorylates PIPKIγ661 under this condition (data not shown). These combined results suggest that Shp-1 dephosphorylates PIPKIγ661 in vitro and that expression of Shp-1 attenuates Src-mediated tyrosine phosphorylation of PIPKIγ661 in HEK293 cells.

To confirm that Shp-1 is capable of dephosphorylating PIPKIγ661, phosphorylated PIPKIγ661 was immunoprecipitated from HEK293 cells co-transfected with PIPKIγ661 and c-Src and used as a substrate for phosphatase assays. After washing with phosphatase buffer, the immunoprecipitates were either mock-treated or treated with recombinant Shp-1 phosphatase. As seen in Fig. 3B, Shp-1 dephosphorylated PIPKIγ661 in vitro, whereas mock-treated PIPKIγ661 remained strongly tyrosine-phosphorylated. To ensure that these results were due to direct dephosphorylation by Shp-1, we expressed recombinant phosphorylated PIPKIγ661 via co-expression with Shp-1 and the HA epitope. The precipitated PIPKIγ661 was resolved by SDS-PAGE and analyzed by Western blot with a phosphotyrosine-specific antibody. The precipitated PIPKIγ661 was resolved by Western blot and analyzed by Western blot as indicated (pY, anti-phosphotyrosine antibody). B, Shp-1 dephosphorylates PIPKIγ661 in vitro. Src-phosphorylated PIPKIγ661 was immunoprecipitated as described in A. The precipitated PIPKIγ661 was washed with phosphatase buffer and either mock-treated or treated with recombinant Shp-1 for 1 h at 37°C. The level of tyrosine phosphorylation was determined by Western blot with a phosphotyrosine-specific antibody. C, recombinant Src-phosphorylated PIPKIγ661 is dephosphorylated by Shp-1 in vitro. T7-tagged PIPKIγ661 and PIPKIγ635 were co-expressed with Src in E. coli. The resulting tyrosine-phosphorylated PIPKIγ661 was purified and either mock-treated or treated with recombinant Shp-1. The level of tyrosine phosphorylation was determined as described in A.
PIPKIγ661 Associates with Shp-1 Phosphatase

**Fig. 4.** Shp-1 attenuates the FAK-mediated enhancement of the PIPKIγ661-talin association. HEK293 cells were co-transfected with 2 μg of each of the indicated constructs using calcium phosphate. After 48 h, PIPKIγ was immunoprecipitated with polyclonal antibodies specific for the C terminus of PIPKIγ661. The immunoprecipitates were resolved by SDS-PAGE and analyzed by Western blot. HA-tagged FAK was detected using a mouse monoclonal anti-HA antibody. All other proteins were detected as indicated.

Expression of PIPKIγ661 or PIPKIγ635 with c-Src in E. coli. The purified phosphorylated PIPKIγ was then mock-treated or treated with Shp-1 in vitro in the same manner used with immunoprecipitated PIPKIγ661. The results were similar because Shp-1 was able to effectively dephosphorylate PIPKIγ661 (Fig. 3C). Interestingly, PIPKIγ635 was weakly phosphorylated upon co-expression with c-Src in bacteria, which has also been observed in mammalian cells (data not shown). This result indicates that residues other than Tyr644 may be phosphorylated by c-Src under these conditions and that Shp-1 may be able to dephosphorylate other tyrosine-phosphorylated residues within the C terminus of PIPKIγ.

**Shp-1 Attenuates the FAK-mediated Association between PIPKIγ661 and Talin** —We have shown that FAK signaling via Src results in a dramatic increase in the phosphorylation level of PIPKIγ661 in our previous work (21, 23). This increased level of phosphorylation leads to a corresponding increase in the affinity of PIPKIγ661 for talin, which has been observed both in vivo and in vitro. To determine whether Shp-1 might play a role in regulation of this association in vivo, we co-expressed PIPKIγ661, FAK, and phosphatase-active or -inactive Shp-1 in HEK293 cells. Upon co-expression with FAK, PIPKIγ661 becomes strongly tyrosine-phosphorylated and binds to talin with much higher affinity (Fig. 4), which is consistent with our previous results (21, 23). However, upon co-expression with active Shp-1, both the level of PIPKIγ661 phosphorylation and the amount of associated talin are decreased to basal levels. This effect is not seen, however, when co-expressing the phosphatase-inactive Shp-1 C455S. These results indicate that Shp-1 is able to modulate the affinity of PIPKIγ661 for talin in vivo.

**Shp-1 Disrupts Targeting of PIPKIγ661 to Focal Adhesions in NRK Cells** —Expression of PIPKIγ661 in NRK cells results in dramatic targeting of PIPKIγ661 to talin at FAs (21). This targeting requires both Tyr644 of the PIPKIγ661 C terminus and Src kinase activity (23). To address a role for Shp-1 in the regulation of the PIPKIγ661-talin association in vivo, NRK cells were co-transfected with PIPKIγ661 and wild type Shp-1. Expression of wild type Shp-1 resulted in a strong nuclear staining and diffuse cytosolic staining, which has been previously observed in other cell lines (26, 27). At low levels of Shp-1 expression, the targeting of PIPKIγ661 to focal adhesions was unchanged, possibly due to an accumulation of Shp-1 in the nucleus. However, at higher levels of Shp-1 expression, the cytosolic staining of Shp-1 was predominantly increased. These cells also displayed a more diffuse cytosolic staining of PIPKIγ661, with a diminished focal adhesion staining pattern (Fig. 5A). Additionally, the observed PIPKIγ661 focal adhesion staining was only partially maintained at the cell periphery. These results were not seen, however, in cells expressing high levels of the phosphatase-inactive mutant Shp-1 C455S (Fig. 5B) because those cells displayed a strong targeting of PIPKIγ661 to FAs throughout the cell surface. The homologous phosphatase Shp-2 also did not have an impact on the targeting of PIPKIγ661 to focal adhesions (Fig. 5B). To ensure that the disrupted targeting of PIPKIγ661 was not due to a general reduction of cellular phosphotyrosine levels, we expressed Shp-1 in NRK cells and visualized phosphotyrosine levels with a phosphotyrosine-specific antibody. Under these conditions, Shp-1 expression did not affect the overall phosphotyrosine staining pattern as compared with adjacent nontransfected cells (Fig. 5C). This likely indicates that Shp-1 dephosphorylates only a specific subset of targets at FAs, including PIPKIγ661.

Because variable expression levels resulting from transient transfection could be attributed to the change in PIPKIγ661 targeting, we generated an NRK cell line stably expressing PIPKIγ661. These cells, when transiently transfected with Shp-1, showed a phenotype similar to that observed when PIPKIγ661 was transiently transfected (Fig. 5A). As seen in Fig. 5D, in cells in which Shp-1 expression was elevated, PIPKIγ661 staining was diffusely cytosolic, with some focal adhesion staining at the cell periphery. These peripheral focal adhesions are likely sites of elevated tyrosine kinase activity because FA assembly would be most highly active on the spreading edges of the cell. It is therefore possible that this observed phenotype might simply be the result of kinase activity prevailing over phosphatase activity at these sites. Still, the combined results from Fig. 5 suggest that Shp-1 may serve as an important regulator for the targeting of PIPKIγ661 to talin in FAs via dephosphorylation of Tyr644 in the C terminus of PIPKIγ661.

**PI4,5P2 Inhibits Shp-1 Phosphatase Activity in Vitro** —Previous work has shown that Shp-1 phosphatase activity can be modulated by lipids in vitro (28, 29). Specifically, Shp-1 was found to be stimulated up to 25-fold by low micromolar concentrations of phosphatidic acid. These same studies reported that Shp-1 was capable of binding to PI4,5P2. Because Shp-1 directly interacts with PIPKIγ661, it is possible that PI4,5P2 generated by PIPKIγ661 might serve to regulate the catalytic activity of Shp-1. To test this hypothesis, in vitro p-NPP phosphatase assays were performed in the presence of PI4,5P2 micelles. As shown in Fig. 6A, a 10 μM concentration of PI4,5P2 lowered Shp-1 phosphatase activity to <5% of the activity in the absence of lipid. Because previous experiments have shown that some negatively charged lipids have an inhibitory effect on Shp-1 activity, the effect of other phosphoinositides was examined (28). As shown in Fig. 6B, the substrate for PIPKIγ661, phosphatidylinositol 4-phosphate, did not have a significant effect on Shp-1 phosphatase activity with concentrations of lipid up to 20 μM. However, the substrate for the type II phosphatidylinositol phosphate kinases, phosphatidylinositol 5-phosphate, did have an inhibitory effect, but at approximately a 10-fold higher concentration as compared with PI4,5P2. Because phosphatidylinositol 5-phosphate is one of the rarest phosphoinositides in vivo, it is not likely that it would serve as a significant regulator of Shp-1 activity. Nevertheless, these combined results suggest that Shp-1 phosphatase activity may be regulated by PIPKIγ661 kinase activity via direct inhibition by PI4,5P2.
Tyrosine phosphorylation serves as a major regulatory mechanism for PIPKIγ/H9253661 function at FAs (21, 23). It both enhances PIPKIγ/H9253661 targeting to talin at FAs and stimulates PI4,5P2 production at these sites. However, the specific phosphatase responsible for dephosphorylation of PIPKIγ/H9253661 remained to be determined. Here we have shown that the tyrosine phosphatase Shp-1 directly interacts with PIPKIγ/H9253661 and is capable of dephosphorylating PIPKIγ/H9253661 both in vitro and in vivo. In addition, Shp-1 effectively disrupts the enhanced association between PIPKIγ/H9253661 and talin mediated by FAK in vivo.

Incorporating both our previous and current results, the following model for regulation of PIPKIγ/H9253661 function via tyrosine phosphorylation can be drawn (Fig. 7).

Upon FAK-dependent Src activation, PIPKIγ/H9253661 becomes tyrosine-phosphorylated, and its affinity for talin becomes greatly enhanced. This results in a strong targeting of PIPKIγ/H9253661 to talin-containing FAs (21, 23). Phosphotyrosine was visualized with an antibody specific for phosphotyrosine (red), and Shp-1 (green) was visualized as described in A. D, NRK cells stably expressing PIPKIγ/H9253661 were transfected with 1 µg of Shp-1 as described in A. PIPKIγ/H9253661 and Shp-1 were visualized with antibodies specific for PIPKIγ/H9253661 (green) or Shp-1 (red).

FIG. 5. Shp-1 expression disrupts targeting of PIPKIγ/H9253661 to focal adhesions. A, NRK cells were co-transfected with 1 µg of Shp-1 and 0.1 µg of PIPKIγ/H9253661 via FuGENE 6 transfection reagent for 16 h. PIPKIγ/H9253661 and Shp-1 were visualized with antibodies specific for PIPKIγ/H9253661 (red) or Shp-1 (green). Shp-1 expression level was assessed by fluorescence intensity. Scale bar, 10 µm. B, NRK cells were co-transfected as described in A with 0.1 µg of PIPKIγ/H9253661 and 1 µg of phosphatase-inactive Shp-1 C455S or wild type Shp-2. PIPKIγ/H9253661 and Shp-1 C455S were visualized as described in A. Shp-2 was visualized with a monoclonal antibody specific for Shp-2 (green).

FIG. 6. PI4,5P2 inhibits Shp-1 phosphatase activity in vitro. A, in vitro phosphatase assay using recombinant Shp-1 and p-NPP as the substrate. PI4,5P2 micelles were added over a range of 0.1–13 µM. The data were normalized to the amount of p-NPP cleaved by Shp-1 without lipid. The results were plotted against the log of the PI4,5P2 concentration, and the data were fit using GraphPad Prism3. B, in vitro phosphatase assay performed as described in A. PI4,5P2, phosphatidylinositol 4-phosphate (PI4P), and phosphatidylinositol 5-phosphate (PI5P) were added over a range of 0.1–20 µM. The triplicate results were normalized, plotted, and fit as described in A.

FIG. 7. Tyrosine phosphorylation serves as a major regulatory mechanism for PIPKIγ/H9253661 function at FAs (21, 23). It both enhances PIPKIγ/H9253661 targeting to talin at FAs and stimulates PI4,5P2 production at these sites. However, the specific phosphatase responsible for dephosphorylation of PIPKIγ/H9253661 remained to be determined. Here we have shown that the tyrosine phosphatase Shp-1 directly interacts with PIPKIγ/H9253661 and is capable of dephosphorylating PIPKIγ/H9253661 both in vitro and in vivo. In addition, Shp-1 effectively disrupts the enhanced association between PIPKIγ/H9253661 and talin mediated by FAK in vivo.

Incorporating both our previous and current results, the following model for regulation of PIPKIγ/H9253661 function via tyrosine phosphorylation can be drawn (Fig. 7). Upon FAK-dependent Src activation, PIPKIγ/H9253661 becomes tyrosine-phosphorylated, and its affinity for talin becomes greatly enhanced. This results in a strong targeting of PIPKIγ/H9253661 to talin-containing FAs (21, 23). Phosphotyrosine was visualized with an antibody specific for phosphotyrosine (red), and Shp-1 (green) was visualized as described in A. D, NRK cells stably expressing PIPKIγ/H9253661 were transfected with 1 µg of Shp-1 as described in A. PIPKIγ/H9253661 and Shp-1 were visualized with antibodies specific for PIPKIγ/H9253661 (green) or Shp-1 (red).
phospholipase C, which generates inositol 1,4,5-trisphosphate and diacylglycerol, leading to stimulation of other downstream targets. Shp-1 attenuates Src signaling on PIPKI-661 via dephosphorylation of the C-terminus of PIPKI-661, which results in diminished affinity of PIPKI-661 for talin. Consequently, this would have the net effect of lowered PI4,5P2 generation at focal adhesions, possibly promoting FA disassembly or turnover. This mechanism would result in a highly dynamic regulation of the PIPKI-661-talin interaction via the opposing activities of Src and Shp-1.

Localized PI4,5P2 generation might also serve as positive feedback for PIPKI-661 targeting to talin because Shp-1 activity was inhibited in the presence of low micromolar amounts of PI4,5P2 in vitro. This was surprising, considering that the majority of work to date has documented a stimulatory role for phospholipids in terms of regulation of Shp-1 activity. Specifically, phosphatidic acid has been shown to have a stimulatory effect both in vitro and in vivo (28, 29, 34). However, previous in vivo observations by our laboratory may offer an explanation for a possible physiological role for the observed inhibition by PI4,5P2.

We have demonstrated that FAK expression results in a strong tyrosine phosphorylation of wild type PIPKI-661 via activation of Src (21). Interestingly, the kinase-inactive PIPKI-661kd showed a >20-fold lower level of tyrosine phosphorylation either with endogenous FAK expression or when co-expressed with dominant-active FAK (21). A possible explanation for these results could be regulation of PIPKI-661 dephosphorylation by Shp-1. Upon targeting to focal adhesions, PIPKI-661 would produce a highly localized pool of PI4,5P2. This might directly inhibit Shp-1 phosphatase activity and would consequently favor the phosphorylated state of PIPKI-661. Because PIPKI-661kd is unable to produce PI4,5P2, Shp-1 associated with PIPKI-661kd would have higher phosphatase activity as compared with Shp-1 associated with wild type PIPKI-661, and this would diminish PIPKI-661kd tyrosine phosphorylation. The low micromolar range of PI4,5P2 inhibition of Shp-1 observed is also similar to other proteins directly modulated by PI4,5P2, such as phospholipase D, gelsolin, and α-actinin (31, 33, 35, 36). Moreover, masking of this lipid by PI4,5P2-binding proteins, such as components of the actin cytoskeleton, conversion into phosphatidylinositol 3,4,5-trisphosphate by phosphatidylinositol 3-kinase, or dephosphorylation by lipid phosphatases could be other possible mechanisms for attenuation of this inhibition.

The role we have proposed for Shp-1 is also consistent with several other lines of evidence implicating Shp-1 as a possible regulator of focal adhesion dynamics. Macrophages derived from Shp-1-deficient “motheaten” mice show significantly increased adhesion to extracellular matrix ligands (10). Similarly, neutrophils derived from these mice have impaired cell motility as compared with normal cells in chemotactic assays (11). These observations would seem to indicate that regulation of FA turnover is disrupted in the absence of Shp-1 activity. Shp-1 has also been implicated in modulation of the actin cytoskeleton, a key component of FA regulation. Recent work has shown that Shp-1 directly dephosphorylates the actin cross-linker α-actinin (15). In the phosphorylated state, α-actinin shows a markedly lower affinity for actin filaments. This results in a more dynamic actin network, which is vital for reorganization of actin structures in motile cells. However, upon dephosphorylation by Shp-1, α-actinin becomes strongly associated with actin, which favors formation of a more static actin skeletal structure. These results, in addition to our observations, collectively suggest a role for Shp-1 in FA dynamics and turnover, possibly via regulation of PIPKI-661 tyrosine phosphorylation.

Cellular functions such as motility and chemotaxis are highly dynamic processes requiring an elevated rate of FA turnover. Highly localized and regulated PI4,5P2 generation, which results from specific targeting of PIPKI-661 to FA, is essential for regulation of FA turnover. We have offered a mechanism for how the PIPKI-661-talin association can be dynamically regulated by tyrosine phosphorylation of PIPKI-661 via the reciprocal actions of Src and Shp-1. Further characterization of the functional interaction between Shp-1 and PIPKI-661 may lead to a greater understanding of how complex functions such as cell motility are regulated.

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