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Regulation of SHP-1 Tyrosine Phosphatase in Human Platelets by Serine Phosphorylation at Its C Terminus*

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SHP-1 is a Src homology 2 (SH2) domain-containing tyrosine phosphatase that plays an essential role in negative regulation of immune cell activity. We describe here a new model for regulation of SHP-1 involving phosphorylation of its C-terminal Ser^{591} by associated protein kinase Cα. In human platelets, SHP-1 was found to constitutively associate with its substrate Vav1 and, through its SH2 domains, with protein kinase Cα. Upon activation of either PAR1 or PAR4 thrombin receptors, the association between the three proteins was retained, and Vav1 became phosphorylated on tyrosine and SHP-1 became phosphorylated on Ser^{591}. Phosphorylation of SHP-1 was mediated by protein kinase C and negatively regulated the activity of SHP-1 as demonstrated by a decrease in the *in vitro* ability of SHP-1 to dephosphorylate Vav1 on tyrosine. Protein kinase Cα therefore critically and negatively regulates SHP-1 function, forming part of a mechanism to retain SHP-1 in a basal active state through interaction with its SH2 domains, and phosphorylating its C-terminal Ser^{591} upon cellular activation leading to inhibition of SHP-1 activity and an increase in the tyrosine phosphorylation status of its substrates.

The reversible phosphorylation of Tyr residues is an important mechanism regulating protein function. The Tyr phosphorylation status, and subsequently the activity, of many proteins is determined by the opposing actions of kinases and phosphatases that are in many cases themselves regulated by phosphorylation. There are large numbers of protein-tyrosine kinases and protein-tyrosine phosphatases with distinct substrate specificity and modes of regulation. The SH2 [1] domain containing tyrosine phosphatases SHP-1 and SHP-2 are members of the protein-tyrosine phosphatase superfamily (1) and possess a catalytic domain with two SH2 domains at the N terminus and a C-terminal tail of poorly characterized function. Although closely related in structure, the roles of SHP-1 and SHP-2 in cells have been shown to be quite different. SHP-1 plays a largely negative signaling role (2) maintaining proteins in an inactive un-phosphorylated state, whereas SHP-2 plays a largely positive signaling role leading to cellular activation (3). The underlying reason for their different roles, despite structural similarity, is still not clear but may partially reside in different mechanisms of regulation of the phosphatases.

Solving the crystal structures of the C-terminally truncated versions of, first, SHP-2 (4) and, second, SHP-1 (5) has lead to the proposal of a model where the inactive conformation of SHPs is maintained by the N-SH2 domain, which occludes the active site of the enzyme, thereby inhibiting the phosphatase activity. The C-SH2 domain, which has minimal interaction with the catalytic domain, is then responsible for selecting substrates (6). This model was supported by earlier mutagenesis studies of SHP-1 showing that removal of the SH2 domains increased the phosphatase activity at physiological pH (7), and another study refined this further by proposing that only the N-SH2 domain was necessary for autoinhibition (8). These early studies also revealed a role for the C-terminal tail of SHP-1, absent from the crystal structure, in regulating phosphatase activity since truncation of the C terminus also stimulated phosphatase activity (7). Phosphorylation of Tyr residues within the C-terminal tail has been shown for both SHP-1 and SHP-2 although the function of these phosphorylation events is debatable (3). However, studies incorporating site-specific phosphotyrosine mimetics have attempted to address the contributions of the SH2 domains and C-terminal Tyr phosphorylation in the regulation of SHP-2 phosphatase activity (9) and SHP-1 phosphatase activity (10). These studies indicated that phosphorylated Tyr residues within the C-terminal tail interact with both the N-SH2 and C-SH2 domain *in vitro* and through these interactions the phosphorylated Tyr residues modestly stimulate phosphatase activity (9).

In addition to Tyr phosphorylation of SHP-1 and SHP-2, other studies have demonstrated Ser phosphorylation of SHP-1 in murine T cells (11), in human platelets (12) and in human neutrophils (13). Also, overexpression studies using insulin receptor, PKC isoforms, and SHP-2 have indicated that SHP-2 is phosphorylated on Ser residues in response to insulin receptor stimulation (14).

Here we hypothesized that regulation of SHP-1 may fundamentally differ from that of SHP-2 in that, because of its negative signaling role, SHP-1 is more likely than SHP-2 to have activity under basal conditions. The implication for SHP-1 is that upon cellular activation, the basal activity may be switched off, allowing Tyr phosphorylation of proteins to proceed without opposing Tyr phosphatase activity. We show that SHP-1, through its SH2 domains, constitutively binds both PKCα and the SHP-1 substrate Vav1. Upon cellular activation,

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[1] The abbreviations used are: SH2, Src homology 2; BIM, bisindolylmaleimide 1; FITC, fluorescein isothiocyanate; GST, glutathione *S*-transferase; HEK, human embryonic kidney; PAR, protease-activated receptor; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; SHP, SH2 domain-containing tyrosine phosphatase; BSA, bovine serum albumin; PBS, phosphate-buffered saline; GFP, green fluorescent protein; WT, wild type.

Ser^{591} in the C-terminal tail of SHP-1 is phosphorylated by PKC, leading to inhibition of its activity. Cellular activation leading to tyrosine phosphorylation of proteins therefore involves not only any increase in kinases activity but also a decrease in phosphatase activity. In the case of SHP-1, phosphorylation of its C-terminal tail Ser^{591} is a critical negative regulator of its activity, leading to enhanced tyrosine phosphorylation of it substrate Vav1.

EXPERIMENTAL PROCEDURES

Preparation and Stimulation of Human Platelets—Platelets were prepared exactly as described previously (15). In brief, venous blood was obtained from volunteers with acid citrate dextrose as an anticoagulant, used at a 1:7 v/v ratio. Platelet-rich plasma was obtained by centrifugation at 200 × *g* for 17 min. Platelets were then isolated by centrifugation of the platelet-rich plasma in the presence of 0.14 μM of prostaglandin E_1 and 10 μM indomethacin. The pellet was resuspended in a modified Tyrode's-HEPES buffer to a density of 4×10^8 platelets/ml. Platelets (400 μl) were preincubated with 1 mM EGTA for 1 min before stimulation with either 0.1 unit/ml bovine thrombin (Sigma), 10 nM phorbol 12-myristate 13-acetate (PMA; Tocris-Cookson), the PAR1 peptide SFLLRN (Bachem, Merseyside, UK), or the PAR4 peptide AYPGKF in an aggregometer at 37 °C with continuous stirring. Where indicated, the platelets were preincubated with bisindolylmaleimide I (BIM I; Tocris-Cookson, Bristol, UK) for 15 min at 30 °C before stimulation.

Antibodies—The polyclonal anti-SHP-1 antibody (C-19), the anti-SHP-2 antibody (C-18), and the donkey anti-rabbit IgG FITC (sc-2090) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The monoclonal anti-SHP-1 and anti-PKCα antibodies and the anti-BD Living Colors™ full-length polyclonal (anti-GFP) antibody were purchased from BD Biosciences. The polyclonal anti-Ser(P) (PKC) substrate antibody was purchased from Cell Signaling Technology (New England Biolabs, Hertfordshire, UK). The anti-phosphotyrosine (4G10) antibody was purchased from Upstate Biotechnology, Inc. (Milton Keynes, UK). The anti-Vav1 polyclonal antiserum was a gift from Dr. Martin Turner (Babraham Institute, UK). Horseradish peroxidase-conjugated secondary antibodies were purchased from Amersham Biosciences.

Immunoprecipitation—Reactions were stopped by lysis of platelets with an equal volume of 2× neutral detergent lysis buffer (100 mM Tris, 300 mM NaCl, and 2% (v/v) Nonidet P-40, pH 7.5, plus complete protease inhibitors (Roche Applied Science) and phosphatase inhibitor mixture (either mixture I for preservation of Ser(P)/Thr(P) or mixture II for preservation of Tyr(P) (Sigma)) or 2× RIPA buffer (100 mM Tris, 300 mM NaCl, 2 mM EDTA, 2% (v/v) Nonidet P-40, 1% (w/v) deoxycholic acid, and 0.2% (w/v) SDS, pH 8.0, plus complete protease inhibitors and phosphate inhibitor mixture). Insoluble debris was cleared from the lysate by centrifugation at 13,000 × *g* for 10 min at 4 °C. Supernatants were precleared with either protein-A-Sepharose (rabbit polyclonal antibodies) or protein-G-agarose (mouse monoclonal antibodies) beads for 1 h. Antibody-protein-A/G complexes were formed by incubation of protein-A-Sepharose with 2 μg of antibody for 30 min at room temperature. Precleared lysates were added to the antibody-protein-A/G complexes and incubated at 4 °C with constant rotation. Immunoprecipitates were washed twice, and proteins were eluted from the beads by incubation of the immunoprecipitates with 60 μl of 2× SDS sample buffer (24 mM Tris, pH 6.8, 10% (v/v) glycerol, 0.8% (v/v) SDS, 6 mM 2-mercaptoethanol, and 0.04% bromphenol blue) at 90 °C for 3 min.

Electrophoresis of Proteins and Immunoblotting—SDS-PAGE and immunoblotting were carried out as described (15). In summary, proteins were separated by discontinuous SDS-PAGE on 10% slab gels and transferred to polyvinylidene difluoride membrane by semi-dry transfer (15 V for 1 h). Membranes were blocked either by incubation with either 5% (w/v) BSA (phosphorylation-specific antibodies) in TBSt (150 mM NaCl, 10 mM Trizma (Tris base), and 0.1% (v/v) Tween 20, pH 7.6) or 5% (w/v) nonfat milk powder in TBSt. Primary antibodies were diluted in either 5% (w/v) BSA (phosphorylation-specific antibodies) in TBSt or 2.5% (w/v) nonfat milk in TBSt. After incubation with primary antibodies, membranes were washed with TBSt and incubated with horseradish peroxidase-conjugated secondary antibodies, diluted in 3% (w/v) BSA, for 1 h at room temperature. After further washing of the membrane, signals were detected by enhanced chemiluminescence. When necessary, membranes were stripped using Restore Western blot stripping buffer (Pierce). Membranes were immersed in stripping buffer and incubated at room temperature for 30 min before extensive washing and reprobing with the appropriate antibody.

Mutagenesis of Human SHP-1—The *Escherichia coli* strain JM109 was transformed with hSHP-1.pGEX-2T DNA (A gift of Dr. Benjamin Neel, Harvard), and plasmid DNA was prepared using a QIAprep miniprep kit (Qiagen). A single point mutation was introduced into the Ser^{591} codon, converting it to an Ala codon, by PCR using the primer extension overlap method and inserted into pGEX-4T-3 vector for expression as a GST-tagged protein in *E. coli* or into pEGFP-C3 vector for expression as a GFP-tagged protein in HEK cells. The primer sequences for this mutation are 5′-gagaagagcaagggtgccctcaagaggaagtga-3′ (forward mutagenesis primer) and 5′-cacttcctcttgagggcaccctgctcttctc-3′ (reverse mutagenesis primer; this construct was named SHP-1 S591A). Deletion of the tandem SH2 domains of human SHP-1 was achieved by PCR using the following primers, 5′-ccagggaattcccaagaagacggggattgag-3′ (forward primer) and 5′-cggcctcgagtcacttcctcttgagggaacc-3′ (reverse primer), and was inserted into the pGEX-4T-3 vector for expression as a GST-tagged protein in *E. coli*. This construct was named ΔT-SH2 SHP-1. Constructs were sequenced to ensure that undesirable mutations were not present.

Expression of GFP-tagged Proteins in HEK-293 Cells—HEK-293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with serum and penicillin/streptomycin at 37 °C. 35-mm dishes were seeded with HEK-293 cells and transfected with 1 μg of plasmid DNA (GFP-full-length human SHP-1 wild type or S591A) using Genejuice transfection reagent (Novagen) when at ~75% confluency. 48 h after transfection, cells were washed twice with Krebs-Ringer bicarbonate buffer (Sigma) and then incubated in 1 ml of Krebs-Ringer bicarbonate buffer for 1 h at 37 °C. After 1 h, where indicated, PMA was added to a final concentration of 100 nM and incubated for a further 1 h at 37 °C. Cells were lysed using neutral detergent lysis buffer and GFP-tagged proteins immunoprecipitated with anti-GFP antibody.

Expression and Purification of GST Fusion Proteins—The *E. coli* strain BL21 Rosetta (Novagen and Merck) was transformed with plasmid DNA encoding either wild type human SHP-1, ΔT-SH2 SHP-1, or SHP-1 S591A as GST fusion proteins. Single colonies were picked and used to inoculate 5-ml cultures of LB broth, grown overnight at 37 °C with constant shaking. The following morning the overnight culture was diluted (1:50) into fresh LB broth and grown at 37 °C until the $A_{600\ nm} = 0.5$. Expression of fusion proteins was then induced by the addition of isopropyl-1-thio-β-D-galactopyranoside to 0.1 mM, and incubation was continued at 30 °C for another 4 h. Bacterial cells were harvested by centrifugation, and pellets were resuspended in 25 ml/1 liter of original culture of PBS supplemented with 0.1% (v/v) Nonidet P-40 and complete protease inhibitors. Cells were lysed by sonication and clarified by centrifugation. The clarified bacterial cell lysates were incubated with glutathione-agarose for 3 h at 4 °C with constant rotation. The agarose beads were then washed with PBS, and bound proteins were eluted with 5 mM reduced glutathione in 50 mM Tris, pH 8.0. The eluted proteins were buffer-exchanged into PBS by gel filtration using Bio-Rad P-6DG gel and concentrated using Millipore Ultrafree 15-centrifugal filter devices (30 NMWL). The protein concentration was then determined using the Bradford Assay by using BSA as standard.

GST Pulldown Assay—Platelets were stimulated as required and lysed in neutral detergent lysis buffer containing phosphatase inhibitor mixture II (Sigma), centrifuged, and pre-cleared with 50 μl of glutathione-Sepharose (Sigma) by incubation at 4 °C with constant rotation for 1 h. Meanwhile, 10 μg of purified GST fusion protein or GST alone was incubated with 50 μl of glutathione-Sepharose at room temperature for 1 h. After pre-clearing the platelet, lysates were incubated with the GST/GST fusion proteins coupled to beads at 4 °C overnight and washed twice with 1× neutral detergent lysis buffer. Proteins were eluted from the beads by incubation of the GSH-Sepharose beads with 60 μl of 2× SDS sample buffer at 90 °C for 3 min.

In Vitro Kinase Assay—The PKCα isoform was immunoprecipitated from stimulated platelet lysates as described earlier under "Experimental Procedures" using a monoclonal anti-PKCα antibody (BD Biosciences). The immunoprecipitates were washed with lysis buffer and resuspended in 50 μl of kinase assay buffer (20 mM HEPES, pH 7.2, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 100 mM NaCl, and 0.15 mM ATP). GST fusion proteins were added, and the samples were mixed and incubated at 37 °C for 30 min with occasional mixing. The reaction was terminated by the addition of SDS sample buffer. Phosphorylation of GST fusion proteins was determined by immunoblotting with the anti-phospho-(Ser) PKC substrate antibody.

In Vitro Protein-tyrosine Phosphatase Assay—Tyrosine-phosphorylated Vav1 was immunoprecipitated from pervanadate-treated Jurkat cells using anti-Vav1 antiserum. For each time point 2.5 μl of antiserum was used to immunoprecipitate Vav1 from ~10^8 cells/ml. SHP-1 was immunoprecipitated from basal or stimulated human platelets as de-

scribed in this section and incubated with Vav1 immunocomplexes. Dephosphorylation reactions were carried out in 40 μl of 20 mM imidazole HCl, pH 7.0, containing 0.1% (v/v) 2-mercaptoethanol, at 37 °C. Reactions were stopped by the addition of 10 μl of 5× sample buffer and incubation at 95 °C for 5 min. 10-μl aliquots were subjected to SDS-PAGE and immunoblotting with the anti-Tyr(P) (4G10) antibody. Immunoblots were scanned and analyzed by densitometry.

Immunofluorescence Confocal Imaging of Platelets—Immunostaining of platelets was carried out exactly as described previously (16). Platelets were stimulated with either thrombin or PMA, and reactions were terminated by the addition of 4% (v/v) paraformaldehyde in phosphate-buffered saline (PBS: 147 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4) and left at room temperature for 30 min. Platelets were pelleted by centrifugation at 500 × *g* for 5 min and washed twice with PBS. Platelets were immobilized on poly-L-lysine-coated coverslips overnight, permeabilized by incubation of coverslips with PBST (PBS plus 0.5% (v/v) Triton X-100) at room temperature for 10 min, and then incubated with PBST plus 1% (w/v) BSA for 30 min at room temperature to block nonspecific binding. Immobilized platelets were then incubated with the polyclonal anti-SHP-1 in PBST plus 1% (w/v) BSA at 4 °C for 24 h. Coverslips were then washed three times with PBST before incubation with fluorescein isothiocyanate (FITC)-labeled secondary antibody diluted in PBST plus 1% (w/v) BSA. Coverslips were again washed three times with PBST before incubation with 4% (v/v) paraformaldehyde in PBS for 10 min; coverslips were mounted onto slides using a 13.5% Mowiol solution containing 2.5% (w/v) 1,4-diazobicyclo- [2,2,2]octane. Platelets were imaged using a Leica TCS-NT confocal laser scanning microscope equipped with Kr/Ar laser (488, 568, and 647 nm lines) attached to a Leica DM IRBE inverted fluorescence microscope.

RESULTS

SHP-1 Is Phosphorylated on Ser^{591} by PKC Upon Cellular Activation—It had already been shown that SHP-1 may be phosphorylated on Ser (12), although the function of this phosphorylation, the kinase responsible, and the site of phosphorylation have not been determined. Li *et al.* (12) had provided pharmacological evidence that a PKC isoform may be responsible for phosphorylating SHP-1 in platelets, although Gaits *et al.* (17) had shown in DAMI cells that SHP-1 Ser phosphorylation was not inhibited by the PKC inhibitor GF109203X (also called bisindolylmaleimide I; BIM I). Given the discrepancy in the literature, we were interested first to determine whether SHP-1 was a PKC substrate *in vivo* by using an anti-phosphopeptide antibody-based approach.

Given the close homology, but distinct functional differences between the two family members SHP-1 and SHP-2, we decided initially to identify putative PKC-mediated phosphorylation sites in SHP-1. This analysis indicated that Ser^{591} of SHP-1 was the most prominent PKC phosphorylation consensus sequence in SHP-1, and this site had no match within the SHP-2 sequence (Fig. 1*A*). Serine 591 in SHP-1 lies just five residues from the C terminus of the phosphatase and lies perfectly within a consensus sequence of a commercially available antibody (anti-Ser(P) (PKC) substrate antibody) that has been generated against a set of peptides that correspond to PKC consensus phosphorylation sites, particularly those phosphorylated by conventional PKC isoforms. We were thus able to address if SHP-1 was a PKC substrate *in vivo* by using an anti-phosphopeptide antibody-based approach. First, we mutated Ser^{591} in SHP-1 to Ala and expressed the mutant (S591A) as a GST fusion protein. *In vitro* phosphorylation of purified GST fusion proteins (wild type (WT) and S591A) by immunoprecipitated PKCα from activated platelets (Fig. 1*B*) revealed that Ser^{591} was phosphorylated by PKCα, and this residue was indeed the only residue within the SHP-1 sequence to be recognized by the anti-Ser(P) (PKC) substrate antibody as being phosphorylated. This finding was confirmed *in vivo* by expressing GFP-SHP-1 (wild type and S591A) in HEK cells (Fig. 1*C*). Phorbol ester activation of the cells leads to detection of phosphorylation by the PKC substrate antibody only in cells expressing wild-type SHP-1, and not in the point mutant Ser^{591}.

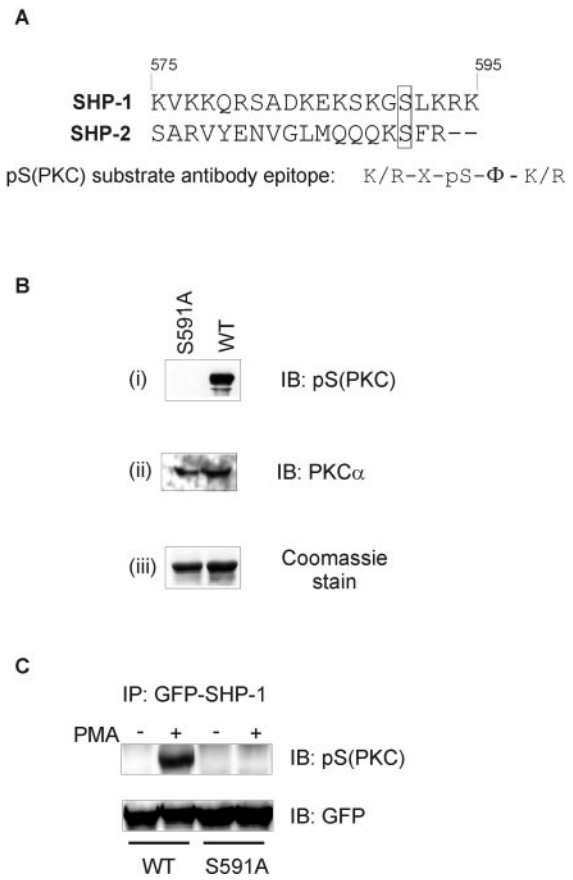

FIG. 1. **Anti-Ser(P) (PKC) substrate antibody specifically recognizes Ser^{591} in SHP-1, which is phosphorylated by PKCα *in vitro*.** *A*, human SHP-1 and SHP-2 sequences were aligned using ClustalW. Part of the alignment covering the extreme C terminus is shown. A Ser residue is conserved between both SHP-1 and SHP-2 (*boxed*), but on SHP-1 this residue (Ser^{591}) is within a consensus sequence for phosphorylation by PKC and recognition by the anti-Ser(P) (PKC) substrate antibody. *X* indicates any amino acid, and Φ indicates amino acid with a hydrophobic side chain. *B*, PKCα was immunoprecipitated from human platelet lysates by using monoclonal anti-PKCα antibody. Washed PKCα immunoprecipitates were used in an *in vitro* kinase assay with either GST-SHP-1 (*WT*) or GST-SHP-1 (*S591A*). Reactions were stopped by the addition of SDS sample buffer, subjected to SDS-PAGE, and immunoblotting (*IB*) with the anti-Ser(P) (PKC) antibody (*i*) and anti-PKCα antibody (*ii*). Coomassie stain of purified GST fusion proteins (*iii*) shows that equivalent amounts of each fusion protein were included in each reaction. *C*, HEK cells were transfected with either GFP-SHP-1 (*WT*) or GFP-SHP-1 (*S591A*), stimulated with PMA as indicated by +, and GFP-tagged proteins were immunoprecipitated with anti-GFP antibody. The phosphorylation of fusion proteins was assessed by immunoblotting with anti-Ser(P) (PKC) substrate antibody. Data shown are representative of three independent experiments.

By having shown then that the anti-Ser(P) (PKC) substrate antibody could act as a specific anti-phosphopeptide antibody for Ser^{591} within SHP-1, we addressed whether this site was phosphorylated *in vivo* in platelets upon cellular activation. Fig. 2*A* shows SHP-1 and SHP-2 immunoprecipitated from basal and thrombin-stimulated human platelet lysates immunoblotted with anti-Ser(P) (PKC) substrate antibody. The data show that SHP-1 but not SHP-2 reacted with the antibody in stimulated conditions (Fig. 2*A*), indicating that SHP-1 is indeed phosphorylated on Ser^{591} *in vivo* in platelets. Lack of SHP-2 detection by the antibody validates the specificity of the approach. In addition, membranes were probed with a context independent anti-phosphothreonine (anti-Thr(P)) antibody,

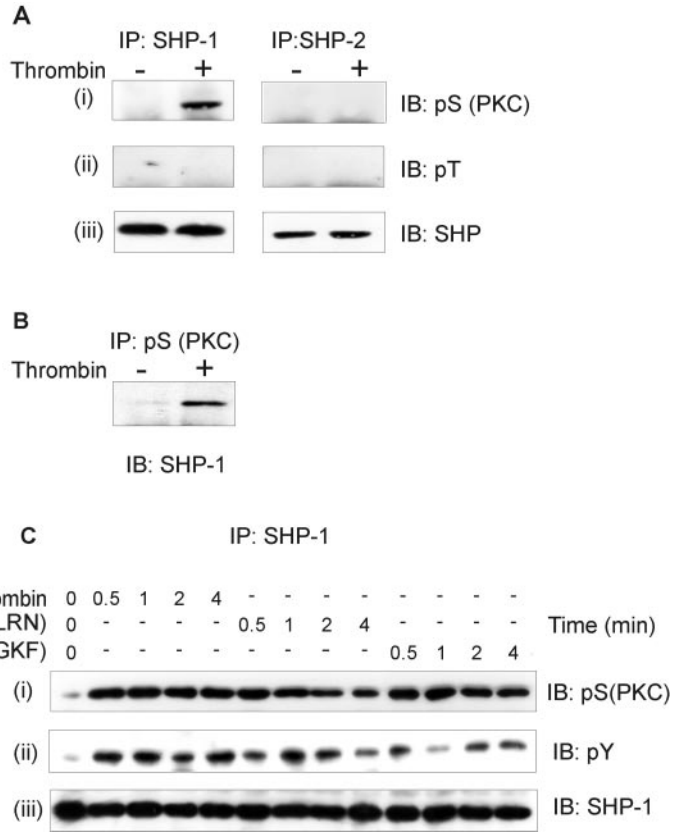

FIG. 2. **Activation of PAR receptors elicits phosphorylation of Ser^{591} on SHP-1.** *A*, SHP-1 and SHP-2 were immunoprecipitated (*IP*) from basal lysates (−) or lysates prepared from human platelets stimulated with thrombin (0.1 units/ml) for 1 min in the presence of EGTA (+). Immunoprecipitates were subjected to SDS-PAGE and immunoblotted (*IB*) with anti-Ser(P) (PKC) substrate antibody (*i*) or context-independent anti-phosphothreonine antibody (*ii*). Immunoblots were stripped and reprobed with the immunoprecipitating antibody, either anti-SHP-1 or anti-SHP-2 (*iii*). *pS*, phosphoserine; *pT*, phosphothreonine. *B*, immunoprecipitation was performed with anti-Ser(P) (PKC) substrate antibody from basal (−) and thrombin-stimulated (+) human platelet lysates. The immunoprecipitate was subjected to SDS-PAGE and immunoblotted with anti-SHP-1 antibody. *C*, SHP-1 was immunoprecipitated from lysates prepared from human platelets stimulated with either thrombin (0.1 unit/ml), PAR1-specific agonist peptide SFLLRN (100 μM), or PAR4-specific agonist peptide AYPGKF (500 μM) for the times indicated. Immunoprecipitates were subjected to SDS-PAGE and immunoblotted with anti-Ser(P) (PKC) substrate antibody (*i*) or 4G10 anti-phosphotyrosine monoclonal antibody (*ii*). Immunoblots were stripped and reprobed with anti-SHP-1 antibody (*iii*). Data shown are representative of at three independent experiments.

which detected no phosphorylation of either SHP-1 or SHP-2 on Thr. This is consistent with Li *et al.* (12) who also showed no phosphorylation of SHP-1 on Thr. Immunoprecipitations were also performed with the polyclonal SHP-1 antibody in the presence of blocking peptide. This confirmed that the immunoreactive band, which was obtained when immunoblotted with anti-Ser(P) (PKC) substrate antibody, was indeed SHP-1 because no immunoreactivity was observed when blocking peptide was included (data not shown). Also, SHP-1 was found to be present in anti-Ser(P) (PKC) substrate antibody immunoprecipitates from thrombin-stimulated human platelet lysates (Fig. 2*B*), again indicating that SHP-1 was genuinely phosphorylated on Ser^{591} *in vivo* upon cellular activation.

In human platelets thrombin has been shown to elicit signal transduction through both PAR1 and PAR4 receptors (18), so we determined if activation of one or both of these receptors was required for either Ser^{591} or Tyr phosphorylation of SHP-1. Human platelets were stimulated with either 0.1 unit/ml thrombin, 100 μM PAR1 peptide (SFLLRN) (19), or 500 μM PAR4 peptide (AYPGKF) (20) for the times indicated in Fig. 2*C*. SHP-1 was immunoprecipitated, subjected to SDS-PAGE, and immunoblotted with anti-Ser(P) (PKC) substrate antibody (Fig. 2*C, i*) or 4G10 anti-phosphotyrosine (Fig. 2*C, ii*). Ser^{591} phosphorylation of SHP-1 was rapid, reaching a maximum within 30 s. When human platelets were stimulated with thrombin and the PAR4-specific peptide (AYPGKF), phosphorylation of Ser^{591} was sustained over the period of the time course. However, Ser^{591} phosphorylation of SHP-1 in response to the PAR1-specific peptide (SFLLRN) was maximal by 30 s and decreased slightly after 2 min. Thus activation of either PAR1 or PAR4 causes phosphorylation of SHP-1 on Ser^{591} with subtly different dynamics. In agreement with previous reports (21), thrombin induced phosphorylation of SHP-1 on Tyr also (Fig. 2*C*). Phosphorylation on Tyr was rapid and sustained for thrombin, PAR1, and PAR4 peptides, indicating that either receptor may couple to this phosphorylation event, as for phosphorylation of Ser^{591}.

To confirm that phosphorylation of SHP-1 on Ser^{591} was because of PKC activity, human platelets were stimulated with the PKC activator phorbol 12-myristate 13-acetate (PMA). As shown in Fig. 3*A*, stimulation with 10 nM PMA was sufficient to induce phosphorylation of SHP-1 on Ser^{591}, and experiments with the specific PKC inhibitor BIM I demonstrated that the phosphorylation event was dose-dependently abolished by pretreatment of human platelets with this inhibitor (Fig. 3*B*). Pretreatment of human platelets with 5 μM BIM I for 15 min before thrombin stimulation was sufficient to partially reduce Ser phosphorylation of SHP-1; treatment with higher concentrations of BIM I completely abolished immunoreactivity of SHP-1 with the antibody. These experiments indicated that PKC is responsible for phosphorylation of SHP-1 on Ser^{591} in human platelets.

It was important to determine whether Ser^{591} phosphorylation was dependent upon Tyr phosphorylation of SHP-1. Fig. 3*C* shows that in the presence of the Src family kinase inhibitor PP1, thrombin-induced Ser^{591} phosphorylation of SHP-1 is unaffected although Tyr phosphorylation is ablated. This demonstrates that Ser^{591} phosphorylation of SHP-1 is not dependent upon Tyr phosphorylation of the molecule and that the two phosphorylation events are independent modifications.

Thrombin-induced Translocation of SHP-1 Is Not Dependent upon Phosphorylation of Ser^{591}—It was important to ascertain the functional role of Ser^{591} phosphorylation of SHP-1 by PKC. One possible role was to direct localization of SHP-1 within the cell. We investigated SHP-1 localization in human platelets by immunostaining of fixed platelets with the polyclonal anti-SHP-1 antibody (Fig. 4). In basal platelets (Fig. 4*A*), SHP-1 was localized to the cytoplasm, in agreement with previous studies using ectopically expressed GFP-SHP-1 (22). Upon platelet activation with thrombin (Fig. 4*B*), SHP-1 translocated to the cell

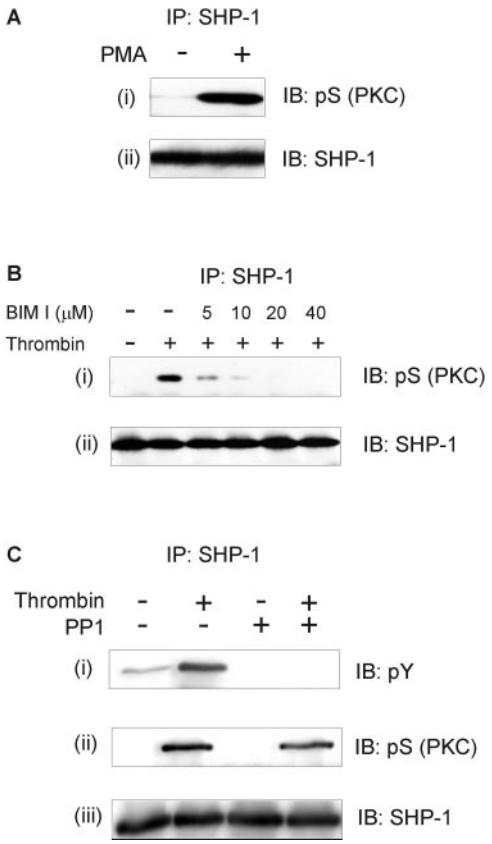

FIG. 3. **Ser[591] phosphorylation on SHP-1 is mediated by PKC.** *A*, human platelets were stimulated with 10 nM PKC activator PMA in the presence of 1 mM EGTA for 15 min and lysed, and SHP-1 was immunoprecipitated. The immunoprecipitate (*IP*) was subjected to SDS-PAGE and immunoblotted (*IB*) with anti-Ser(P) (PKC) substrate antibody (*i*). The immunoblot was stripped and reprobed with anti-SHP-1 antibody (*ii*). *B*, human platelets were treated with the indicated concentrations of the PKC inhibitor BIM I at 30 °C for 15 min before incubation with EGTA for 1 min and stimulation with 0.1 unit/ml thrombin for 1 min as indicated. Platelets were then lysed immediately, and SHP-1 was immunoprecipitated. The immunoblot was probed with anti-Ser(P) (PKC) substrate antibody (*i*) before being stripped and reprobed with anti-SHP-1 antibody (*ii*). *C*, platelets were pretreated with the Src family kinase inhibitor PP1 (10 μM) for 15 min, or Me_2SO control as indicated, prior to incubation with EGTA for 1 min and then stimulation with thrombin (0.1 unit/ml) as indicated. Platelets were then lysed; SHP-1 was immunoprecipitated, and immunoblots were probed with either 4G10 anti-phosphotyrosine mAb (*i*) or anti-Ser(P) (PKC) substrate antibody (*ii*). The immunoblot was stripped and reprobed with anti-SHP-1 antibody (*iii*). All data shown are representative of at least three independent experiments.

periphery of the activated platelet. This translocation was unaffected by the PKC inhibitor BIM I (Fig. 4*C*). The PKC activator PMA was also unable to induce translocation of SHP-1 (data not shown), indicating that the translocation event was independent of PKC activity. These data indicate that agonist-dependent translocation of SHP-1 to the cell periphery is independent of phosphorylation by PKC on Ser[591].

Phosphorylation of SHP-1 Ser[591] Negatively Regulates Its Catalytic Activity—A role for Ser phosphorylation in regulating the activity of SHP-1 has been postulated by several groups in different cell types. Li *et al.* (12) had shown that thrombin was able to induce a small increase in activity of SHP-1 in platelets, whereas Gaits *et al.* (17) had shown that lysophosphatidic acid was unable to induce an increase in the SHP-1 activity in DAMI cells. In human neutrophils (13), and also in the cellular response to DNA damage (24), it was found that *in vitro* PKC-mediated phosphorylation of SHP-1 caused inhibition of SHP-1 activity, in contrast with the results of Li *et al.* (12) in platelets.

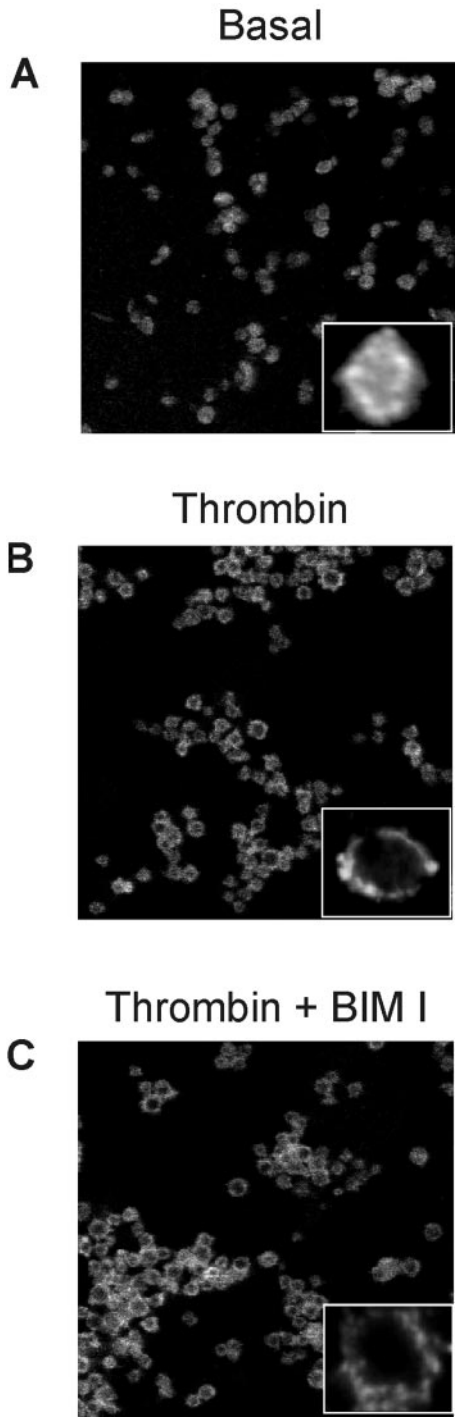

FIG. 4. **SHP-1 translocates to the periphery of the platelet independently of phosphorylation of Ser[591].** Human platelets were either basal (*A*) or stimulated with thrombin (0.1 unit/ml, 60 s) (*B*). A sample of platelets was also incubated with 10 μM BIM I for 15 min prior to stimulation with thrombin (0.1 unit/ml, 60 s) (*C*). Samples were fixed with paraformaldehyde and an aliquot adhered to poly-L-lysine-coated coverslips, which were then immunostained with polyclonal anti-SHP-1 antibody, FITC-conjugated secondary antibody, and imaged by confocal immunomicroscopy. *Insets* are of single cells selected from the wider field of view and magnified using Leica confocal software. Data shown are representative of four independent experiments.

We sought to address the effect of PKC-mediated Ser[591] phosphorylation on endogenous SHP-1 phosphatase activity by assessing the role of PKC activity in regulating the Tyr phosphorylation status of a known SHP-1 substrate. Vav1 has been shown to be a SHP-1 substrate in NK cells (25) and also to be Tyr-phosphorylated in thrombin-activated platelets (26–28).

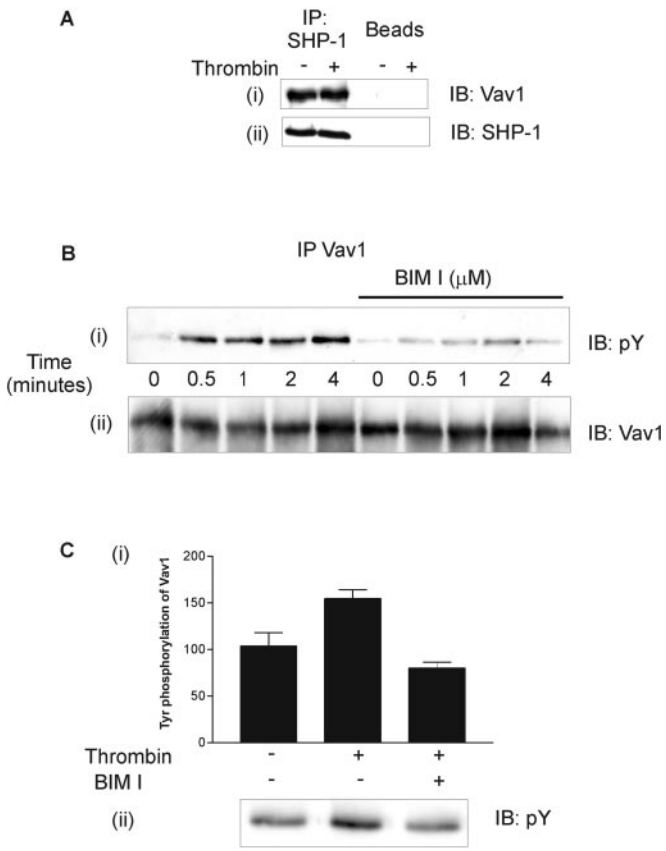

FIG. 5. **PKC-dependent phosphorylation of SHP-1 Ser^{591} negatively regulates SHP-1 activity.** *A*, basal platelets or platelets stimulated with 0.1 unit/ml thrombin for 1 min were lysed in neutral lysis buffer containing phosphatase inhibitor mixture I (Ser/Thr protein phosphatase inhibitors) and SHP-1 immunoprecipitated with 5 μg of monoclonal anti-SHP-1 antibody. Mock immunoprecipitations were also carried out with just protein-G beads as a negative control. Immunoprecipitates (*IP*) were washed with 1× Nonidet P-40, subjected to SDS-PAGE and immunoblotting (*IB*) with anti-Vav1 antibody (*i*), and re-probed for SHP-1 (*ii*). Data are representative of three independent experiments. *B*, platelets were treated 10 μM BIM I for 15 min or Me_2SO alone for 15 min before being stimulated with 0.1 unit/ml thrombin for the times indicated and lysed in 2× RIPA buffer containing phosphatase inhibitors. Vav1 was immunoprecipitated from lysates using anti-Vav1 antiserum, washed with 1× RIPA buffer, and subjected to SDS-PAGE and immunoblotting with anti-Tyr(P) (4G10) antibody (*i*). Immunoblots were then stripped and reprobed with anti-Vav1 antiserum (*ii*). Data shown are representative of at least three independent experiments. *C*, dephosphorylation of tyrosine-phosphorylated Vav1 by SHP-1 immunoprecipitated from either basal platelets, thrombin-stimulated platelets (thrombin 0.1 units/ml, 60 s), or platelets pretreated with BIM I (10 μM) followed by thrombin stimulation (0.1 units/ml, 60s) was carried out as described under "Experimental Procedures." The *bar graph* (*i*) represents densitometry data from three anti-Tyr(P) immunoblots (mean ± S.E., $n = 3$). A representative antiphosphotyrosine immunoblot showing tyrosine phosphorylation status of Vav1 is shown in (*ii*).

Fig. 5*A* shows by co-immunoprecipitation that, in platelets, SHP-1 is constitutively bound to Vav1 and that the association is not altered upon stimulation of platelets with thrombin. Experiments using GST fusions of either full-length SHP-1 or SHP-1 lacking its tandem SH2 domains (ΔT-SH2-SHP1) revealed that the interaction takes place partially through the catalytic and C-terminal tail domains because there is a weak interaction seen with ΔT-SH2-SHP1 (data not shown). The interaction with full-length GST-SHP-1 is stronger however, indicating that the SH2 domains of SHP-1 may stabilize Vav1 interaction. In Fig. 5*B*, Vav1 was immunoprecipitated from platelet lysates stimulated with thrombin for the times indicated. The effect of PKC inhibition on the Tyr phosphorylation of Vav1 showed a marked reduction in stimulated Tyr phosphorylation of Vav1 in the presence of BIM I, indicating that PKC may positively regulate Vav1 Tyr phosphorylation. Fig. 5*C* shows that the regulation of Vav1 Tyr phosphorylation by PKC is likely to be mediated by negative regulation of SHP-1 activity by PKC. The Tyr phosphorylation status of Vav1 *in vitro* was assessed as an assay for SHP-1 activity. SHP-1 was immunoprecipitated from either basal platelets or platelets activated with thrombin in the absence or presence of the PKC inhibitor BIM I. These immunoprecipitates were incubated *in vitro* with Vav1 that had been immunoprecipitated from peroxovanadate-treated Jurkat T-cells, to ensure maximal tyrosine phosphorylation of Vav1. The data show that the activity of SHP-1 is decreased upon thrombin stimulation, as detected by a greater Tyr phosphorylation status of Vav1 when incubated with SHP-1 taken from stimulated compared with basal conditions. This reduction in activity induced by thrombin was reversed however in the presence of BIM I, indicating that the inhibitory influence of thrombin on SHP-1 is mediated by PKC.

SHP-1 Constitutively Associates with PKCα through Its SH2 Domains—It was important to identify the PKC isoform responsible for phosphorylation and regulation of SHP-1 on Ser^{591}. We approached this by assessment of PKC isoforms that physically associate with SHP-1 by co-immunoprecipitation. These experiments revealed that from among the PKCα, PKCβ, and PKCδ isoforms present in platelets, only PKCα associated with SHP-1 immunoprecipitates (Fig. 6*A*). Fig. 6, *B–D*, shows the results of experiments exploring the interaction of SHP-1 with PKCα. Immunoprecipitations were performed from basal or activated platelet lysates with a polyclonal anti-SHP-1 antibody (Fig. 6*B*) or a monoclonal anti-SHP-1 antibody (Fig. 6*C*), antibodies reactive against different epitopes on human SHP-1. Immunoblotting with anti-PKCα revealed constitutive association with SHP-1, which was unaffected by stimulation of platelets with thrombin. Also, immunoblotting of anti-PKCα immunoprecipitates with polyclonal anti-SHP-1 antibody demonstrated the presence of SHP-1 in these immunoprecipitates (Fig. 6*D*). To confirm the specificity of the interaction, Fig. 6*B* also shows an immunoprecipitation performed with polyclonal anti-SHP-1 in the presence of excess blocking peptide; this confirmed that PKCα was a true SHP-1 interacting protein. We sought to address which region of SHP-1 was required for interaction with PKCα by pulldown assays with human platelet lysates using GST full-length human SHP-1 (WT) and the truncated version lacking the tandem SH2 domains (ΔT-SH2) (Fig. 6*E*). Most importantly, this revealed that the SH2 domains of SHP-1 were required for interaction of SHP-1 with PKCα, because PKCα was only found in a complex with full-length SHP-1 (WT) (Fig. 6*E*). Because occupation of the N-terminal SH2 domain has been shown to induce activation of SHP-1, this finding may explain the high basal activity state of SHP-1. Also, if Tyr(P) phosphatase inhibitors were excluded from the lysis buffer, no interaction of SHP-1 with PKCα was observed (data not shown), suggesting that the interaction may be mediated through a Tyr-phosphorylated intermediate protein, because PKCα itself is not Tyr-phosphorylated under basal conditions (data not shown).

DISCUSSION

This study has identified two important mechanisms regulating SHP-1 activity in human platelets. First, we have shown that upon cellular activation SHP-1 is phosphorylated on Ser^{591} in its C terminus by PKC, inhibiting the ability of SHP-1 to dephosphorylate its associating substrate Vav1. Second, we have shown that SHP-1 constitutively binds to PKCα and that this interaction requires SHP-1 SH2 domains. This has impli-

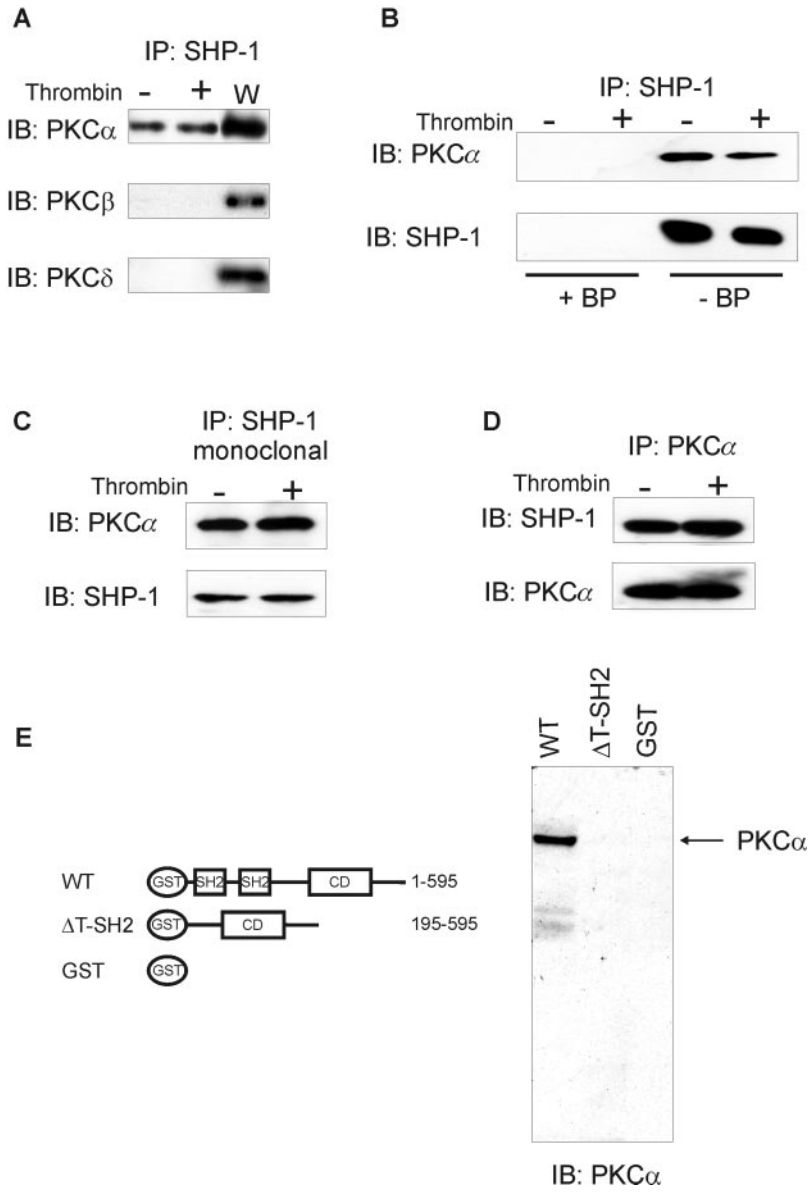

FIG. 6. **PKCα physically associates with SHP-1 in human platelets.** Lysates were prepared from basal (−) or thrombin-stimulated (+) human platelets. *A*, SHP-1 was immunoprecipitated from platelet lysates using a polyclonal SHP-1 antibody. Immunoprecipitates (*IP*), or whole cell lysates (*W*), were immunoblotted (*IB*) with monoclonal anti-PKC isoform-specific antibodies as indicated. *B*, SHP-1 was immunoprecipitated from platelet lysates using polyclonal anti-SHP-1 antibody. The anti-peptide polyclonal SHP-1 antibody was incubated with a 10-fold excess of blocking peptide (+*BP*) or equivalent volume of PBS (−*BP*) before being used in immunoprecipitations. The immunoprecipitates were immunoblotted with monoclonal anti-PKCα antibody (*top panel*) before being stripped and reprobed with the polyclonal anti-SHP-1 antibody (*bottom panel*). *C*, SHP-1 was immunoprecipitated from platelet lysates using a monoclonal anti-SHP-1 antibody, which has a different epitope to the polyclonal anti-SHP-1 antibody used in this study. Immunoprecipitates were immunoblotted with monoclonal anti-PKCα antibody (*top panel*) before being stripped and reprobed with the immunoprecipitating monoclonal anti-SHP-1 antibody (*bottom panel*). *D*, PKCα was immunoprecipitated from platelet lysates using a monoclonal anti-PKCα antibody. Immunoprecipitates were immunoblotted with anti-SHP-1 antibody (*top panel*); immunoblots were stripped and reprobed with monoclonal anti-PKCα antibody (*bottom panel*). *E*, purified GST fusion proteins, full-length SHP-1 (*WT*), and deletion mutant lacking the tandem SH2 domains (ΔT-SH2) were coupled to GSH-Sepharose beads and incubated with basal (−) or stimulated lysates (+). Samples were subjected to SDS-PAGE and immunoblotting with the monoclonal anti-PKCα antibody. All data shown are representative of at least three independent experiments.

cations for constitutive activation of SHP-1 in the cell, as discussed below. Fig. 7 illustrates the proposed model for regulation of SHP-1.

Protein tyrosine phosphorylation is a central mechanism in all mammalian cell types for regulation of protein function and is controlled by the balance between the activities of kinases and phosphatases. Under basal conditions the majority of proteins are not phosphorylated, and although there are important exceptions to this rule, such as members of the Src family of kinases, most proteins are kept in a nonphosphorylated state in basal cells. In order to maintain this state, it would be predicted therefore that the basal phosphatase activity outweighs the basal kinase activity. It follows also that there may be two ways in which protein phosphorylation may increase upon cellular activation: (i) activation of kinases and (ii) inactivation of phosphatases. It is likely, however, that both mechanisms operate simultaneously. The roles of SHP-1 and SHP-2 in cells have been shown to be quite different, with SHP-1 playing a largely negative signaling role (2) maintaining signaling proteins in an inactive un-phosphorylated state, whereas SHP-2 plays a largely positive signaling role leading to cellular activation. It would therefore be appropriate that SHP-1 is maintained in a high basal activity state which could be switched off rapidly upon cell activation, whereas the converse would be the case for SHP-2. In agreement with this hypothesis, we show here for human platelets, as others have shown in other cell types (29), that under basal conditions the activity of SHP-1 is already high and that upon cellular activation with thrombin this activity is diminished (Fig. 5*C*). Indeed, mice lacking functional SHP-1, the motheaten phenotype, exhibit severe autoimmune diseases of multiple organs, leading to death within 3 weeks of birth. It may be inferred from this phenotype that SHP-1 plays a constitutive role in maintaining low levels of protein Tyr phosphorylation within immune cells.

SHP-2 and then SHP-1 structures have been determined over the last 7 years, with both members of the family showing similar conformations (4, 5). These structural models, based upon purified recombinant proteins lacking the C-terminal tail, provide insight into how the domains of the phosphatase interact and suggest a mechanism by which phosphatase activity might be regulated. They indicate that proteins that engage the SH2 domains of SHPs are likely to lead to enzymatic activation and subsequent dephosphorylation of substrates *in vivo*. Our evidence that SHP-1 is maintained in a constitutively active state leads us to propose that this may be mediated by consti-

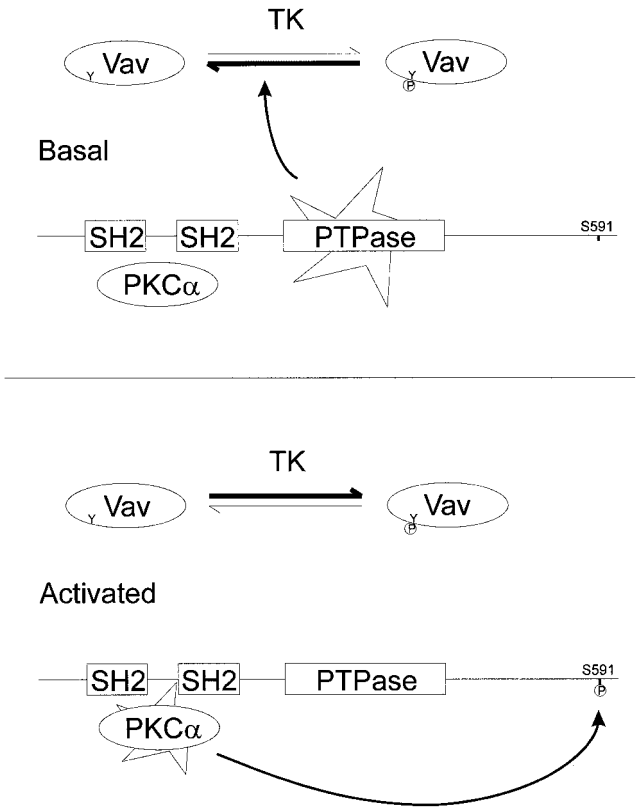

FIG. 7. **Model of regulation of SHP-1 by associated PKCα and phosphorylation of C-terminal Ser^{591}.** The diagram shows basal and activated cell conditions. Under basal conditions, SHP-1 is maintained in an active state by constitutive coupling of its SH2 domains to a protein complex that includes PKCα. SHP-1 also associates constitutively with its substrate Vav1, maintaining the basal nonphosphorylated state of its substrate. Upon activation of the cell, PKCα is activated leading to phosphorylation of SHP-1 on Ser^{591} at the C terminus and inhibition of its phosphatase activity. This event allows unhindered Tyr phosphorylation of Vav1 by tyrosine kinases (*TK*). Phosphorylation of Ser^{591} is therefore a critical negative regulator for SHP-1 and plays an important role in allowing positive tyrosine phosphorylation signals to proceed unimpaired by opposing phosphatase activity.

tutive binding of its SH2 domains to a protein complex that includes PKCα (Fig. 6*E*), as illustrated in the model diagram shown in Fig. 7. The nature of the interaction between SHP-1 and PKCα is not presently known, although it is likely to involve an intermediate protein because PKCα is not Tyr-phosphorylated under basal conditions in platelets.[2] The intermediate protein or proteins that comprise the complex are also not known, although SHP-1 has been found previously to constitutively associate with Jak2 kinase (30), with Tyk-2 kinase (31), and also with the SH2 domain containing protein SLP-76 (32) *in vivo*. It is possible that these proteins or others form part of the SHP-1-PKCα complex.

SHP-1 basal activity may be switched off upon cellular activation, thereby releasing proteins from the inhibitory effect of its basal phosphatase activity and allowing rapid Tyr phosphorylation of signaling proteins. The mechanism underlying this regulation of SHP-1 activity was important to determine. The C-terminal tail has been proposed to play a role in regulating the activities of both SHP-1 and SHP-2, although the nature of this role remains controversial. Phosphorylation of tyrosine residues within the C terminus have been shown possibly to play a role. Protein ligation techniques have recently shown for SHP-2 that phosphorylation of Tyr-542 or Tyr-580 may directly stimulate catalysis 2- or 3-fold (9). A similar approach has shown that Tyr-536, and to lesser extent Tyr-564, may also directly stimulate catalysis of SHP-1 *in vitro* (10). We chose to investigate whether phosphorylation on serines or threonines by PKC may also play a role in regulating SHP-1.

The antibody-based approach used in the present study for detection of Ser phosphorylation of SHP-1 was advantageous for a number of reasons. It allowed us to analyze Ser phosphorylation of SHP-1 *in vivo* indicating that the phosphorylation event was physiologically relevant and thus avoiding potential problems associated with interpretation of data from overexpression systems. Most importantly, however, knowledge of the antibody epitope showed that only Ser^{591} of SHP-1 was in an appropriate motif for phosphorylation by PKC that could be recognized by the Ser(P) (PKC) substrate antibody. Mutational analysis of this residue in a GST fusion protein *in vitro*, or as a GFP construct in HEK cells, demonstrated definitively that this antibody would only recognize phosphorylation of this site in PKC, and therefore the antibody could be used effectively as a site-specific anti-phosphoSer^{591} antibody. Moreover, Ser^{591} was within a consensus sequence for phosphorylation by classical PKC isoforms primarily, based upon determined PKC isoform-specific substrate motifs (33). Our finding of phosphorylation of SHP-1 at Ser^{591} was therefore consistent with the finding that PKCα selectively associated with SHP-1.

The role of phosphorylation of Ser^{591} by PKC was important to determine, and we speculated that it may either determine the localization of SHP-1 within the cell or regulate its activity. We were first able to show that localization of SHP-1 was not regulated by phosphorylation of Ser^{591}, as shown by the lack of effect of BIM I on thrombin-induced translocation of SHP-1 (Fig. 4). However, the activity of SHP-1 was controlled by PKC-mediated phosphorylation, as its ability to dephosphorylate its substrate Vav1 is regulated by this event. Vav1 is a large scaffolding protein expressing multiple protein interaction domains and an intrinsic Rho/Rac GEF activity. Vav1 has been shown to be phosphorylated on Tyr, including in response to thrombin in human platelets (26–28), and has been shown recently (25) to be a substrate for SHP-1 in NK cells. It has also been shown to associate with SHP-1 in lymphocytes and macrophages, and Kon-Kozlowski *et al.* (23) showed this to be mediated by an interaction between the SH3 and SH2 domain of Vav and phosphorylated Tyr in SHP-1, possibly within the C-terminal tail. Although the authors could not conclude that the interaction was direct, they demonstrated that it was constitutive in these cells. Our data showing Vav1-SHP-1 interaction is in agreement with this. Therefore, we propose that phosphorylation of SHP-1 at Ser^{591} by PKC inhibits SHP-1, allowing greater Tyr phosphorylation of Vav1 as illustrated diagrammatically in Fig. 7. This does not involve disruption of the physical interaction between SHP-1 and Vav1 (Fig. 5*B*), and the regulation of SHP-1 by Ser^{591} phosphorylation is independent of any regulation by tyrosine phosphorylation of SHP-1 (Fig. 3*C*). The nature of inhibition of SHP-1 activity by Ser^{591} phosphorylation may be direct or it may be that it provides a recruitment site for a protein (or proteins) that interferes with the ability of SHP-1 to dephosphorylate Vav1.

In conclusion, we propose a model for regulation of SHP-1 where it is held in a basally active state by occupation of its SH2 domains by a protein complex that includes PKCα. Upon cellular activation, this associating kinase phosphorylates SHP-1 on Ser^{591} in its C terminus, leading to inactivation of SHP-1, allowing Tyr phosphorylation of the associated Vav1 to proceed unopposed.

[2] G. Pula, M. L. Jones, and A. W. Poole, unpublished observations.

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Mechanisms of Signal Transduction:

Regulation of SHP-1 Tyrosine Phosphatase in Human Platelets by Serine Phosphorylation at Its C Terminus

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