Tyrosine Phosphorylation and Relocation of SHIP Are Integrin-mediated in Thrombin-stimulated Human Blood Platelets*

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The SH2 domain-containing inositol 5-phosphatase, SHIP, known to dephosphorylate inositol 1,3,4,5-tetraakisphosphate and phosphatidylinositol 3,4,5-trisphosphate has recently been shown to be expressed in a variety of hemopoietic cells. This 145-kDa protein is induced to associate with Shc by multiple cytokines and may play an important role in the negative regulation of immunocompetent cells mediated by FcγRIIB receptor. We report here that SHIP is present in human blood platelets and may be involved in platelet activation evoked by thrombin. Platelet SHIP was identified by Western blotting as a single 145-kDa protein. Both phosphatidylinositol 3,4,5-trisphosphate and inositol 1,3,4,5-tetraakisphosphate 5-phosphatase activities could be demonstrated in anti-SHIP immunoprecipitates of platelet lysate. Thrombin stimulation induced a tyrosine phosphorylation of SHIP, this effect being prevented if platelets were not shaken or if RGD-containing peptides were present, indicating an aggregation-dependent, integrin-mediated event. Moreover, although the intrinsic phosphatase activity of SHIP did not appear to be significantly increased, tyrosine-phosphorylated SHIP was relocated to the actin cytoskeleton upon activation in an aggregation- and integrin engineering-dependent manner. Finally, the striking correlation observed between phosphatidylinositol 3,4-bisphosphate production and the tyrosine phosphorylation of SHIP, as well as its relocation to the cytoskeleton upon thrombin stimulation, suggest a role for SHIP in the aggregation-dependent and GpIIb-IIIa-mediated accumulation of this important phosphoinositide.

Phosphoinositide 3-kinases (PI 3-kinases) play an important role in signal transduction induced by various growth factors (1, 2). In vitro, this lipid kinase activity is able to specifically phosphorylate the D3-position of the inositol ring of various phosphoinositides, thus producing phosphatidylinositol 3-monophosphate (PtdIns(3)P), phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P 2), and phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P 3). However, except for h-Vps34, the activity of which is restricted to PtdIns (3), the major substrate for PI 3-kinases in stimulated, intact mammalian cells is phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P 2) (2, 4, 5). This leads to the formation of PtdIns(3,4,5)P 3, which is thought to be an important lipid mediator of cellular signaling (1, 2, 6–8). So far, the only degrading enzyme discovered for this particular phosphoinositide is a 5-phosphatase producing PtdIns(3,4)P 2 (4, 9–11). Recently, a direct regulation of the Akt proto-oncogene product by PtdIns(3,4,5)P 3 has been reported, suggesting a possible second messenger function for this lipid (12).

In platelets, the production of these D3-phosphorylated phosphoinositides upon stimulation has been well documented (5, 13, 14). PtdIns(3,4,5)P 3 appears rapidly and transiently upon thrombin stimulation, whereas PtdIns(3,4)P 2 accumulates more slowly and for a large part in an aggregation-dependent manner (14, 15). Mass determination has revealed that up to 180 pmol/10^9 platelets of the latter is produced upon stimulation with the thromboxane receptor agonist U46619, representing 9.3% of the total phosphatidylinositol bisphosphates (16). Interestingly, PtdIns(3,4)P 2 has been shown to mediate specific effects in human platelets including actin assembly for filopodial growth (17), activation of the serine-threonine kinase Akt (12), and sustained exposure of the integrin GpIIb-IIIa, leading to irreversible aggregation (17, 18). Therefore, it is crucial to understand the mechanisms involved in the regulation of PtdIns(3,4,5)P 3 production. One obligatory step in its biosynthesis is the phosphorylation at the D3-position of the inositol ring. The activation of the two forms of PI 3-kinases (p55/p110 and p110γ) found in platelets has been studied by different groups (reviewed in Ref. 5). Activation of p55/p110 (PI 3-kinase α) has been shown to involve the small G-protein RhoA (19, 20) and our recent results suggested that p125^AKT may play a role in the aggregation- and integrin-dependent activation of this PI 3-kinase (15). Interestingly, it has been proposed that PI 3-kinase α is the PI 3-kinase mainly involved in the sustained activation of GpIIb-IIIa in stimulated platelets (20). The p110γ-
related form of PI 3-kinase seems to be activated via the βγ complex of dissociated heterotrimeric G-proteins (20, 21). Although a putative PI 4-kinase phosphorylating PtdIns(3)P (22) or a PI 3-kinase phosphorylating PtdIns(4)P may play a role in the formation of PtdIns(3,4)P2 in platelets, it has been suggested that the main part of PtdIns(3,4)P2 is formed by 5-dephosphorylation of newly generated PtdIns(3,4,5)P3 (23). Therefore, in addition to PI 3-kinase activation, a key regulatory step would appear to be the involvement of a PtdIns(3,4,5)P3 5-phosphatase (SHIP) (28, 29), the substrate specificity of which is peptide before thrombin stimulation as reported previously (14, 15).

In contrast, the PI 3-kinase lets were not shaken or were preincubated for 1 min with 500 munits of SHIP in vitro, as it is also a PtdIns(4,5)P2, inositol 1,4,5-trisphosphate, and Ins(1,3,4,5)P4 5-phosphatase (25–27). In contrast, the PI 3-kinase–associated 5-phosphatase seems to be specific for PtdIns(3,4,5)P3 (26). Although the preferential in vivo substrates for these 5-phosphatases and their regulation are still poorly known, they are potentially important elements in the control of intracellular level of PtdIns(3,4,5)P3 and PtdIns(3,4)P2.

The recently cloned SH2 domain-containing inositol 5-phosphatase (SHIP) (28, 29), the substrate specificity of which is restricted to Ins(1,3,4,5)P4 and PtdIns(3,4,5)P3, has been shown to be involved in signal transduction of immunoreceptors (30–33) and to be expressed in a variety of hemopoietic cells (34, 35). In B and mast cells, SHIP could act as a link to mediate the pleiotropic inhibitory effects induced by the FcγRIIB receptor (31, 36). These observations prompted us to investigate whether SHIP could also be present in human blood platelets and could be involved during platelet activation by thrombin. Indeed, thrombin-stimulated platelets provide an interesting model to study the different pathways leading to PtdIns(3,4,5)P3 accumulation, since the production of this phosphoinositol is high and sustained, and has been shown to be mainly, but not exclusively, aggregation-dependent and integrin-mediated (5, 14).

**EXPERIMENTAL PROCEDURES**

Reagents—An antipeptide antiserum to murine SHIP was raised in rabbits.2 For analysis in platelets, the serum was employed at a dilution of 1 in 20 for immunoprecipitation and at 1 in 2000 for Western blot analysis. The mouse anti-phosphotyrosine 4G10 antibody was purchased from the Upstate Biotechnology, Inc. The rabbit anti-phosphotyrosine (Y-15) was obtained from Promega. The 75-kDa enzyme has a broad substrate specificity which was cloned into the pcDNA3 expression vector (Invitrogen) as described previously (28). Using the unique internal BamHI site in human SHIP was prepared using a C-terminally truncated construct, which was cloned into the pcDNA3 expression vector (Invitrogen) as described previously (28). The 75-kDa enzyme has a broad substrate specificity which was cloned into the pcDNA3 expression vector (Invitrogen) as described previously (28). Using the unique internal BamHI site in human SHIP was prepared using a C-terminally truncated construct, which was cloned into the pcDNA3 expression vector (Invitrogen) as described previously (28). Using the unique internal BamHI site in human SHIP was prepared using a C-terminally truncated construct, which was cloned into the pcDNA3 expression vector (Invitrogen) as described previously (28). Using the unique internal BamHI site in human SHIP was prepared using a C-terminally truncated construct, which was cloned into the pcDNA3 expression vector (Invitrogen) as described previously (28).

**Preparation of Platelet Cytosolic and Particulate Extracts—**Washed platelets (1.5 × 10^9) resuspended in buffer A (100 mM Tris-HCl, pH 7.4, 10 mM EGTA, 1 mM Na3VO4, 1 mM PMSF, 4 μg/ml aprotinin and leupeptin) were disrupted by sonication at 4 °C (40 kHz for 3 × 5 s) using an ultrasonic cell disrupter (Branson Sonifier 250). Cytosolic and membrane fractions were separated by centrifugation at 120,000 × g for 40 min at 4 °C. The particulate pellet was resuspended in buffer A. Protein concentrations were determined using the Bio-Rad protein assay system using bovine serum albumin as standard.

**Gel Electrophoresis and Immunoblotting—**Proteins from the different fractions were resuspended in the electrophoresis sample buffer, boiled for 5 min, separated by 7.5% SDS/PAGE, and transferred onto a nitrocellulose membrane (Gelman Sciences) as reported previously (15). The blots were blocked with 1% milk powder, 1% bovine serum albumin in a buffer containing 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20. Immunodetection was performed with the relevant antibodies, and the reactions were visualized using peroxidase-conjugated secondary antibodies and the ECL chemiluminescence system. The quantification of the different bands was performed by a densitometric analysis, which determines the pixel volume in each area (Gel Doc 1000, Bio-Rad).

**Immunoprecipitation—**Reactions (1.5 × 10^9) cells in 0.5 ml were stopped by addition of one volume of ice-cold lysis buffer containing 80 mM Tris-HCl, pH 7.5, 200 mM NaCl, 20 mM EDTA, 2% Triton X-100, 200 mM NaF, 80 mM Na3PO4, 4 mM Na3VO4, 2 mM PMSF, and 10 μg/ml each of aprotinin and leupeptin. After gentle shaking during 20 min at 4 °C and centrifugation (12,000 × g for 10 min at 4 °C), the soluble fraction was collected and preincubated for 30 min at 4 °C with protein A-Sepharose CL4B. The preincubated suspensions were then incubated for 1 h at 4 °C with the adequate antibodies, and the immune complexes were precipitated by addition of 50 μl of 10% (v/v) protein A-Sepharose CL4B for 1 h. After centrifugation (6000 × g for 5 min at 4 °C), the immunoprecipitates were washed once in lysis buffer diluted in one volume of distilled water, then washed with a buffer (containing 10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.5% Triton X-100, 100 μg/ml Na3VO4, 1 mM PMSF, 1 μg/ml each of aprotinin and leupeptin), and finally washed twice with the same buffer without Triton X-100 and Na3VO4. Immunoprecipitates were then used for electrophoresis. For enzyme assays, one half of the immunoprecipitate was used.

**Inositol Polynosphate 5-Phosphatase Assays—**PtdIns(3,4,5)P3 was prepared using PtdIns(4,5)P2, (–1)32P[2]PPI (9000 Ci/mmol, NEN Life Science Products, France), and immunopurified as described previously (9). The 5-phosphatase activity was measured essentially as described elsewhere (30) using sonicated vesicles that contained 30,000 dpm of TLC-purified PtdIns(3,4,5)P3 [2]PPI, together with 50 μg of phosphatidyserine in 50 mM Tris-HCl, pH 7.5. Reaction mixtures (25 μl) containing immunoprecipitated SHIP, 50 mM Tris-HCl, pH 7.5, 10 mM MgCl2, and substrate were mixed for 30 min at 37 °C. Reactions were stopped by addition of chloroform/methanol (v/v) and the lipids extracted following a Bligh and Dyer modified procedure (41). PtdIns(3,4,5)P3 and its degradation product PtdIns(3,4)P2 were separated by TLC as described previously (42), and the radioactive spots were visualized by a PhosphorImager 445 SI (Molecular Dynamics, Inc) or autoradiography. Ins(1,3,4,5)P4, 5-phosphatase assays were done with [3H]Ins(1,3,4,5)P4 (1 μM) as described previously (28, 43, 44).

**Lipid Extraction and Analysis—**The production of PtdIns(3,4,5)P3 was analyzed in platelets labeled with 32P (Amersharm International) as described previously (14, 15). Briefly, reactions were stopped by addition of chloroform/methanol (v/v), and the lipids were extracted following a Bligh and Dyer modified procedure (41), deacylated, and finally analyzed by HPLC on a Partisphere SAX column (Whatman International Ltd, United Kingdom) (14, 15).

**RESULTS**

**Expression of 145-kDa SHIP in Human Blood Platelets—**To determine whether the SH2 domain-containing inositol phosphatase SHIP was expressed in human platelets, a specific

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2 E. Radley, R. Woscholski, and P. J. Parker, unpublished results.
antibody raised against a C-terminal peptide sequence of mouse SHIP was used. No signal could be detected in COS cells transfected with the vector alone (Fig. 1A, lane 1), whereas a single 145-kDa protein was detected in SHIP-transfected cells (Fig. 1A, lane 2). As shown in Fig. 1A (lane 4), an identical single 145-kDa protein was detected in human blood platelet lysate, whereas a normal rabbit serum (NRS) gave no signal (Fig. 1A, lane 3). This antibody, but not the NRS, could also immunoprecipitate a 145-kDa protein from a platelet lysate that was recognized by the antiserum (Fig. 1A, lanes 6 and 7). This 145-kDa protein was mainly found in the soluble fraction of platelet lysates (Fig. 1A, lanes 8 and 9). To further establish that the platelet 145-kDa protein recognized by the antiserum was indeed an inositol and phosphatidylinositol polyphosphate 5-phosphatase, phosphatase activities were determined in anti-SHIP immunoprecipitates. As clearly shown in Fig. 1B and C, the 145-kDa immunoprecipitated protein exhibited both PtdIns(3,4,5)P$_3$ and Ins(1,3,4,5)P$_4$ 5-phosphatase activities, respectively. Altogether, these data indicated that the 145-kDa SHIP was present in human blood platelets.

Tyrosine Phosphorylation and Relocation of SHIP Were Integrin-mediated—To see whether SHIP could be involved in thrombin-induced platelet activation, anti-phosphotyrosine immunoprecipitates from resting or activated platelets were probed with the anti-SHIP antibody. As shown in Fig. 2A, SHIP was weakly present in anti-phosphotyrosine immunoprecipitates obtained from resting platelets or platelets stimulated with thrombin for a short period of time (30 s), whereas SHIP was clearly detected after longer stimulation (3 and 5 min). The specificity of the immunoprecipitation reaction was assessed by using protein A-Sepharose alone as a control, which did not precipitate SHIP. These data suggest that SHIP was mainly recruited to multienzymatic complexes, which are recovered in anti-phosphotyrosine immunoprecipitates, during later steps of platelet activation. In agreement with this, we found that this event was inhibited if platelets were not shaken or if the binding of fibrinogen to the integrin GpIIb-IIIa was prevented (Fig. 2B), indicating an aggregation- and integrin-dependent mechanism.

To determine the tyrosine phosphorylation state of SHIP during thrombin activation, anti-SHIP immunoprecipitates were prepared and probed with an anti-phosphotyrosine antibody. As shown in Fig. 2B, SHIP was weakly tyrosine-phosphorylated in resting conditions or after 30 s of stimulation, whereas its tyrosine phosphorylation was clearly increased upon 3 and 5 min of stimulation. Again, this effect was blocked if platelets were not shaken or if RGDS peptide was present (Fig. 2B, right panel). As a control, antibodies were stripped off the same nitrocellulose, and this was reprobed with the anti-SHIP antibody to check the amount of immunoprecipitated proteins which was similar over the different conditions (data not shown).

To check whether SHIP 5-phosphatase activity was increased under these conditions, we determined the enzymatic activity in anti-SHIP immunoprecipitates obtained from resting or activated platelets. In vitro assays indicated that the activity of SHIP was not significantly increased upon stimulation, when either PtdIns(3,4,5)P$_3$ (Fig. 3A) or Ins(1,3,4,5)P$_4$ (Fig. 3B) was used as substrate.

We then addressed the question of a possible thrombin stimulation-dependent relocation of SHIP as a potential regulatory event. We and others have shown previously that stimulation of platelets by thrombin resulted in the redistribution of several enzymes involved in signal transduction, including PI 3-kinases, to the cytoskeleton (5, 15, 45). The translocation of the PtdIns 3-kinase α to the cytoskeleton has been correlated with stimulation of platelets by thrombin.
with the production of PtdIns(3,4)P$_2$ (15). We therefore examined whether SHIP could translocate to the cytoskeleton. Interestingly, SHIP was weakly present in the cytoskeleton of resting platelets but was clearly relocated to the cytoskeleton upon thrombin stimulation (Fig. 4A). Fig. 4A shows the time course for SHIP translocation to the cytoskeleton, and it is seen that this relocation was a late event. It was dependent on aggregation and integrin engagement as it was inhibited when the platelets were not shaken or when RGDS peptide was used. To determine the localization of SHIP to the cytoskeleton, we have performed a selective extraction to obtain the actin filament system (15, 40). Fig. 4B shows that SHIP was relocated to the actin filament system upon thrombin stimulation. It is noteworthy that the cytoskeletal pool of SHIP, which represented 9% ± 2 of the total amount of SHIP, was tyrosine-phosphorylated as determined by immunoprecipitation of SHIP from the cytoskeleton and Western blot analysis using the anti-phosphotyrosine antibody as a probe (data not shown).

**Correlation between PtdIns(3,4)P$_2$ Accumulation and SHIP Mobilization**—We then addressed the question of a correlation between relocation of SHIP and production of PtdIns(3,4)P$_2$ in thrombin-stimulated platelets. The kinetics of PtdIns(3,4)P$_2$ accumulation in whole platelets have been compared with the time course over which SHIP was (i) tyrosine-phosphorylated and (ii) translocated to the cytoskeleton. A striking temporal correlation between these different events was observed (Fig. 5A) and also with the appearance of SHIP in anti-phosphotyrosine immunoprecipitates (see Fig. 2A). Moreover, these events were similarly inhibited when integrin-mediated signals were abolished in the absence of shaking or in the presence of the RGDS peptide (Fig. 5B).

**DISCUSSION**

With regard to the pivotal role of PI 3-kinase in signal transduction, it is important to underline the route of synthesis and the regulatory factors involved in the generation of the different D3-phosphorylated polyphosphoinositides. In thrombin-stimulated platelets, PtdIns(3,4)P$_2$ appears to be an important messenger (5, 12, 17, 18), and its accumulation is known to be, in a large part, a relatively late event that depends on aggregation and integrin engagement (5, 12–15). The molecular mechanisms regulating the production of this phosphoinositide are still unknown. The binding of fibrinogen to its integrin receptor GpIIb-IIIa has been shown to be necessary (14) but probably not sufficient for a full response of PtdIns(3,4)P$_2$ accumulation (20, 46). A PI 3-kinase is obviously involved in the formation of this phosphoinositide; however, several other enzymes might be implicated, for instance a PI 4-kinase acting on PtdIns(3)P (22) or a PtdIns(3,4,5)P$_3$ 5-phosphatase. To unravel the underlying mechanisms, the different enzymes that may participate in the accumulation of PtdIns(3,4)P$_2$ must be identified. In this report, we have addressed the question of whether the recently described inositol 5-phosphatase SHIP would be present in platelets and could be involved during activation of platelets by thrombin in a way compatible with the production of PtdIns(3,4)P$_2$. In other cells, SHIP has been shown to be tyrosine-phosphorylated in response to several stimuli and to form complexes with the signaling molecules Shc and Grb2 (29, 30, 34, 47). It also binds to tyrosine-phosphorylated immunoreceptor tyrosine-based activation motif of FcεRI (30) or immunoreceptor tyrosine-based inhibition motif of FcγRIIB (31). We demon-
strate here that SHIP is present in human blood platelets as a 5-phosphatase active toward both PtdIns(3,4,5)P$_3$ and Ins(1,3,4,5)P$_4$. SHIP was identified by Western blotting as a single 145-kDa protein. Other species (i.e. 110-kDa or 130-kDa proteins), as reported recently in mature blood cells (47), have not been detected. It is known that thrombin induces several waves of tyrosine phosphorylation in platelets, the latest being dependent upon aggregation and integrin engagement (48). Here we show that SHIP is mainly recovered in anti-phospho-tyrosine immunoprecipitates obtained at a late stage of platelet stimulation. This observation suggests that SHIP may belong to multienzymatic complexes formed during an aggregation- and integrin-dependent signaling mechanism. This is confirmed in the absence of shaking or by the use of the RGDS peptide, which competes with fibrinogen for binding to GpIIb-IIIa. We further show that SHIP itself is weakly tyrosine-phosphorylated, both in resting and in the early phase of platelet stimulation, but becomes clearly tyrosine-phosphorylated after 3 min of stimulation, this tyrosine phosphorylation being aggregation-dependent and integrin-mediated. Despite the care taken during platelet preparation, one cannot fully exclude the occurrence of a minor nonspecific platelet activation that may explain the weak basal tyrosine phosphorylation level observed in resting conditions. However, in some cells, SHIP has been found to be constitutively tyrosine-phosphorylated (30). An important point that we are currently investigating is the identification of the tyrosine kinase responsible for its phosphorylation. In this respect, pp60$^*$src or p125$^*$FAK are potential kinases as they are known to be recruited to adhesion plaque-like areas formed during platelet aggregation (15).

SHIP has been shown to associate with Shc following interleukin-3 stimulation (35), and it has been postulated that SHIP can compete with Grb2 for binding to the activated form of Shc, thus modulating the Ras pathway. In RBL-2H3 cells, Shc, Grb2, and SHIP are constitutively associated (30), while in other cells SHIP becomes associated with Shc only upon stimulation (35, 47). It was reported recently that during T cell receptor signaling, the phosphotyrosine binding domain of Shc is necessary and sufficient for its association with tyrosine-phosphorylated SHIP (49). In RBL-2H3 cells, SHIP has been shown to directly bind to FcRI via its SH2 domain (30). Therefore, depending on the cell type, SHIP seems to be associated with a different subset of phosphotyrosyl proteins, and we are currently investigating the proteins that can associate with SHIP in activated platelets.
When the 5-phosphatase activity was assayed in SHIP immunoprecipitates obtained from platelets stimulated for different times, we could not detect any significant increase in its specific activity, even though we occasionally noticed a slight increase after 3 and 5 min of stimulation. We cannot exclude the possibility of an essential cofactor or protein that would be required to see any change in activity. Osborne et al. (30) have found that SHIP does not require tyrosine phosphorylation to be active, and they even showed that its \textit{in vitro} phosphorylation by Lck leads to a decreased activity. Our data also indicate that SHIP immunoprecipitated from resting cells can be active toward PtdIns(3,4,5)P$_3$ and Ins(1,3,4,5)P$_4$, \textit{in vitro}. However, another essential regulatory event in the metabolism of phoshoisostes is the localization of implicated enzymes. The cytosolic PLC$_\gamma$ or PI 3-kinase, for instance, must translocate to the membrane or to membrane/cytosol interface elements to reach their lipid substrates. Here, we show that tyrosine-phosphorylated SHIP is also relocated to the actin cytoskeleton of thrombin-activated platelets in an aggregation-dependent and integrin-mediated mechanism. A number of observations have already suggested a role for cytoskeleton in the assembly of platelet signaling complexes that may localize activated enzymes close to their relevant substrates (5, 45, 50). We have shown previously that PtdIns(3,4)P$_2$ accumulation parallels the aggregation and the translocation of PI 3-kinase to the cytoskeletal matrix (15). It is noteworthy that we found here a temporal correlation between the mobilization of SHIP in the pool of tyrosine-phosphorylated proteins, its tyrosine phosphorylation, its relocation to the cytoskeletal matrix, and the production of PtdIns(3,4)P$_2$ occurring upon thrombin stimulation. Moreover, these mechanisms are inhibited to a comparable extend when aggregation and integrin GpIb-IIIa engagement are blocked. However, further work will be necessary to quantify the relative impact of the tyrosine phosphorylation and/or the relocation of SHIP on its own activation and on PtdIns(3,4)P$_2$ appearance. Using different agonists, we have recently shown that a large part of the accumulation of PtdIns(3,4)P$_2$ was correlated with the irreversible aggregation mechanism (51). Interestingly, this phosphoinositide has been shown to play an essential role in the organization of some specific actin assembly (17) and to sustain the activation of integrins leading to irreversible aggregation (17, 18). It has also been shown recently to be able to stimulate the serine-threonine kinase Akt (12).

Besides SHIP, two other PtdIns(3,4,5)P$_3$ 5-phosphatases have been described in platelets, which may also participate in the regulation of the level of these important phosphoinositides (25, 26). Interestingly, one of these phosphatases has been shown to be associated with PI 3-kinase $\alpha$ in resting platelets, and thrombin stimulation leads to the dissociation of the two enzymes (26). Therefore, this novel phosphatase and SHIP may be involved at different stages of platelet activation. The relative effect of these phosphatases on the cellular level of PtdIns(3,4,5)P$_3$ and PtdIns(3,4)P$_2$ is, however, difficult to predict precisely and must await further investigation. Indeed, although their regulation is still poorly known, 3- and/or 4-phosphatases may also play important roles in the more or less rapid degradation of intracellular PtdIns(3,4)P$_2$ (4, 9, 52). Furthermore, an independent pathway for PtdIns(3,4)P$_2$ production may exist via a PI 4-kinase phosphorylating PtdIns(3)P or via a recently described C2 domain-containing PI 3-kinase that phosphorylates primarily PtdIns and PtdIns(4)P (53). Nevertheless, so far, the latter enzyme has not been described in platelets.

In addition, one should note that SHIP is also able, \textit{in vitro}, to hydrolyze Ins(1,3,4,5)P$_4$ to a soluble inositol phosphate that has been shown to bind and activate a GTPase-family member that regulates Ras negatively (54). Therefore, SHIP could down-regulate GTP activity and increase Ras activity in certain cells. The Ins(1,3,4,5)P$_4$ generated may also be involved in cell signaling, as it is a potent competitive inhibitor of the inositol 3,4,5,6-tetrakisphosphate 1-kinase (55). However, the very rapid kinetics (10–20 s) of Ins(1,3,4,5)P$_4$ and Ins(1,3,4,5)P$_4$ production in thrombin-stimulated platelets (56) do not correlate with the late tyrosine phosphorylation and relocation of SHIP that we have observed. Therefore, if SHIP rapidly hydrolyzes this soluble compound, it may be independently of the late recruitment that we have observed.

Altogether, our results indicate that SHIP is recruited down-
stream of adhesion-receptor engagement during platelet activation and provide evidence that SHIP is a potential candidate for the hydrolysis of PtdIns(3,4,5)P$_3$ producing a significant part of the PtdIns(3,4,5)P$_3$ that accumulates. Thus, in addition to its down-regulation of PtdIns(3,4,5)P$_3$-dependent mechanisms, SHIP may generate PtdIns(3,4)P$_2$, another important messenger triggering specific platelet functions (12, 17, 18). However, the relative impact of SHIP on PtdIns(3,4)P$_2$ degradation in platelets and downstream essential physiological responses further investigations to determine the contribution of SHIP and other 5-phosphatases in the accumulation of PtdIns(3,4)P$_2$ in platelets and downstream essential physiological responses.

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