SHIP-2 FORMS A TETRAMERIC COMPLEX WITH FILAMIN, ACTIN AND GPIb-IX-V. Localization of SHIP-2 to the activated platelet actin cytoskeleton.


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ABSTRACT

The platelet receptor for von Willebrand factor (vWF) glycoprotein (GP)Ib-IX-V complex mediates platelet adhesion at sites of vascular injury. The cytoplasmic tail of the GPIbα subunit interacts with the actin-binding protein, filamin, anchoring the receptor in the cytoskeleton. In motile cells, the second messenger PtdIns(3,4,5)P₃ induces submembraneous actin remodelling. The inositol polyphosphate 5-phosphatase, SHIP-2, hydrolyzes phosphoinositide 3,4,5 trisphosphate (PtdIns(3,4,5)P₃) forming PtdIns(3,4)P₂, and regulates membrane ruffling via complex formation with filamin (Dyson et al., J. Cell. Biol 2001 155(6) 1065-1079). In this study we investigate the intracellular location and association of SHIP-2 with filamin, actin, and the GPIb-IX-V complex in platelets. Immunoprecipitation of SHIP-2 from the Triton-soluble fraction of unstimulated platelets demonstrated association between SHIP-2, filamin, actin and GPIb-IX-V. SHIP-2 associated with filamin or GPIb-IX-V, was active, and demonstrated PtdIns(3,4,5)P₃ 5-phosphatase activity. Following thrombin or vWF-induced platelet activation, detection of the SHIP-2, filamin and receptor complex decreased in the Triton-soluble fraction, although in control studies the level of SHIP-2, filamin, or GP1b-IX-V immunoprecipitated by their respective antibodies did not change following platelet activation. In activated platelets spreading on a vWF matrix, SHIP-2 localized intensely with actin at the central actin ring and co-localized with actin and filamin at filopodia and lamellipodia. In spread platelets, GPIb-IX-V localized to the centre of the platelet and showed little co-localization with filamin at the plasma membrane. These studies demonstrate a functionally active complex between SHIP-2, filamin, actin and GPIb-IX-V which may orchestrate the localized hydrolysis of PtdIns(3,4,5)P₃ and thereby regulate cortical and submembraneous actin.
INTRODUCTION

Following vascular injury, platelet adhesion and aggregation mediate the immediate arrest of bleeding; however, in pathological states this response may contribute to arterial blockage leading to cerebrovascular and coronary arterial occlusion. The interaction of the platelet membrane glycoprotein (GP)Ib-IX-V complex with its adhesive ligand, von Willebrand factor (vWF), in the subendothelial matrix initiates platelet adhesion under conditions of rapid blood flow in the arterial circulation. The GPIb-IX-V complex can also bind plasma vWF under conditions of pathological shear stress, leading to platelet aggregation and thrombosis. 1-3 Following binding of vWF, the GPIb-IX-V complex initiates intracellular signals inducing platelet activation, spreading and activation of the integrin $\alpha_{IIb}\beta_3$ receptor from a low to high affinity state (inside out signalling). The molecular mechanisms regulating vWF-GPIb-IX-V-induced signaling, however, remain to be fully delineated. 4

The GPIb-IX-V complex is composed of four subunits; GPIb$\alpha$ disulphide-linked to GPIb$\beta$, GPV and GPIX. The extracellular N-terminal globular domain of GPIb$\alpha$ contains the binding site for vWF. The 96 amino acid cytoplasmic tail of GPIb$\alpha$ contains no overt signaling domains, but directly binds the actin binding–protein, filamin 1a, and the signaling adapter protein, 14-3-3$\zeta$. 5,6 Filamins are large actin-binding proteins that stabilize submembraneous actin webs and link them to cellular membranes. Filamins promote high angle actin filament branching and thereby actin gelation, although the molecular mechanisms mediating this effect are incompletely understood. Three filamin isoforms A, B and C have been identified, with A and B expressed in platelets. 7 The interaction of filamin with GPIb-IX-V is essential for regulating the shape of resting
platelets, the spreading of activated platelets and the anchoring of the receptor complex to the membrane skeleton.\textsuperscript{5,8-10} The stabilization of the platelet membrane resulting from the interaction of filamin with GPIb-IX-V may not only prevent platelet membrane deformation under conditions of high fluid shear stress, but also regulates the adhesive function of the receptor complex itself and the ability of the receptor complex to maintain adhesion to vWF under conditions of high shear stress.\textsuperscript{10,11} Following platelet adhesion, vWF binding to GPIb-IX-V induces the cytoskeletal translocation and activation of the p85/p110 form of phosphoinositide 3-kinase (PI 3-kinase).\textsuperscript{12} Under shear conditions, PI 3-kinase is essential for vWF-induced calcium release.\textsuperscript{13} A potential mechanism for activation of PI 3-kinase via GPIb-IX-V has been demonstrated in studies from our laboratory, which have shown that the p85 subunit of PI 3-kinase forms a complex with both 14-3-3\(\zeta\) and the receptor complex.\textsuperscript{6}

The inositol lipids phosphatidylinositol 4,5 bisphosphate (PtdIns(4,5)P\(_2\)) and phosphatidylinositol 3,4,5 trisphosphate (PtdIns(3,4,5)P\(_3\)), play a central role in the regulation of actin polymerisation and thereby cell motility.\textsuperscript{14,15} Many recent studies have demonstrated in motile cells that PtdIns(3,4,5)P\(_3\) spatially localizes to the leading edge of the cell, at membrane ruffles (lamellipodia). Production of this lipid determines where and when submembraneous actin polymerization takes place. PtdIns(3,4,5)P\(_3\) localizes many signaling and actin-regulatory proteins to the inner wall of the plasma membrane and allosterically regulates their function. (reviewed by \textsuperscript{16}) In this manner, PtdIns(3,4,5)P\(_3\) mediates agonist-induced actin-dependent extension of lamellipodia and cell migration. The agonist-induced transient accumulation of PtdIns(3,4,5)P\(_3\) at the leading edge of motile cells results from a balance between its synthesis by the PI 3-kinase and hydrolysis by specific lipid phosphatases.\textsuperscript{17} Recent studies have demonstrated
the SH2-inositol polyphosphate 5-phosphatase, SHIP-2, hydrolyzes the 5-position phosphate from PtdIns(3,4,5)P₃ forming PtdIns(3,4)P₂ and is localized to the leading edge of the cell, whereby the 5-phosphatase regulates submembraneous actin and membrane ruffling. This 5-phosphatase is a widely expressed enzyme that contains an amino-terminal SH2 domain, a central 5-phosphatase domain, and a carboxyl-terminal proline-rich domain. It bears significant sequence identity with the hematopoietic specific 5-phosphatase, SHIP-1, except in the proline-rich domain where the two 5-phosphatases demonstrate significant sequence diversity. SHIP-2 localization to membrane ruffles is mediated via complex formation through its C-terminal proline-rich domain with the actin-binding protein, filamin. Further evidence for the role SHIP-2 plays in regulating submembraneous actin has been demonstrated by studies which have shown SHIP-2 forms a complex with p130Cas at focal adhesions and regulates cell adhesion. Collectively these studies suggest that SHIP-2 regulates the localized changes in PtdIns(3,4,5)P₃ that instruct submembraneous actin remodelling, membrane ruffling and promote cell adhesion.

As SHIP-2 appears to significantly influence cytoskeletal remodelling, we investigated the localization and expression of SHIP-2 in platelets. Platelets are highly motile cells with a complex cytoskeleton. At early time points in platelets spreading on a VWF matrix, SHIP-2 localized initially to filopodia and the central actin ring, and with filamin and submembraneous actin at lamellipodia. Actin, GPIb-IX-V, SHIP-2 and filamin were shown in complex in the Triton-soluble fraction of platelets, however, following platelet activation the level of this complex decreased significantly. No complex was detected in the detergent extracted actin cytoskeleton of resting or activated platelets, however, immunofluorescence co-localization studies demonstrate SHIP-2 and
filamin colocalized at the submembraneous actin cytoskeleton of activated platelets spreading on a vWF matrix, suggesting the SHIP-2 filamin complex may relocate to the submembraneous actin cytoskeleton upon platelet activation. Collectively these studies provide evidence that SHIP-2 localizes in spreading platelets to sites of active actin remodelling and thereby may regulate localized concentrations of PtdIns(3,4,5)P₃.
MATERIALS AND METHODS

Reagents.

Monoclonal antibodies raised to human platelet filamin, SHIP-1, cortactin, and HA were obtained from Chemicon (Temecula, U.S.A), Santa Cruz (Santa Cruz, U.S.A), Auspep (Parkville, Australia), Molecular Probes (Eugene, U.S.A), and Silenus (Melbourne, Australia). Rabbit polyclonal antibodies to glycocalicin (a soluble fragment of GPIbα) and SHIP-2 were raised as previously described.6,18 von Willebrand Factor (vWF) and botrocetin were purified as previously described.9 [α-32P]dCTP and [γ-32P]ATP were from NEN Life Science products (Boston, U.S.A) HA-SHIP-1 cDNA was a kind gift from Dr. Gerry Krystal (British Columbia Cancer Agency, Canada). All other reagents were purchased from Sigma (St. Louis, U.S.A) unless otherwise stated.

Northern Blot Analysis.

A membrane containing approximately 20 µg poly(A) RNA isolated from various cell lines was probed with the C-terminal proline-rich domain of human SHIP-2 cDNA (encompassing nucleotides 3017-3989) labelled with [α-32P]dCTP by random priming (Prime-a-gene, Promega) and hybridised to the membrane overnight at 42°C. Blots were washed using standard procedures.22 The membranes were allowed to decay and subsequently hybridized to an actin probe.

Platelet Immunoprecipitations.

Platelets obtained from healthy volunteers who were medication free for at least ten days were washed using a previously described method.23 Washed platelets were either not stimulated or stimulated with 1 U/ml of thrombin or vWF, 10 µg/ml, and botrocetin, 3 µg/ml, for 5 minutes. Following activation with thrombin or vWF, the
platelets were lysed with 1 volume of Triton X-100 lysis buffer (200 mM Tris-HCl, pH 7.4, 10% Triton X-100, 50 mM EGTA, 4 mM leupeptin, 4 mM aprotinin, 2.5 mg/ml PMSF) to 9 volumes of platelets and rocked at 4°C for 1 hour. Lysates were centrifuged at 15,400 x g for 10 minutes separating the Triton X-100-soluble and insoluble (actin cytoskeletal) extracts. The supernatant represents the Triton-soluble lysate. The cytoskeletal pellet was washed twice with 20 mM Tris, pH 7.4, 150 mM NaCl, 400 µM leupeptin, 400 µM aprotinin, and 250 µg/ml PMSF, solubilized by incubation with RIPA buffer (10 mM NaH₂PO₄, pH 7.0, 150 mM NaCl, 2 mM EDTA, 250 µg/ml PMSF, 400 µM leupeptin, 400 µM aprotinin, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS) for 1 hour at 4°C and centrifuged at 15,400 x g for 10 minutes. The supernatant represents the RIPA-extracted actin cytoskeleton. Immunoprecipitation of GPIb-IX, filamin or SHIP-2 was performed as follows; 5 µg of polyclonal antisera directed against GPIb (anti-glycocalicin antibody), or 5 µg of monoclonal filamin antibody, or 5 µg of polyclonal antisera against SHIP-2, or 5 µl of pre-immune or non-immune sera were incubated overnight at 4°C with 600 µl of Triton-soluble lysate and 60 µl of a 50% slurry of protein-A-Sepharose that was pre-equilibrated with 20 mM Tris, pH 7.4, 150 mM NaCl. The protein-A-Sepharose pellets were washed extensively with 20 mM Tris, pH 7.4, 150 mM NaCl and either analyzed by SDS-PAGE and immunoblotting, or used for 5-phosphatase assays as described below. For DNase I treatment experiments, platelets were lysed with 1 volume of Triton X-100 lysis buffer (without EGTA) and fractionated in the presence of DNase I (final concentration of 2 mg/ml). The Triton X-100-soluble fraction was isolated and immunoprecipitated as described above with either non-immune sera, or affinity-purified SHIP-2 antibodies.
Intracellular localization in platelets.

Coverslips were coated with vWF (10 µg/ml) blocked as previously described.²⁴ Platelets obtained from healthy volunteers as previously described,²³ were applied to vWF coated coverslips and incubated at 37°C. Following this, coverslips were washed with Tyrodes buffer (12 mM NaHCO₃, 10 mM Heps, 137 mM NaCl, 2.7 mM KCl, 5.5 mM glucose), fixed with 4 % formaldehyde and permeabilized with 0.5 % Triton X-100. Polymerized actin was detected using phalloidin-Texas Red conjugate. Proteins were detected using polyclonal antibodies to; GPIbα diluted 1:300 (final concentration of 0.76 µg/ml), GPIbβp diluted 1:1000 (final concentration of 0.40 µg/ml), and affinity-purified SHIP-2 used neat, detected with FITC-conjugated goat anti-rabbit IgG. Filamin, cortactin and vinculin were localized using monoclonal antibodies diluted 1:1000, 1:100 and 1:800, respectively, and detected with FITC/TRITC-conjugated goat anti-mouse IgG.

PtdIns(3,4,5)P₃ 5-Phosphatase Assays.

The preparation of ³²P-labeled PtdIns(3,4,5)P₃ substrate was undertaken as follows: 25 µg of phosphatidylserine and 65 µg of PtdIns(4,5)P₂ were mixed, dried under nitrogen, then resuspended in 400 µl of lipid resuspension buffer (20 mM Heps, pH 7.5, 1 mM MgCl₂, 1 mM EGTA) and sonicated for 5 min. 50 µl of this suspension was added to 5 nmol of unlabeled ATP and 20 µl of [³²P]ATP (2 mCi, 3000 mCi/mmol), 5 µl of 20x kinase buffer (400 mM Heps, pH 7.5, 100 mM MgCl₂, 20 mM EGTA) and 1 µg of affinity-purified recombinant PI 3-kinase, in a final reaction volume of 100 µl.²⁵ Following incubation overnight at room temperature, the reaction was terminated by the addition of 1 M HCl, and following the addition of 5 µg of phosphatidylserine, the lipid
was extracted with chloroform/methanol (1:1) and 2 M KCl saturated with chloroform and dried. PtdIns(3,4,5)P₃ 5-phosphatase assays were performed on immunoprecipitates in a buffer containing 50 mM Tris, pH 7.2 and 5 mM MgCl₂ as described previously.²⁶ Following the reaction, lipids were extracted and analyzed by TLC. The phosphoinositide standards used for the migration of PtdIns(3,4,5)P₃ and PtdIns(3,4)P₂ on TLC were verified by deacylation and HPLC analysis.²⁷

**Immunoblotting HA-tagged SHIP-2 and SHIP-1 recombinant proteins in COS-1 cells.**

COS-1 cells were transiently transfected with either HA empty vector, HA-SHIP-2, or HA-SHIP-1 as previously described¹⁸ and lysates immunoblotted with either affinity-purified polyclonal SHIP-2 antibodies, or monoclonal SHIP-1 antibodies.
RESULTS

SHIP-2 is expressed in platelets

Although SHIP-2 has been strongly implicated in regulating actin dynamics at the leading edge of the cell, this signal-terminating enzyme has not been characterized in highly motile cells, such as platelets. Analysis of the expression profile of SHIP-2, has revealed widespread expression in skeletal muscle, heart, brain, lung and some hematopoietic cells including T and B cells. However, expression of SHIP-2 in platelets has yet to be fully characterized. Northern analysis was therefore performed using a probe encoding sequences unique to SHIP-2 (nucleotides 3017 to 3989) that have no homology to SHIP-1. Analysis of various cancer cell lines including adenocarcinoma (Hela), a human megakaryocytic cell line (MEG-01), T cell leukemia (Jurkat), lung cancer (A549), neuroteratocarcinoma (NT) and glioma (C6) demonstrated expression of a 4.75 kb message, consistent with the predicted size of the SHIP-2 transcript (Figure 1A). The expression level of SHIP-2 in the megakaryocyte cell line, MEG-01, when standardized by reprobing the Northern blot with an actin probe, was comparable to that observed in the other cell lines.

To further characterize SHIP-2 in platelets, we immunoblotted and immunoprecipitated SHIP-2 from Triton-soluble platelet lysates using a previously characterized affinity-purified SHIP-2-specific anti-peptide antibody. This SHIP-2 antibody is raised to peptide sequences, unique to SHIP-2, not found in the highly related 5-phosphatase SHIP-1, or any other 5-phosphatase. To confirm specificity of the SHIP-2 antibody, SHIP-1 or SHIP-2 immunoprecipitates from Triton-soluble platelet lysates, were immunoblotted with specific antibodies to each 5-phosphatase. SHIP-2 anti-peptide antibodies detected a 148 kDa polypeptide in SHIP-2, but not SHIP-1,
immunoprecipitates, consistent with SHIP-2 expression in platelets (Figure 1B). Furthermore SHIP-1 antibodies were not immunoreactive with SHIP-2 immunoprecipitates. SHIP-2 antibodies also did not detect SHIP-1 when hemagglutinin tagged (HA) SHIP-1 was expressed at high levels in COS-7 cells (Figure 1C).

We also investigated if SHIP-2 translocated to the actin cytoskeleton in thrombin-stimulated platelets, as has been shown for SHIP-1. The subcellular compartmentalization of SHIP-2, filamin and GPIb in resting versus thrombin-stimulated platelets was compared to that of the p85 subunit of PI 3-kinase. In unstimulated platelets, SHIP-2 was detected in the Triton-soluble fraction, but not in the actin cytoskeleton (Figure 1D). Minor proteolysis of SHIP-2 was demonstrated by the presence of a slightly faster migrating polypeptide, which we and others have previously detected in various cell lysates. In resting platelets, both filamin and GPIb were present in the unstimulated platelet actin cytoskeleton. Following thrombin activation, the level of SHIP-2 expression in the Triton-soluble fraction remained relatively unchanged and SHIP-2 was only detected very faintly in the actin cytoskeleton (Figure 1D). However, we noted increased amounts of GPIb and filamin in the actin cytoskeleton following 5 minutes thrombin stimulation.
Figure 1A, B, C and D
Intracellular localization of SHIP-2 in vWF stimulated platelets.

Many motile cells respond to agonists and chemokines by localized actin remodelling or membrane ruffling (lamellipodia) in the direction of the stimulus. Platelets are terminally differentiated anucleate cells, with a highly specialized cytoskeleton, and the formation of filopodia and lamellipodia in response to platelet activation is well described. We investigated the intracellular localization of SHIP-2 in platelets spreading on a vWF matrix using confocal microscopy. In addition, in order to improve the size of the platelet images and the resolution, high-powered confocal microscopy was performed using 400 x magnification.

The intracellular localization of SHIP-2 in platelets spreading on a vWF matrix was dynamic with respect to specific phases of platelet activation and was different to that we previously reported in resting and EGF-stimulated COS-7 cells. At early phases of platelet adhesion, SHIP-2 staining was intense in small unspread platelets and did not specifically localize to any discernable structure however, staining was absent from the centre of the platelet (Figure 2Aa). As platelets spread, SHIP-2 localized to filopodia (Figure 2Ab see arrow), and also SHIP-2 antibodies stained a central ring structure, with no staining within the ring (Figure 2Bc see arrow). SHIP-2 also localized to lamellipodia (Figure 2A see arrow in d). In spread platelets SHIP-2 staining was diffuse at the plasma membrane and staining at the central ring was maintained, but was less clearly demarcated (Figure 2Ae). Pre-immune staining was non-reactive. The outline of the platelets used for preimmune staining was demonstrated by phalloidin staining.
Figure 2A
To compare the localization of SHIP-2 with respect to actin in platelets spreading on a vWF matrix, we co-stained for SHIP-2 and polymerized actin, the latter was detected using phalloidin. In partially spread platelets, the staining of SHIP-2 at filopodia co-localized intensely with actin, (see arrow Figure 2Ba and b) and at the central actin ring (also called the granulomere) 31 (Figure 2Ba and b). With increased platelet spreading, SHIP-2 co-localization with the central actin ring was predominantly on the inner aspect of the actin ring (Figure 2Bc see arrow in merged images). In some platelets as the actin ring enlarged, SHIP-2 staining was predominantly internal to the actin ring, and was diffuse rather than “ring-like” at this site (Figure 2Bd arrow in SHIP-2 staining). Localization of SHIP-2 to lamellipodia was demonstrated in the merged images by the eccentric co-localization of SHIP-2 with actin staining at one side of the platelet (Figure 2Bd, see arrow in merged image). These studies demonstrate a novel localization of SHIP-2 in spreading platelets, to filopodia and the central actin ring, in addition to lamellipodia. The localization of SHIP-2 to these sites, may serve to regulate PtdIns(3,4,5)P3 hydrolysis and thereby actin remodelling.
Figure 2B
We have recently shown that the SHIP-2 proline-rich domain binds the actin-binding protein, filamin, at the C-terminal immunoglobulin repeats 22-24. In COS-7 cells, co-immunoprecipitation of SHIP-2 and filamin demonstrates the two species form a constitutive complex \textit{in vivo}, which is unaltered by EGF stimulation. We further pursued the investigation of this association in human platelets to reveal other potential components in this complex ie. actin and GPIbα. We determined the complexes SHIP-2 forms with filamin, actin and GPIbα in both unstimulated and thrombin- or vWF-stimulated platelets. Filamin binds polymerized actin \textit{via} its N-terminal actin-binding domain (Figure 3A). In platelets the calcium-dependent protease calpain, cleaves filamin into two fragments, one of approximately 190 kDa, which contains the actin-binding domain, and another of 100 kDa, with the latter further hydrolyzed to 90 kDa and 10 kDa polypeptides (Figure 3A). The SHIP-2 binding site on filamin is present in both the 100 and 90 kDa fragments, but not the 190 kDa polypeptide (Figure 3A). To eliminate the possibility that actin may act as an intermediate in the association between SHIP-2 and filamin, SHIP-2 immunoprecipitations were performed in the presence or absence of DNase I, a potent inhibitor of actin polymerization. Platelet Triton-soluble lysates with or without EGTA (DNase I enzyme activity is dependent on the presence of divalent cations), were immunoprecipitated using affinity-purified antibodies specific for SHIP-2 and immunoblotted with filamin antibodies, which recognize both intact filamin as well as calpain-cleaved filamin polypeptides. In the presence of EGTA and without DNase-1, SHIP-2 immunoprecipitates, but not pre-immune immunoprecipitates, demonstrated a 250 kDa polypeptide recognised by filamin antibodies (Figure 3B, lanes 4 and 1 respectively, upper panel), consistent with association between SHIP-2 and filamin in unstimulated platelets. Under these conditions, SHIP-2 immunoprecipitates also
contained actin (Figure 3B, lane 4, lower panel). In the absence of EGTA and or with
DNase I, SHIP-2 immunoprecipitates immunoblotted with filamin antibodies
demonstrated 100 and 90 kDa polypeptides, consistent with calpain-cleaved filamin
fragments which have lost the actin-binding domain. (Figure 3B, lanes 5 and 6, upper
panel). However, the level of association of the 100 and 90 kDa filamin fragments with
SHIP-2 appeared decreased, compared to intact filamin. No actin was demonstrated in
SHIP-2 immunoprecipitates under these conditions (Figure 3B lanes 5 and 6, lower
panel). In control studies we demonstrated equal immunoprecipitation of SHIP-2 in
platelet lysates in the presence or absence of EGTA (Figure 3B). Collectively these
studies demonstrate a trimeric complex between SHIP-2, filamin and actin in the Triton-
soluble lysate from resting platelets. Under these conditions association between SHIP-2
and calpain-cleaved filamin appears independent of actin.
Figure 3A and B
SHIP-2, filamin and GPIb form a complex in the Triton-soluble compartment of resting platelets, but not stimulated platelets.

The intracellular domain of GPIbα associates with filamin, which in turn directly binds actin.\(^5,8\) In resting platelets, this interaction stabilizes the platelet membrane. In addition, under conditions of high shear the interaction between GPIbα and filamin is essential for receptor anchorage to vWF matrix.\(^4,8\) As we have shown SHIP-2 associates with filamin and actin, we determined if GPIbα was also present in this complex. This was also of interest, as we have previously shown that PI 3-kinase binds to GPIbα via an interaction with 14-3-3, and therefore may generate PtdIns(3,4,5)P\(_3\), the substrate for SHIP-2, in close association with the receptor.\(^6\) Triton-soluble platelet lysates were isolated in the presence of EGTA (to minimize calpain cleavage of filamin) from resting or thrombin-activated platelets and immunoprecipitated with SHIP-2 specific antibodies and immunoblotted with antibodies to filamin, or anti-glycocalcicin (Figure 4A). The latter antibody recognizes the extracellular domain of GPIbα.\(^6\) Both intact filamin and cleaved-filamin were detected in SHIP-2 immunoprecipitates from the Triton-soluble fraction of unstimulated platelets. Following platelet activation, the level of filamin detected in SHIP-2 immunoprecipitates significantly decreased (Figure 4A). SHIP-2 immunoprecipitates were probed with anti-GPIb and detected a 125 kDa polypeptide, consistent with association of GPIb and SHIP-2, in the Triton-soluble fraction of unstimulated platelets. Detection of the GPIb-SHIP-2 complex reduced significantly in this fraction following thrombin activation. These results are in contrast to the constitutive association of SHIP-2 and filamin we have previously reported in the Triton-soluble fraction of EGF-stimulated COS-7 cells.\(^18\)
vWF mediates platelet adhesion through the binding of matrix-bound vWF to GPIb-IX-V, which then transmits signals leading to platelet activation. We investigated whether the association of SHIP-2 and filamin was also influenced by signaling, specifically mediated through the GPIb-IX-V complex. Anti-SHIP-2 immunoprecipitates from the Triton-soluble fraction of untreated or vWF-stimulated platelets were immunoblotted with filamin or GPIb antibodies (Figure 4A, lower panels). These studies demonstrated that SHIP-2 immunoprecipitates contained both filamin and GPIb. However, following vWF treatment, the level of the SHIP-2/filamin/GPIb complex detected in the Triton-soluble fraction decreased significantly.

To confirm interactions between SHIP-2, filamin and GPIb, reciprocal immunoprecipitation experiments were performed in which filamin was immunoprecipitated from the Triton-soluble fraction, derived from resting or thrombin-stimulated platelets, and probed using affinity-purified polyclonal antibodies to SHIP-2 or GPIb (Figure 4B). SHIP-2 was present in anti-filamin immunoprecipitates of the Triton-soluble fraction, of resting or thrombin-stimulated platelets (Figure 4B), but decreased significantly following thrombin stimulation. We also noted some proteolysis of SHIP-2 in filamin immunoprecipitates. Filamin immunoprecipitates demonstrated GPIb in complex in the Triton-soluble fraction of unstimulated platelets. However, the level of GPIb detected in filamin immunoprecipitates decreased following thrombin activation.

We also performed anti-GPIb immunoprecipitations of the Triton-soluble fraction of resting or thrombin-stimulated platelets and probed the immunoprecipitates with polyclonal antibodies to SHIP-2 or monoclonal antibodies to filamin (Figure 4C). SHIP-2 was present in anti-GPIb immunoprecipitates of the Triton-soluble platelet fraction of
unstimulated platelets, and decreased in this fraction following thrombin stimulation. Association between GPIb and filamin was detected in the Triton-soluble fraction of unstimulated platelets. We noted, however, that the detection of this complex decreased upon thrombin activation. In control studies we immunoprecipitated SHIP-2, filamin, or GPIb from the Triton-soluble fraction of resting, thrombin- or vWF-activated platelets and immunoblotted with the immunoprecipitating antibody. The level of SHIP-2, filamin and GPIb immunoprecipitated did not change following platelet activation, demonstrating that the relative amount of immunoprecipitated antigen did not change as a result of platelet activation, although the level of the SHIP-2, filamin and or GPIb detected in complex decreased (Figure 4D).

**SHIP-2 in the SHIP-2/filamin/GPIb complex in resting platelets is catalytically active.**

SHIP-2 hydrolyzes the 5-position phosphate from PtdIns(3,4,5)P$_3$ generating PtdIns(3,4)P$_2$ and thereby regulates PtdIns(3,4,5)P$_3$-induced actin remodelling. We investigated in unstimulated platelets whether SHIP-2 bound to filamin/actin and GPIb was enzymatically active. Anti-SHIP-2, anti-filamin and anti-GPIb immunoprecipitates from the Triton-soluble fraction of unstimulated, or thrombin-stimulated platelets were bound to protein-A-Sepharose, and washed extensively. The protein-A-Sepharose pellets were incubated with PtdIns(32P-3,4,5)P$_3$ and standard 5-phosphatase enzyme assays were performed as described under Materials and Methods. PtdIns(3,4,5)P$_3$ and the lipid products of the 5-phosphatase assays were extracted and analyzed by thin layer chromatography (Figure 4E). Incubation of pre-immune immunoprecipitates with PtdIns(3,4,5)P$_3$ resulted in little formation of PtdIns(3,4)P$_2$. In contrast, the SHIP-2, filamin and GPIb immunoprecipitates all demonstrated evidence of significant
PtdIns(3,4,5)P₃ 5-phosphatase activity. In these immunoprecipitates, but not pre-immune immunoprecipitates, the addition of PtdIns(3,4,5)P₃ resulted in the formation of PtdIns(3,4)P₂, consistent with expression of functionally active SHIP-2 enzyme in complex with both filamin/actin and GPIb in unstimulated platelets. There was no change in the relative amount of SHIP-2 PtdIns(3,4,5)P₃ 5-phosphatase activity immunoprecipitated following thrombin stimulation (Figure 4E).
A

Filamin immunoblot

GPIb immunoblot

Thrombin (min) 0 5 0 5

IP: Pre I  SHIP-2

vWF: - + - +

IP: Pre I  SHIP-2

B

SHIP-2 immunoblot

GPIb immunoblot

Thrombin (min) 0 5 0 5

IP: Non I  Filamin

IP: Non I  Filamin

C

SHIP-2 immunoblot

Filamin immunoblot

Thrombin (min) 0 5 0 5

IP: Non I  GPIb

IP: Non I  GPIb
Figure 4 A,B,C,D,E
Localization of SHIP-2, filamin and GPIb with actin in spreading platelets.

The results of the immunoprecipitation analysis demonstrate SHIP-2 in a complex with filamin, actin and GPIbα. Following thrombin-stimulation the level of the SHIP-2 and filamin, and filamin and GPIb complex significantly decreased in the Triton-soluble fraction. We investigated the association between SHIP-2, filamin, actin and GPIb-IX-V in the RIPA-extracted cytoskeletal fraction using immunoprecipitation of each species and immunoblot analysis. A complex was not reproducibly detected between SHIP-2 and filamin and/or GPIb-IX-V in the RIPA-extracted cytoskeletal fraction, from either unstimulated or activated platelets (data not shown). There are several possible interpretations of this result. The SHIP-2/GPIb/filamin complex may disassociate following platelet activation, or the complex may incorporate into the activated actin cytoskeleton and due to the technical constraints of the harsh extraction conditions using RIPA extraction buffer, which contains 1% NP40 and 0.1% SDS, we cannot demonstrate this complex in the activated actin cytoskeleton. Therefore to investigate these species in the activated cytoskeleton, the intracellular localization of SHIP-2, filamin, and GPIb (detected using specific antibodies) was co-localized with actin (detected by phalloidin staining, which stains polymerized actin) in platelets spreading on a vWF matrix (Figure 5). The co-localization of filamin and GPIb with actin in a functional model of spreading platelets to our knowledge has not been previously reported.

In partially spread platelets, filamin localized in the cytosol and plasma membrane staining was prominent (Figure 5Aa). Patchy co-localization with actin was demonstrated in between filamin and actin at the central actin ring (Figure 5Aa merged image). With increased platelet spreading, intense filamin staining was demonstrated at the plasma membrane, with little cytosolic staining (Figure 5Ab). Filamin staining co-localized with
submembraneous actin at the plasma membrane lamellipodia (Figure 5Ab, see arrows in merged images). In spread platelets there was little co-localization between filamin and actin at the central actin ring.

To determine the intracellular localization of GPIb, we used two distinct antibodies, polyclonal antibodies to GPIbβp, which recognizes cytoplasmic sequence within the GPIbβ chain (data not shown), and polyclonal antibodies to the extracellular domain of GPIbα (Figure 5B). Results with both antibodies were comparable. GPIbβp antibodies stained intensely a central region of the platelet, which was localized predominantly internal to the central actin ring. Only very faint plasma membrane staining was detected using this antibody (data not shown). Antibodies to the extracellular region of GPIbα demonstrated intense staining at the centre of the spreading platelet, which localized internal to the central actin ring and demonstrated patchy co-localization with actin at this site. No plasma membrane staining was demonstrated using this antibody (Figure 5B). We also examined whether GPIb co-localized with filamin (Figure 5C). Consistent with the immunoprecipitation data demonstrating decreased detection of the filamin and GPIb complex with platelet activation in the Triton-soluble fraction, patchy co-localization between these species was observed. In spread platelets, filamin localized predominantly at the plasma membrane, whilst the receptor localized to the centre of the platelet. Patchy co-localization was detected at the centre of the spread platelet, with no co-localization detected at the plasma membrane (Figure 5C). In control studies, to demonstrate specificity of the antibodies we examined the intracellular localization of vinculin and cortactin. Vinculin localized to the platelet membrane, and diffusely in the cytosol with no staining detected at the central actin ring, whereas
cortactin localized to lamellipodia at the plasma membrane, as has recently been reported in platelets (Figure 5D).
Figure 5A, B, C and D

We also determined the co-localization of SHIP-2 with filamin in spreading platelets (Figure 6). SHIP-2 and filamin co-localized intensely at filopodia and also demonstrated co-localization at the central actin ring (Figure 6a and b). In spread platelets filamin localized most intensely at the plasma membrane (Figure 6c). Colocalization between filamin and SHIP-2 was demonstrated at plasma membrane lamellipodia (Figure 6c merged images). In the cytosol SHIP-2 staining was detected, but filamin staining was less intense and the two species demonstrated only patchy co-localization at this site. These studies suggest the SHIP-2-filamin complex may be incorporated in to the submembraneous actin cytoskeleton, as platelets are activated by spreading on a vWF matrix.
Figure 6
DISCUSSION

SHIP-2 is a PtdIns(3,4,5)P₃ 5-phosphatase that has recently been shown to regulate submembraneous actin, lamellipodia formation and integrin-dependent cell adhesion. The function of this signal-terminating enzyme, however, has not been characterized in highly motile cells. The results of this study demonstrate a number of significant novel findings. First, in human platelets, SHIP-2 forms an indirect complex with actin via interaction with the actin-binding protein, filamin. In spreading platelets, SHIP-2 co-localizes with actin at filopodia, lamellipodia and the central actin ring. The enzyme also dynamically relocates in spread platelets internal to the central actin ring. Second, SHIP-2 forms a functionally active complex with filamin, and the level of this complex decreases in the Triton-soluble fraction following platelet adhesion and activation. This is in contrast to adherent COS-7 cells in which the complex between these proteins is constitutive in the Triton-soluble fraction following EGF stimulation. Third, SHIP-2 also complexes with GPIb-IX-V and following platelet activation less complex is detected in the Triton-soluble fraction. Fourth, we have also investigated the relationship between SHIP-2/filamin/actin and GPIb-IX-V and co-localized these species by imaging platelets spreading on a vWF matrix. These studies indicate GPIb-IX-V is primarily localized to the centre of the platelet, whilst filamin localizes with SHIP-2 at lamellipodia at the submembrane actin cytoskeleton, suggesting the complex between SHIP-2 and filamin may relocalize to this site, rather than disassociate upon platelet activation.

PtdIns(3,4,5)P₃ is a critical regulator of many intracellular signaling pathways. Recent studies have highlighted the role this lipid signaling cascade, in particular PtdIns(3,4,5)P₃, plays in instructing actin remodelling.¹⁴,¹⁵,¹⁷ PtdIns(3,4,5)P₃ localizes to
lamellipodia and regulates Rac and Rho activation. The metabolism of PtdIns(3,4,5)P$_3$ has emerged as molecular mechanism regulating cell migration. SHIP-2 localizes to lamellipodia and inhibits membrane ruffling, via regulation of PtdIns(3,4,5)P$_3$. In this study, we have shown SHIP-2 indirectly binds actin via an association with filamin. The signaling adapter protein, 14-3-3ζ, directly binds the cytoplasmic domain of GPIbα and also PI 3-kinase indicating PtdIns(3,4,5)P$_3$ is generated in close proximity to the receptor. The demonstration of functionally active SHIP-2, a specific PtdIns(3,4,5)P$_3$-5-phosphatase associated with GPIb-IX-V, provides evidence for the localized regulation of this signaling molecule in unstimulated platelets.

The results from the studies reported here showing decreased SHIP-2/filamin complex, in the detergent-soluble fraction of thrombin- or vWF-stimulated platelets is different to the constitutive association of SHIP-2 and filamin in adherent EGF-stimulated COS-7 cells. There are several possible interpretations of these results. In platelets the SHIP-2/filamin/GPIb complex may dissassociate upon thrombin or vWF stimulation, or alternatively the complex may become incorporated into the activated platelet cytoskeleton. The failure to detect the SHIP-2/filamin complex in the actin cytoskeletal fraction may be a consequence of the complex existing below detectable levels, if represents only a minor proportion of each species, or the complex may be highly sensitive to the RIPA extraction buffer, used extract the actin cytoskeletal fraction.

In human platelets which express high levels of calpain, the decreased association of SHIP-2 with filamin in activated platelets may result from calpain-mediated cleavage of filamin at the hinge region, thereby disrupting the SHIP-2 binding site. Previously we have shown using yeast two-hybrid analysis that the association between SHIP-2 and filamin is maximal when mediated by filamin repeats 22-24, which includes the calpain-
cleaved hinge II region, located between repeats 23 and 24 (see Figure 3A). Repeats 22 and 23 in combination, with or without the hinge region II interacted with SHIP-2, however, this was weaker than with intact repeats 22-24. However, we cannot exclude the possibility that the decreased detection of the complex between SHIP-2 and filamin/GPIb-IX-V reflects the incorporation of this complex from the Triton-soluble fraction into the cytoskeletal fraction. In support of this contention, we have shown using immunoconfocal microscopy that SHIP-2 and filamin, but not GPIb-IX-V, co-localize with each other and submembraneous actin at both filopodia and lamellipodia.

Filamin plays a complex role in regulating platelet activation. In addition to binding GPIb-IX-V, filamin binds various other macromolecules, including engaging the β-chain of β-3 integrin, as well as in other cells the small GTPases, Cdc42, Rac, and their guanine nucleotide exchange factor, Trio. To date, over 20 binding partners have been identified and many of these binding partners facilitate the activation of localized signaling pathways involving actin polymerization. The GPIbα chain binds repeats 17-19 of filamin. The sequences of the GPIbα receptor that mediate this interaction have recently been shown to encompass residues 570-90, which are also essential for membrane anchorage of the receptor complex under conditions of high shear stress. The results of the study reported here demonstrate filamin may also provide a scaffold for the assembly of signaling proteins, such as SHIP-2, to facilitate signaling via the GPIb-IX-V receptor. We also demonstrated in spread platelets that the filamin and the GPIb-IX-V complex are localized in distinct cytoskeletal compartments; filamin at the submembraneous cytoskeleton and the GPIb-IX-V receptor centralized to the inner central actin ring. This receptor localization is consistent with previous studies using electron microscopy that GPIb-IX-V is located centrally, and although receptor
localization with respect to polymerized actin or co-localization with filamin was not demonstrated, receptor centralization was shown to be dependent on an intact actin cytoskeleton. However, in contrast to our findings which show a decrease in the filamin/GPIb-IX-V complex detected in the Triton-soluble fraction of activated platelets, Kovacsovics and Hartwig showed constitutive association between these species by co-immunoprecipitation analysis. The reason for these differences may relate to technical differences in that we did not routinely include DNase-I, which potently inhibits actin polymerization, or phallacidin, which stabilizes existing actin filaments in either the lysis or the immunoprecipitation buffer. The results of our analysis are consistent with the contention that as platelets spread and actin polymerizes, GPIb remains internal to the central actin ring and filamin localizes with SHIP-2 to submembraneous actin, rather than incorporating with a centralized/internalized receptor complex.
ACKNOWLEDGMENTS

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REFERENCES

FIGURE LEGENDS

Figure 1: SHIP-2 is expressed in a megakaryocyte cell line and human platelets

A. Membranes containing approximately 20 µg of mRNA from the indicated cell lines, adenocarcinoma of the cervix (HeLa), megakaryoblast (MEG-01), T cell leukemia (Jurkat), lung carcinoma (A549), neuroteratocarcinoma (NT) and glioma (C6), were hybridized with SHIP-2 cDNA (nucleotides 3017-3989) and washed as described under “Materials and Methods”. After exposure, the membrane was allowed to decay and subsequently hybridized to an actin probe.

B. The Triton X-100-soluble fraction of platelets was isolated and immunoprecipitated with either Non-immune (Non I) sera, affinity purified SHIP-2 anti-peptide, or SHIP-1 antibodies, and immunoblotted with antibodies to SHIP-2 (upper panel), or SHIP-1 (lower panel).

C. COS-1 cells transiently transfected with HA empty vector, or HA-SHIP-1 were harvested and the lysates immunoblotted with either SHIP-1 (left panel), or SHIP-2 (right panel) antibodies.

D. Platelets were either left untreated or treated with thrombin (1 U/ml, final concentration) for 5 minutes at room temperature, and then lysed. The Triton-soluble and actin cytoskeletal (Actin CSK) fractions were isolated and immunoblotted with antibodies to SHIP-2, filamin, glycocalicin (GPIb), or p85 as indicated. Results shown are typical of three separate experiments.

Figure 2: SHIP-2 localizes to actin-rich regions in spreading platelets.

A. Platelets spread on a vWF matrix were fixed, permeabilized, stained with SHIP-2 antibodies, or co-stained with affinity-purified pre-immune sera and phaloidin, and
visualized by confocal microscopy. Arrows indicate SHIP-2 staining at filopodia (b), or the central actin ring (c), or lamellipodia (d). Bar, 10 µm.

B. Platelets spread on a vWF matrix were fixed, permeabilized, co-stained with affinity-purified SHIP-2 antibodies and phalloidin, and visualized by confocal microscopy. Arrows indicate co-localization of SHIP-2 and phalloidin at filopodia (a and b), at the inner actin ring (c) and at lamellipodia (d) (merged images). The arrow showing SHIP-2 staining in (d) indicates diffuse central staining detected in some spread platelets. Bar, 10 µm.

**Figure 3: SHIP-2 associates with filamin independently of actin.**

A. Schematic of the calpain mediated cleavage profile of filamin A. The empty box represents the actin-binding domain (ABD) and the repeats 1-24 of filamin are shown as ellipses. Grey and black filled ellipses represent GPIb and SHIP-2 binding sites, respectively. The calpain-cleavage sites on intact filamin are shown. Calpain-mediated cleavage of filamin results in the production of a 100 kDa and 90 kDa fragments which retain the GPIb and partial SHIP-2 binding sites and a 190 kDa fragment which contains the actin-binding domain, but lacks the SHIP-2 and GPIb binding site.

B. The Triton X-100-soluble fraction of platelets isolated in lysis buffer containing EGTA (lanes 1 and 4), in the absence of EGTA (lanes 2 and 5), or in the presence of DNase I without EGTA (lane 3 and 6) was immunoprecipitated with either Preimmune (Pre I) sera, or SHIP-2 affinity-purified antibodies and immunoblotted with filamin antibodies (upper panel), or with actin antibodies (lower panel), or with SHIP-2 antibodies as shown. Arrows indicate the migration of intact filamin and calpain-cleaved filamin fragments.
Figure 4: Filamin and GPIb co-immunoprecipitate with SHIP-2.

A. The Triton X-100-soluble fraction of resting, thrombin- (1 U/ml, final concentration for 5 minutes), or vWF-stimulated platelets (10 µg/ml, and botrocetin, 3 µg/ml, final concentration for 5 minutes) platelets was isolated. The Triton X-100-soluble fraction was immunoprecipitated with either pre-immune (Pre I) sera, or SHIP-2 antibodies and immunoblotted with filamin, or GPIb antibodies.

B. The Triton X-100-soluble fraction of resting and thrombin-stimulated platelets was isolated as in (A), immunoprecipitated with either non-immune (Non I) sera, or filamin antibodies and immunoblotted with either affinity-purified SHIP-2 antibodies, or GPIb antibodies.

C. The Triton X-100-soluble fraction of resting and thrombin-stimulated platelets was isolated as in (A), immunoprecipitated with either Non-immune (Non I) sera, or GPIb antibodies and immunoblotted with affinity-purified SHIP-2 antibodies, or filamin antibodies.

D. The Triton X-100-soluble fraction of resting, thrombin- (1 U/ml, final concentration for 5 minutes), or vWF-stimulated platelets (10 µg/ml, and botrocetin, 3 µg/ml, final concentration for 5 minutes) was isolated. The Triton X-100-soluble fraction was immunoprecipitated with either Non-immune (Non I) sera, SHIP-2, filamin or GPIb antibodies and immunoblotted with the immunoprecipitating antibody.

E. Triton X-100-soluble fractions were isolated from untreated platelets and immunoprecipitated with either Non-immune (Non I) sera, affinity-purified SHIP-2 antibodies, filamin antibodies, or GPIb antibodies. The Triton X-100-soluble fraction was also isolated from thrombin-stimulated (1 U/ml, final concentration for 5 minutes) platelets and immunoprecipitated with either Non-immune (Non I) sera or affinity-
purified SHIP-2 antibodies. Immunoprecipitates captured on protein-A-Sepharose were subjected to PtdIns(3,4,5)P$_3$ 5-phosphatase assays and the lipid products of the enzyme assay examined by thin layer chromatography. The migration of the phospholipids was compared to known standards PtdIns(3,4,5)P$_3$ and PtdIns(3,4)P$_2$.

**Figure 5: Localization of filamin, GPIb and actin in spreading platelets.**

Platelets spread on a vWF matrix were fixed, permeabilized and co-stained with filamin antibodies and phalloidin (Aa and b), GPIb$\alpha$ antibodies and phalloidin (B), GPIb$\alpha$ and filamin antibodies (C), vinculin antibodies (D) and cortactin antibodies (D). Arrows in merged images in (5Ab) indicate co-localization of filamin and submembraneous actin at lamellipodia. Cells were visualized by confocal microscopy. Bars, 10 $\mu$m.

**Figure 6: SHIP-2 co-localizes with filamin in spreading platelets at lamellipodia.**

Platelets spread on a vWF matrix were fixed, permeabilized, co-stained with filamin antibodies and affinity-purified SHIP-2 antibodies, and visualized by confocal microscopy. Bar, 10 $\mu$m.
SHIP-2 forms a tetrameric complex with filamin, actin, and GPIb-IX-V.

Localization of SHIP-2 to the activated platelet actin cytoskeleton.

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