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The SH2-domain of SHIP1 interacts with the SHIP1 C-terminus: Impact on SHIP1/Ig-α interaction

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ABSTRACT

The SH2-containing inositol 5′-phosphatase, SHIP1, negatively regulates signal transduction from the B cell antigen receptor (BCR). The mode of coupling between SHIP1 and the BCR has not been elucidated so far. In comparison to wild-type cells, B cells expressing a mutant IgD- or IgM-BCR containing a C-terminally truncated Ig-α respond to pervanadate stimulation with markedly reduced tyrosine phosphorylation of SHIP1 and augmented activation of protein kinase B. This indicates that SHIP1 is capable of interacting with the C-terminus of Ig-α. Employing a system of fluorescence resonance energy transfer in S2 cells, we can clearly demonstrate interaction between the SH2-domain of SHIP1 and Ig-α. Furthermore, a fluorescently labeled SH2-domain of SHIP1 translocates to the plasma membrane in an Ig-α-dependent manner. Interestingly, whereas the SHIP1 SH2-domain can be pulled-down with phospho-peptides corresponding to the immunoreceptor tyrosine-based activation motif (ITAM) of Ig-α from detergent lysates, no interaction between full-length SHIP1 and the phosphorylated Ig-α ITAM can be observed. Further studies show that the SH2-domain of SHIP1 can bind to the C-terminus of the SHIP1 molecule, most probably by inter- as well as intra-molecular means, and that this interaction regulates the association between different forms of SHIP1 and Ig-α.

1. Introduction

The hemopoietic-specific SH2-domain-containing inositol 5′-phosphatase 1 (SHIP1) is known as an important negative regulator of the phosphatidylinositol 3-kinase (PI3K) pathway [1]. It comprises an N-terminal SH2-domain, a central catalytic domain, and a proline-rich C-terminus containing two NPXY sequences which, after phosphorylation on tyrosines, can interact with PTB-domain-containing adaptor proteins, like Shc or Dok1 [1]. SHIP1 is critically involved in the downregulation of activation signals from the BCR after coligation of the BCR with the inhibitory receptor, FcγRIIB (CD32), through IgG-antigen immune complexes [2,3]. In such a scenario, the PTK Lyn phosphorylates the tyrosine residue within the FcγRIIB ITIM, thus creating a docking site for the SH2-domain of SHIP1 [4]. By this way SHIP1 is recruited to the plasma membrane and hydrolyzes its substrate, phosphatidylinositol-3,4,5-trisphosphate (PIP_3), thereby inhibiting various BCR-mediated, PIP_3-dependent responses, e.g. calcium mobilization [3]. Moreover, SHIP1 bound to the FcγRIIB can fulfill a phosphatase-independent adaptor role by interacting with Dok1 and RasGAP, thus contributing to the suppression of MAPK activation [5]. However, SHIP1 not only acts downstream from the inhibitory FcγRIIB, but has also been demonstrated to be tyrosine-phosphorylated in response to antigen stimulation and has an attenuating role for the signal emitted from the BCR itself [6–9]. Furthermore, we could establish SHIP1 as a crucial gatekeeper of mast cell degranulation by acting downstream of the activating immunoreceptor FcεR1 and restricting PI3K-mediated signals [10,11]. In the case of the FcεR1, it is thought that SHIP1 binds to the phosphorylated ITAM of the β-subunit of the FcεR1 by means of its SH2-domain [12]. However, it is still not known how SHIP1 function is coupled to the BCR.

Although there is only one gene for SHIP1 multiple forms of this signaling protein can be found in different cells. In cell lineages of the bone marrow and the blood, expression of SHIP1 forms changes according to both the developmental stage and differentiated lineage

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of the mature blood cell [13]. Moreover, spliced forms of SHIP1, 110 and 135 kDa in length with alterations in the C-terminal tail of SHIP1, which is thought to be important for mediating protein–protein interactions, have been identified [14,15]. Complicating the picture, SHIP1 protein has been shown to be C-terminally cleaved yielding additional forms (110, 125, and 135 kDa in length) [16]. Furthermore, a SHIP1 isoform, s-SHIP, particularly expressed in embryonic and hemopoietic stem cells has been described lacking the SH2-domain and being unable, in contrast to full-length SHIP1, to interact with the adaptor protein Shc [17]. Despite this increasing knowledge about different forms of SHIP1 the functional implications of these findings are still lacking.

Signals generated by the BCR are required for the activation, proliferation, and differentiation of B cells [18]. The BCR comprises the membrane-bound immunoglobulin (mIg), which carries the two antigen binding sites and the disulfide-bridged Ig-α/Ig-β heterodimer, which is non-covalently bound to mIg and mediates the signal transduction of the BCR [19]. Due to the predominant activating nature of the BCR and since there is a so-called functional dichotomy between ITAM and ITIM sequences (as mentioned above), it was very intriguing when two groups studying mutant mice which carried a C-terminal truncation of Ig-α published a negative signaling role for Ig-α in immature B cells [20,21]. Immature B cells from these Ig-α mutant mice responded to BCR engagement with an exaggerated and prolonged calcium response and induction of protein tyrosine phosphorylation. These data indicated that in particularly Ig-α was involved in coupling the BCR to negative signaling elements.

In this report we present data demonstrating that the SH2-domain of SHIP1 is capable of binding to the phosphorylated ITAM of the Ig-α subunit of the BCR. However, the C-terminus of SHIP1 can also interact with the SH2-domain of SHIP1, thus enabling intra-/inter-molecular SHIP1 interactions and most likely competition with the Ig-α ITAM. C-terminally truncated versions of SHIP1, which have lost the ability for intra-molecular SHIP1 interaction, however, bind to Ig-α in an enhanced manner. These data indicate that at least part of Ig-α's negative regulatory potential is realized by the association with the well-known negative regulator SHIP1.

2. Materials and methods

2.1. Cell culture

The J558L myeloma cells and transfectants were maintained (37 °C, 5% CO_2) in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 50 units/ml penicillin, 50 mg/ml streptomycin, and 50 μM 2-mercaptoethanol. J558L∂mwt [22] express an IgDwt-BCR and J558L∂mM6 transfectants express an IgD-BCR with an intracellularly truncated Ig-α subunit lacking the ITAM [23]. All mIg molecules of the different J558L cell lines carry the antigen-binding site of the monoclonal antibody B1-8, which is specific for the hapten NP. Schneider S2 cells were grown in Schneider's *Drosophila* medium (Life Technologies, Inc., Karlsruhe, Germany) supplemented with 10% FCS at 27 °C without CO_2. Cells were passaged every 2 to 3 days to maintain exponential growth. Bone marrow-derived mast cells (BMMCs) were differentiated and cultured as described [24].

2.2. Reagents

Monoclonal anti-phosphotyrosine antibodies (4G10) were purchased from Biozol (Eching, Germany) and polyclonal anti-Shc antibodies from BD Transduction Laboratories (Heidelberg, Germany). Polyclonal anti-Syk (N19), monoclonal anti-SHIP1 (P1C1) and polyclonal anti-SHIP1 (N1 and M14) antibodies were obtained from Santa Cruz (Heidelberg, Germany). The monoclonal antibodies against BAP32 have been described previously [25]. Biotinylated and phosphorylated peptide (biotin-TKPEMFENPLpYGSVSS) corresponding to amino acids 1011–1026 of murine SHIP1 was obtained from GL Biochem, Shanghai. Dephosphorylation of the phospho-peptide was achieved using calf intestinal phosphatase from New England Biolabs (1 μl enzyme in a 30 μl volume, 37 °C, 30 min; NEB Buffer 3). Efficiency of dephosphorylation was tested by spotting peptides on PVDF membrane and immunoblot analysis (4 G10). Recombinant GST and GST-SH2(SHIP1) were produced according to Leitges et al. [26]. To obtain the plasmid pD-HASHIP1, the plasmid HASHIP1cDNAmBS (provided by Dr. G. Krystal) was digested with EcoRI/XhoI, blunted, and cloned into the SmaI-digested *Drosophila* expression vector pRmHa-3. The plasmid pD-ΔSH2-SHIP1 was generated from pD-HASHIP1 by QuickChange mutagenesis/deletion using the following primers: sense primer: GCC TGC CAT GGT CCC TGG GCT GGA GGA GGA GGA TGC TAT TG; antisense primer: CAA TAG CAT CCT CCT CCT CCA GCC CAG GGA CCA TGG CAG GC. Using QuickChange mutagenesis, a Stop codon was introduced into pD-HASHIP1 instead of the triplet coding for Y798 yielding pD-HASHIP1 Y798Stop. Primers were the following: sense: CCC ATT ATC TCT GAC CCC GAA TAA TTA CTG GAC CAG C; antisense: G CTG GTC CAG TAA TTA TTC GGG GTC AGA GAT AAT GGG. To obtain the plasmid pD-SH2(SHIP1)-YFPv, the cDNA for the SH2-domain of SHIP1 (sense primer: CGG AAT TCA TGC CTG CCA TGG TCC CTG GG; antisense primer: GGG GTA CCG TCC TTG GCC TCG CTG GG) and the cDNA for YFPv (sense primer: GGG GTA CCA TGG TGA GCA AGG GCG; antisense primer: CGG GAT CCT TAC TTG TAC AGC TCG TCC) were amplified by PCR. The SHIP1-SH2 fragment was cut with EcoRI/KpnI, and the YFPv fragment with KpnI/BamHI, and were ligated into pRmHa-3, which was opened with EcoRI/BamHI. Expression vectors coding for Ig-α-ECFP (pD.LFLAG-mb1-ECFP.II) and Ig-αtl-ECFP (pD.LFLAG-mb1TL-ECFP.II) were cloned using standard recombinant DNA techniques including PCR. The Ig-α-ECFP.II fusion contains, from the N terminus, the following features (fluorophore sequence parts underlined; linker sequences italic): Ig-α-*Linker*-ECFP ({…}AQLEKP-*SGGGGSGGGG*-MVSKGE{…}DELYK). Ig-α-tl-ECFP.II contains Ig-αtl-*Linker*-ECFP ({…}GTLLLFR-*SGGGGSGGGG*-MVSKGE{…}DELYK). All newly cloned plasmids have been verified by DNA sequencing. All other *Drosophila* expression plasmids have been described recently [27].

2.3. Activation of B cells, precipitation, and Western blotting

Cells (2×10^6/ml) were starved for 30 min at 37 °C in RPMI 1640 and stimulated with different concentrations of PV for the indicated times. The PV solution was prepared according to Baumann et al. [28]. After stimulation, cells were pelleted for 20 s in a tabletop centrifuge and solubilized with 0.5–1.0% NP-40 (Calbiochem, Bad Soden, Germany) at 4 °C in phosphorylation solubilization buffer (PSB) [29]. Total cell lysates (TCL) corresponding to 3×10^5 cell equivalents were directly separated by SDS-PAGE and subjected to Western blot analysis as described previously [22]. For immunoprecipitation, 5 μg of the respective antibody was added to the cell lysates and incubated on a rotator at 4 °C for 4 h followed by a 1 h incubation with 10 μl of proteinG-sepharose beads. Precipitates were washed three times with PSB containing one third of the concentration of detergent as for lysis and analyzed by Western blotting.

2.4. Peptide pulldown

Lysates from untreated or PV-stimulated J558L∂m cells (1.5×10^7/point) were prepared as described. Lysates were precleared for 1 h with 13 μl of streptavidin-agarose-beads (Novagen, Darmstadt, Germany) and then subjected to an overnight affinity-purification with 1 μM of the respective N-terminally biotinylated peptide plus 13 μl of streptavidin-agarose-beads. The precipitates were washed twice with PSB containing 0.3% NP-40 and were analyzed by Western blotting. Biotinylated peptides encompass unphosphorylated or doubly-phosphorylated amino acids

179–198 (PepI and P-PepI) or doubly-phosphorylated amino acids 190–209 (P-PepII) of murine Ig-α [30] and unphosphorylated or phosphorylated peptides corresponding to the ITIM sequence of the FcγRIIB C-terminus (ITIM and P-ITIM) [31].

2.5. S2 cell transfections

S2 cell transfections were done as previously described [27]. S2 cells were transfected using the cationic lipid CellFectin (Life Technologies, Inc.). The S2 cells were spread in a 6-well plate (Greiner, Frickenhausen, Germany) at 1×10^6 cells per well in 3 ml medium. Supercoiled plasmids (0.3 μg of each plasmid was adjusted with the *Drosophila* expression vector pRmHa-3 to 3 μg per well) were complexed with lipid (10 μl CellFectin Reagent) in Schneider's *Drosophila* medium. The complex was incubated at room temperature for 15 min, filled up to 1 ml, and then added to cells from which normal growth medium had been removed (cells were washed 1× with medium without FCS). After 18 h, the complex was removed and replaced by Schneider's *Drosophila* medium containing FCS and incubated for further 24 h. To induce the production of proteins, $CuSO_4$ was added to the culture to a final concentration of 1 mM for 24 h. $CuSO_4$-induced cells were then harvested, washed once with PBS, and lysed as described above.

2.6. FRET analysis

FRET studies were performed using a flow cytometric approach previously described [32,33]. Ig-α-ECFP or Ig-α-TL-ECFP where cotransfected with Ig-β or Ig-β-TL, scδm (coding for a single-chain mIgD), SH2(SHIP1)-YFPv and Syk in *Drosophila* S2 cells. After 24 h of induction with $CuSO_4$, protein expression of these constructs was stopped by incubating the cells with $CuSO_4$-free medium for 2 h. Cells were harvested and washed with FACS buffer (PBS containing 3% FCS) before analysis on a BD LSR II cytometer (Becton Dickinson). Excitation was done with a 405 nm coherent VioFlame laser (Becton Dickinson) and a 488 nm air-cooled coherent argon-ion Sapphire Laser (Becton Dickinson) for ECFP or YFPv, respectively. ECFP was detected using a 470/15 nm bandpass filter. YFPv and FRET were detected using a 530/30 nm bandpass filter, after excitation with the 488 nm and 405 nm laser, respectively. Sequential excitation allowed for the detection of all three signals (ECFP, YFPv, and FRET) from a single cell. Compensation was applied so that no FRET signal was detectable in samples transfected with Ig-α-ECFP or SH2(SHIP1)-YFPv alone. In order to normalize for transfection efficiency, 50,000 cells expressing both ECFP and YFPv were analyzed for FRET signals using the FACSDiva Software (Becton Dickinson).

2.7. Confocal microscopy studies

For transfection and inspection, 6×10^5 S2 cells were cultured in glass bottom wells. Fluorescing fusion proteins were visualized with a confocal laser scanner (Leica SP2, Leica Microsystems GmbH, Germany) mounted onto an inverted microscope (Leica DM IRE2, Leica Microsystems GmbH, Germany) using a 63× water immersion lens (HCX PL APOCS 63.0×1.20 W). Excitation was done with a 488 nm laser and a 514 nm laser for YFPv samples and EGFP samples, respectively. Emission of YFPv was detected in the range of 525–600 nm and EGFP in the range of 500–550 nm.

3. Results

3.1. PV-induced tyrosine phosphorylation of SHIP1 is dependent on the C-terminus of Ig-α

In previous experiments we compared J558Lμm cells expressing a wild-type IgM-BCR and J558LμmM6 cells expressing an IgM-BCR with a C-terminally truncated Ig-α with respect to their PTK substrate tyrosine phosphorylation after PV treatment. A 145 kDa protein (pp145) was tyrosine-phosphorylated significantly stronger in the J558Lμm cells compared to J558LμmM6 cells [34]. Comparable results were obtained analyzing J558Lδm and J558LδmM6 cells (Suppl. Fig. 1). Due to size and tyrosine phosphorylation, we tested whether this protein was the SH2-containing inositol 5-phosphatase SHIP1. J558L∂mwt and J558L∂mM6 cells were left untreated or stimulated with a PV dose (10 μM), which resulted in a comparable general substrate tyrosine phosphorylation in both cell types. Postnuclear lysates were then subjected to anti-SHIP1 immunopurifications. The precipitates were analyzed by anti-Ptyr followed by anti-SHIP1 Western blotting. Indeed, two SHIP1 forms (p145 and p135) were strongly tyrosine-phosphorylated in PV-treated wild-type cells, whereas only weak SHIP1 tyrosine phosphorylation was detectable in J558L∂mM6 cells (Fig. 1A). A comparable difference in SHIP1 tyrosine phosphorylation was detected between PV-treated J558LμmwtT and J558LμmM6 cells (Fig. 1B). These data suggest that SHIP1 tyrosine phosphorylation in response to PV stimulation is critically dependent on the presence of the C-terminus of Ig-α. Since SHIP1 is known to suppress activation of the PI3K-dependent kinase PKB by hydrolyzing PIP_3 [35], we assumed a positive effect of truncated Ig-α on PKB phosphorylation. Indeed, it was found that the mutant cells responded in an augmented manner. Activation of PKB in response to PV was faster in the mutant cells (0.5 min) compared to wild-type cells (3 min) and also stronger throughout the time-course experiment (Fig. 1C).

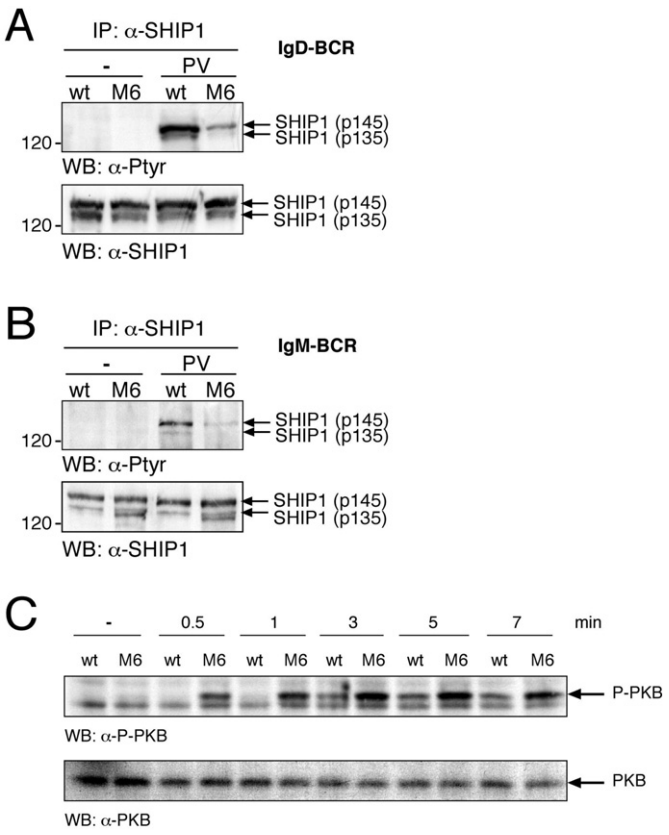

Fig. 1. SHIP1 tyrosine phosphorylation is dependent on the Ig-α C-terminus. A. J558L∂m and J558L∂mM6 cells were left untreated or stimulated with 10 μM PV for 3 min. The cells were lysed and PS were subjected to anti-SHIP1 (P1C1) immunoprecipitation. The precipitates were analyzed by anti-Ptyr (upper panel) and anti-SHIP1 (P1C1) Western blotting (lower panel; loading control). The SHIP1 forms are marked with arrows. B. J558Lμm and J558LμmM6 cells were treated as described in (A). SHIP1 tyrosine phosphorylation was analyzed accordingly. C. J558L∂m and J558L∂mM6 cells were left unstimulated (−) or treated with 3 μM PV for the indicated times. PS were then analyzed for expression of P-PKB (upper panel). The membrane was reprobed with anti-PKB antibodies (lower panel; loading control). P-PKB and PKB are indicated by arrows.

3.2. Ig-α-dependent translocation of the SHIP1 SH2-domain to the plasma membrane

SHIP1 tyrosine phosphorylation was dependent on the presence of the ITAM-containing Ig-α C-terminus. Thus, we reasoned that SHIP1 might bind to the phosphorylated Ig-α ITAM, probably via its SH2-domain, to become phosphorylated by a BCR-proximal PTK. Using the Scoring Matrix-Assisted Ligand Identification (SMALI) web tool, we analyzed ITAM sequences of murine BCR, TCR, and FcεR1 (a total of 10 ITAMs in 8 proteins) for potential interaction with SH2-domain-containing proteins [36]. Interestingly, 4 and 6 ITAM tyrosines were predicted to bind to SHIP1 and SHIP2, respectively (data not shown). Thus, we analyzed the interaction of SHIP1's SH2-domain with Ig-α in living cells. We used the *Drosophila* S2 reconstitution system [27] to monitor translocation of a yellow fluorescence protein (YFPv)-tagged SH2-domain of SHIP1 (SH2(SHIP1)-YFPv) to the BCR at the plasma membrane of S2 cells. As a control, S2 cells were co-transfected with a complete BCR or a BCR containing C-terminally truncated versions of Ig-α and Ig-β (BCRtl) and Syk-GFP. In the case of a complete BCR, Syk-GFP almost exclusively translocated to the plasma membrane (Fig. 2A), whereas Syk-GFP was dispersed throughout the cells when BCRtl was co-expressed (Fig. 2B). Likewise, the SH2(SHIP1)-YFPv protein was evenly distributed in cells in the presence of the BCRtl and Syk (Fig. 2D) whereas, it translocated to patches in the plasma membrane when co-expressed with Syk and the complete BCR (Fig. 2C). Membrane translocation of SH2(SHIP1)-YFPv was still observed with a BCR containing wild-type Ig-α and a C-terminally truncated version of Ig-β (Ig-βtl) (Fig. 2E). This was however completely lost when wild-type Ig-β was paired with a C-terminally truncated Ig-α (Ig-αtl) (Fig. 2F). This was in agreement with the SMALI prediction, since Ig-β was the only murine immunoreceptor subunit, which was predicted to bind neither to SHIP1 nor to SHIP2 (data not shown) [36]. In conclusion, our results obtained by these translocation studies indicate that SHIP1 via its SH2-domain is capable of interacting with the BCR via its signaling subunit Ig-α. It has to be emphasized that the translocation of SH2(SHIP1)-YFPv to Ig-α can be identified in living cells even in the presence of a well-known potent competitor for Ig-α binding, the PTK Syk (see Fig. 2A).

3.3. FRET measurements reveal interaction between the SH2-domain of SHIP1 and Ig-α in living cells

To strengthen the results obtained by translocation studies (Fig. 2A–F), we sought to use a flow cytometric approach to analyze fluorescence resonance energy transfer (FRET) [32,33] between SH2(SHIP1)-YFPv and an Ig-α fused to enhanced cyan fluorescent protein at the C-terminus (Ig-α-ECFP) in the S2 cell system. As a

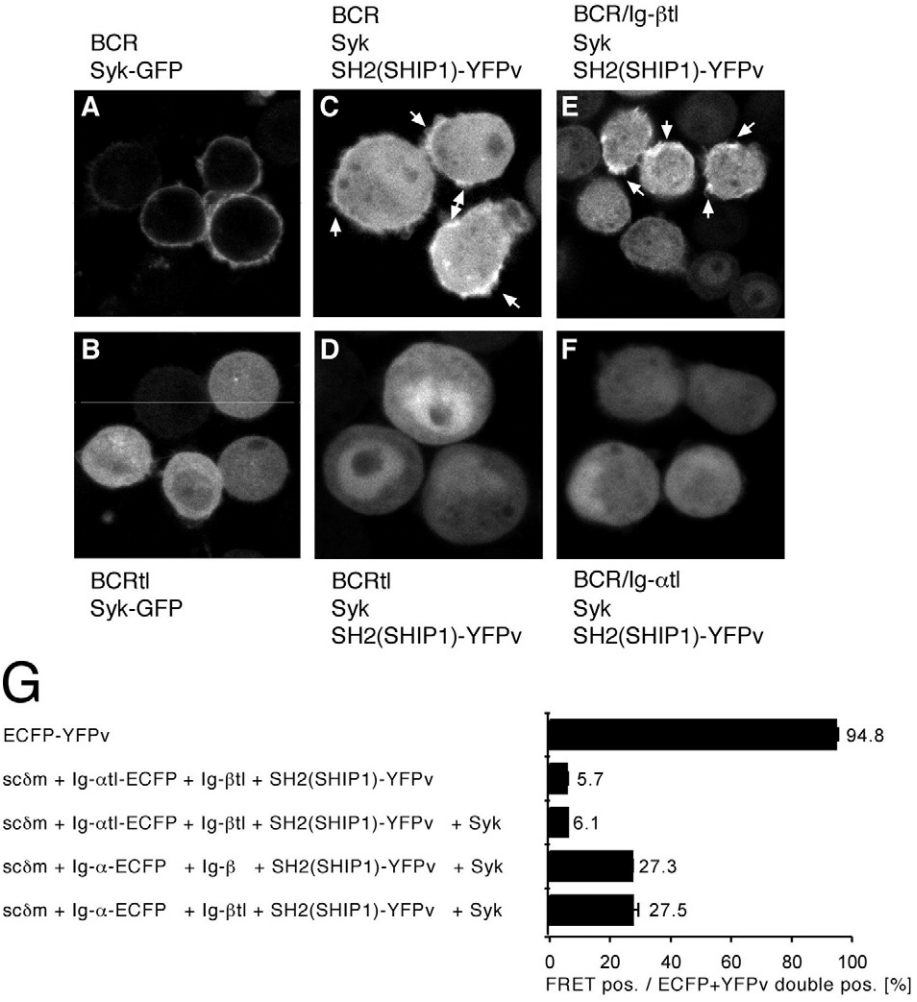

Fig. 2. Ig-α-dependent translocation of SH2(SHIP1)-YFPv to the plasma membrane. A.–F. *Drosophila* S2 cells were transfected with plasmids encoding the indicated proteins. Localization of the fluorescent proteins, GFP-Syk (A, B) and SH2(SHIP1)-YFPv (C–F), was analyzed by confocal microscopy as described in Material and methods. BCR: scδm + Ig-α + Ig-β; BCRtl: scδm + Ig-αtl + Ig-βtl; BCR/Ig-βtl: scδm + Ig-α + Ig-βtl; BCR/Ig-αtl: scδm + Ig-αtl + Ig-β. G. S2 cells transfected as indicated were analyzed for FRET (as described in Material and methods) and the percentage of FRET signal-positive cells per ECFP + YFPv-double positive cells is shown in a bar diagram. Each bar is the mean ± SD. Comparable results were obtained in separate experiments.

positive control, a fusion protein consisting of CFP, a short linker, and YFPv (ECFP-YFPv) was expressed. Cells expressing this fusion protein yielded approximately 95% of FRET signal-positive cells (Fig. 2G). As a negative control, where no specific interaction between the fluorophore-carrying molecules was supposed to occur, SH2(SHIP1)-YFPv and Syk together with a BCRtl containing Ig-αtl-ECFP were expressed. For double-positive cells, about 6% of FRET signal-positive cells (background) were measured (Fig. 2G). When BCRtl was exchanged against the complete BCR containing Ig-α-ECFP, 27% of FRET signal-positive cells were observed, verifying the direct interaction between SH2(SHIP1)-YFPv and Ig-α (Fig. 2G). As observed in our translocation studies, exchanging Ig-β against Ig-βtl did not result in a reduction of interaction (Fig. 2G). It has to be underscored that the FRET signal between SH2(SHIP1)-YFPv and Ig-α-ECFP can be measured in living cells even in the presence of Syk, a potent competitor for Ig-α binding (see Fig. 2A).

3.4. Full-length SHIP1 does not bind to a phosphorylated Ig-α ITAM peptide

In agreement with our translocation and FRET data (Fig. 2), the SH2(SHIP1)-YFPv fusion protein expressed in S2 cells was pulled-down from detergent lysates not only by the FcγRIIB P-ITIM peptide, but also – albeit to a reduced extent – by the Ig-α P-ITAM peptide (P-PepI) (Fig. 3B). The used peptides are depicted in Fig. 3A. The interaction was specific for the P-PepI peptide, since neither unphosphorylated ITAM or ITIM peptides (PepI, ITIM) nor the P-PepII peptide comprising the C-terminal ITAM tyrosine plus Y204 (necessary for the binding of SLP-65 [30]) was effective in this respect (Fig. 3B). This strongly suggests that the SHIP1 SH2-domain interacts with the phosphorylated N-terminal tyrosine of the Ig-α ITAM (the YEGL motif). Intriguingly, using these peptides to pull-down proteins from lysates of unstimulated and PV-stimulated J558Lδm cells no full-length SHIP1 was precipitated by ITAM-related peptides. Whereas the PTK Syk and the adaptor protein Shc bound specifically to the phosphorylated Ig-α ITAM (P-PepI), no binding of SHIP1 to this phosphopeptide was measured (Fig. 3C). SHIP1 also did not interact with P-PepII. However, SHIP1 bound strongly and specifically to the FcγRIIB P-ITIM peptide (Fig. 3C and [31]). In lysates from stimulated cells, Shc also bound to the P-ITIM peptide, probably via its interaction with phosphorylated SHIP1. These data demonstrate that in detergent lysates, full-length SHIP1 neither directly nor indirectly binds to the phosphorylated tail of Ig-α.

3.5. The C-terminus of SHIP1 suppresses the interaction of SHIP1 with Ig-α

In this study so far, a murine monoclonal antibody (P1C1) has been used for SHIP1 detection that binds to SHIP1 within its C-terminus (aa 866–1020) (Fig. 4A). This suggests that full-length

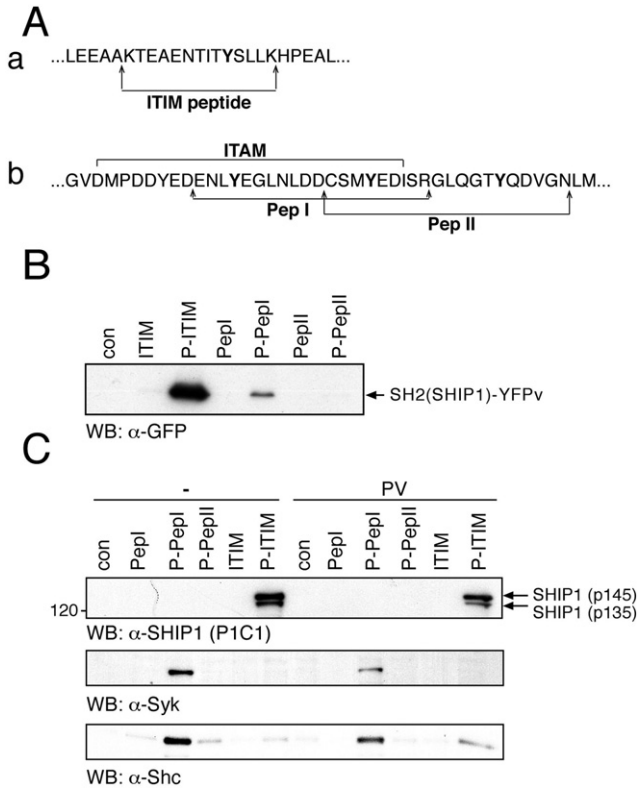

Fig. 3. In the context of full-length SHIP1 the SH2-domain seems to be unable to interact with P-ITAM. A. Schematic view of the ITIM (a) and ITAM (b) peptides used in this study. B. PS from S2 cells expressing SH2(SHIP1)-YFPv were subjected to affinity-purification with biotinylated Ig-α peptides (PepI, P-PepI, PepII, P-PepII) or FcγRIIB peptides (ITIM, P-ITIM). As a control, lysates were incubated only with the precipitating agent, streptavidin-agarose-beads (con). Subsequently, purified proteins were analyzed by anti-GFP Western blotting. SH2(SHIP1)-YFPv is marked by an arrow. C. Proteins from unstimulated (−) or PV-stimulated J558Lδm cells were affinity-purified with biotinylated Ig-α peptides (PepI, P-PepI, P-PepII) or FcγRIIB peptides (ITIM, P-ITIM). As a control, lysates were incubated only with the precipitating agent, streptavidin-agarose-beads (con). Subsequently, purified proteins were analyzed by anti-SHIP1 (P1C1; top panel), anti-Syk (middle panel), and anti-Shc immunoblotting (bottom panel). The SHIP1 forms are marked with arrows.

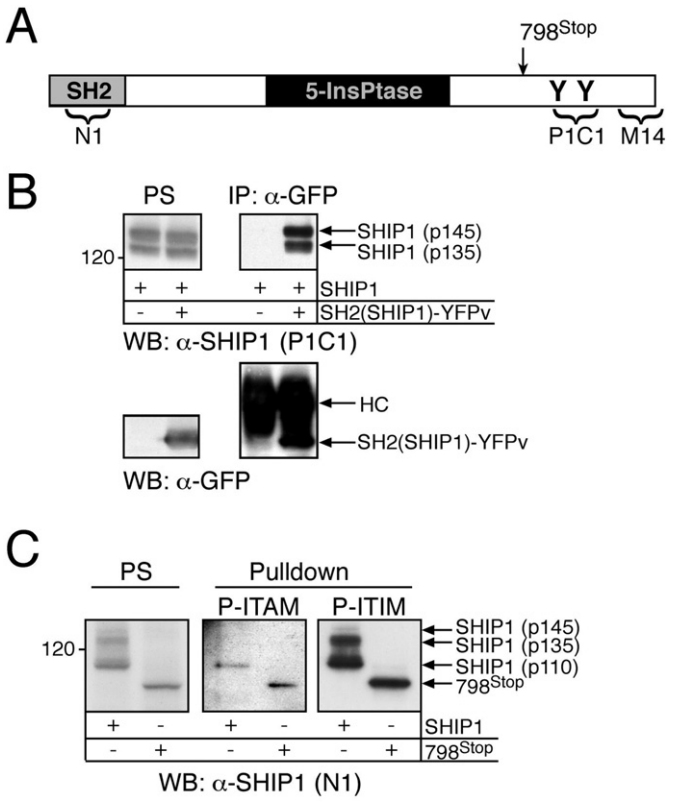

Fig. 4. Interaction between the SH2-domain and the C-terminus of SHIP1. A. Schematic view of SHIP1 and the epitopes of the anti-SHIP1 antibodies N1, P1C1, and M14 (depicted by brackets). The position, where SHIP1 798^{Stop} ends, is indicated. The two NPxY sequences within the C-terminus are marked with Ys. B. S2 cells were transfected either with a plasmid coding for HA-SHIP1 or with plasmids coding for HA-SHIP1 and SH2(SHIP1)-YFPv. PS were either analyzed by direct immunoblotting (left panels) or subjected to immunopurification using anti-GFP antibody (right panels). PS and precipitates were analyzed by anti-SHIP1 (P1C1; upper panels) and anti-GFP Western blotting (lower panels). C. S2 cells were transfected to express either HA-SHIP1 or SHIP1 798^{Stop}. PS were subjected to direct Western blotting (anti-SHIP1 (N1); left panel) or to pulldown using P-ITAM (= P-PepI; middle panel) or P-ITIM (right panel) peptides followed by anti-SHIP1 (N1) immunoblotting. Different SHIP1 forms are indicated by arrows.

SHIP1 or SHIP1 containing most of its C-terminus (and thus being subject to detection by the P1C1 anti-SHIP1 antibody) is unable to bind to the P-PepI peptide (see Fig. 3C). This may be due to the SH2-domain being engaged intra- or inter-molecularly with the C-terminus of the same SHIP1 molecule, since the SH2(SHIP1)-YFPv fusion protein was readily bound by P-ITAM peptide. Thus, we analyzed whether the SH2-domain of SHIP1 can interact with the complete SHIP1 molecule. SHIP1 was expressed in *Drosophila* S2 cells with or without the SH2(SHIP1)-YFPv fusion protein and an immunopurification with anti-GFP antibodies was performed. Indeed, SHIP1 could be co-immunopurified with the SH2(SHIP1)-YFPv fusion protein (Fig. 4B). To test our hypothesis further, we expressed full-length SHIP1 as well as a truncated version of SHIP1 (798^{Stop}), which still contains the catalytic region and cannot be detected by the P1C1 antibody (Fig. 4A), in S2 cells and performed peptide pull-down assays with P-ITIM as well as P-ITAM (= P-PepI) peptides. Precipitated SHIP1 molecules were detected by an anti-SHIP1 antibody (N1), which binds the SH2-domain of SHIP1 (Fig. 4A). SHIP1 798^{Stop} was pulled down by both P-ITAM and P-ITIM peptides with P-ITIM being more effective than P-ITAM (Fig. 4C, compare lanes 4 and 6). This suggests that SHIP1 798^{Stop} is lacking the C-terminal region responsible for intra- or inter-molecular binding of the SH2-domain.

Transfection of S2 cells with SHIP1-encoding cDNA results in expression of different forms of SHIP1, most probably due to post-translational C-terminal proteolytic cleavage [16]. These are full-length SHIP1 (p145) and truncated SHIP1 forms (p135 and p110). Interestingly, in contrast to the longer forms of SHIP1 (p145 and p135), SHIP1 (p110) was able to interact with both P-ITAM of Ig-α and P-ITIM of FcγRIIB (Fig. 4C, compare lanes 3 and 5). Our data suggest that the SH2-domain of SHIP1 can bind to the SHIP1 C-terminus and this interaction might inhibit interaction between SHIP1 and the phosphorylated ITAM sequence of Ig-α.

3.6. SHIP1 molecules are able to dimerize

The results presented so far lead to the conclusion that the SHIP1 SH2-domain interacts intra-molecularly with the SHIP1 C-terminus and/or interacts inter-molecularly with the C-terminus of a second SHIP1 molecule. This would lead to dimerization or even to oligomerization of SHIP1 (exemplarily shown in Fig. 5A). To test the latter hypothesis, full-length SHIP1 was co-expressed with SHIP1 798^{Stop} in S2 cells and immunopurification was carried out with anti-SHIP1 antibody (M14), which is capable of binding to full-length SHIP1 (Fig. 4A), but not to the latter (Fig. 5C, lanes 1 and 2). This resulted in the co-purification of SHIP1 798^{Stop}, which could be detected with the anti-SHIP1 antibody (N1) (Fig. 5C, lanes 4 and 5). Accordingly, a smaller form of SHIP1 (marked by an asterisk) unable to bind to the M14 antibody was co-purified (Fig. 5C, compare lanes 2 and 5). These data indicate that SHIP1 is able to dimerize/oligomerize (Fig. 5A). Next, we sought to determine whether the interaction of the SH2-domain of one SHIP1 molecule with the C-terminus of a second SHIP1 molecule is enough to facilitate the SHIP1–SHIP1 association. To this end, SHIP1 798^{Stop} was co-expressed with a SHIP1 molecule lacking its SH2-domain (ΔSH2-SHIP1) (Fig. 5C, lane 3) and immunopurification with anti-SHIP1 antibody (M14) was performed (a model is shown in Fig. 5B). Indeed, SHIP1 798^{Stop} was co-purified with ΔSH2-SHIP1 as observed by anti-SHIP1 (N1) immunoblotting (Fig. 5C, lane 6).

3.7. The SHIP1 SH2-domain interacts with phosphorylated NPxY[1020]

Next, we aimed at identifying the tyrosine residue in the SHIP1 C-terminus, which upon phosphorylation enables interaction with the SHIP1 SH2-domain. Using the SMALI web tool [36], SHIP1's Y^{1020} was listed as highly potential interaction site for the SH2-domain of

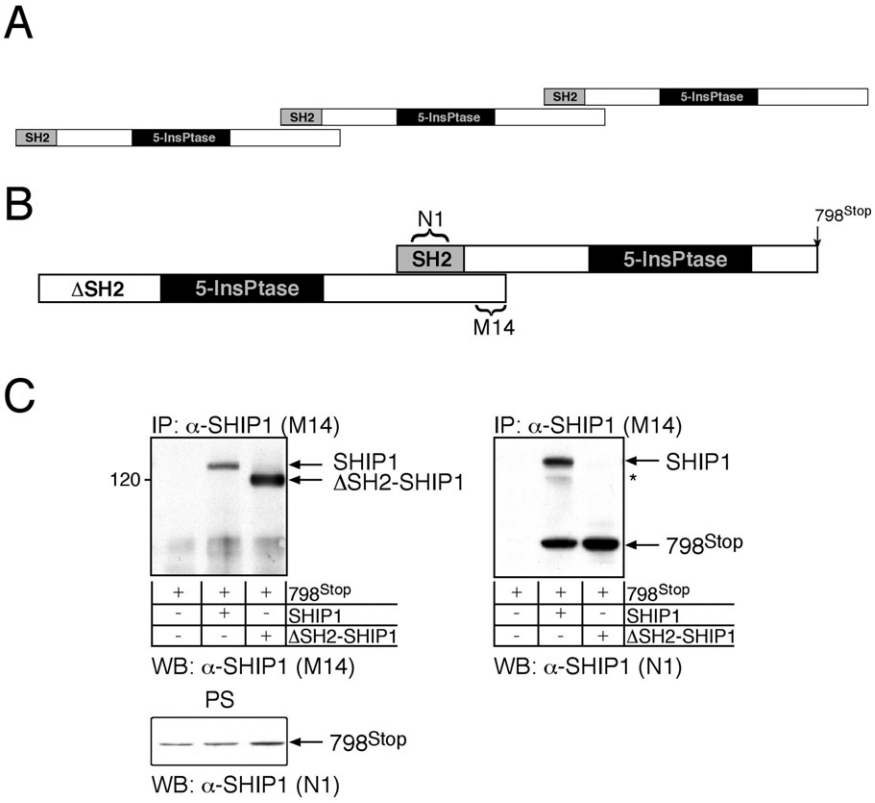

Fig. 5. Dimerization of SHIP1. A. Schematic view of putative SHIP1 oligomerization. B. Schematic view of the interaction between ΔSH2-SHIP1 and SHIP1 798^{Stop}. The epitopes of anti-SHIP1 antibodies, N1 and M14, are indicated by brackets. C. S2 cells were transfected to express SHIP1 798^{Stop}, SHIP1 798^{Stop} and HA-SHIP1, or SHIP1 798^{Stop} and ΔSH2-SHIP1. PS were subjected to immunopurification with anti-SHIP1 antibodies (M14) and precipitates were analyzed by anti-SHIP1 (M14; upper left panel) or anti-SHIP1 (N1; right panel) Western blotting. Moreover, PS were directly analyzed for expression of SHIP1 798^{Stop} by immunoblotting with anti-SHIP1 (N1) antibodies (lower left panel).

SAP (a.k.a. DSHP or SH2D1A), an SH2-domain known to compete for binding to phosphotyrosine motifs with the SH2-domain of SHIP1 [37]. Indeed, SHIP1 could be purified from lysates of unstimulated J558L cells using a phosphorylated peptide corresponding to the amino acid sequence around Y^{1020} (Fig. 6A). In contrast, the dephosphorylated peptide was very inefficient in purifying SHIP1 (Fig. 6A). To rule out that SHIP1 was only pulled down by the phosphorylated peptide via a linker protein binding to both the peptide and SHIP1, the peptide was tested to bind to recombinant GST-SH2(SHIP1). Again, the phosphorylated peptide pulled down GST-SH2(SHIP1) markedly more efficient than the unphosphorylated peptide (Fig. 6B). GST alone, though used in vast excess, was only marginally precipitated and there was no difference between phosphorylated and unphosphorylated peptides (Fig. 6B). Next, SHIP1 dimerization/oligomerization was analyzed in J558L cells. As shown in Fig. 6C, full-length SHIP1 was immunoprecipitated with anti-SHIP1 antibody (M14) from J558L lysates and co-purified SHIP1 molecules of shorter length were detected by Western blotting with anti-SHIP1 antibody (N1). The molecular weight of this band is approximately 110 kDa, which is consistent with the expected size of a shorter form of SHIP1 lacking a part of the C-terminus [15,16]. This indicates presence of at least SHIP1 dimers in J558L cells. Finally, we sought to determine the presence of SHIP1–SHIP1 complexes in a different hemopoietic cell type, in which SHIP1 function has been demonstrated to be of relevance for the control of multiple effects or functions, namely bone marrow-derived mast cells (BMMCs) [10,38]. Immunopurification from detergent lysates was carried out with anti-SHIP1 (M14) and Western blot analysis was performed with M14 as well as N1 anti-SHIP1 antibodies (Fig. 6D). Again, smaller SHIP1 isoforms (detected by N1) were co-purified with the M14 antibody, indicating a general nature of SHIP1–SHIP1 complexes in hemopoietic cells. The high molecular weight form of SHIP1, specifically recognized and precipitated by the M14 antibody, is enriched in the immunoprecipitates. The low molecular weight form of SHIP1 (asterisk), however, seems not to be enriched in a comparable manner, suggesting that the interaction between high and low molecular weight forms of the protein cannot be thus quantitatively correlated.

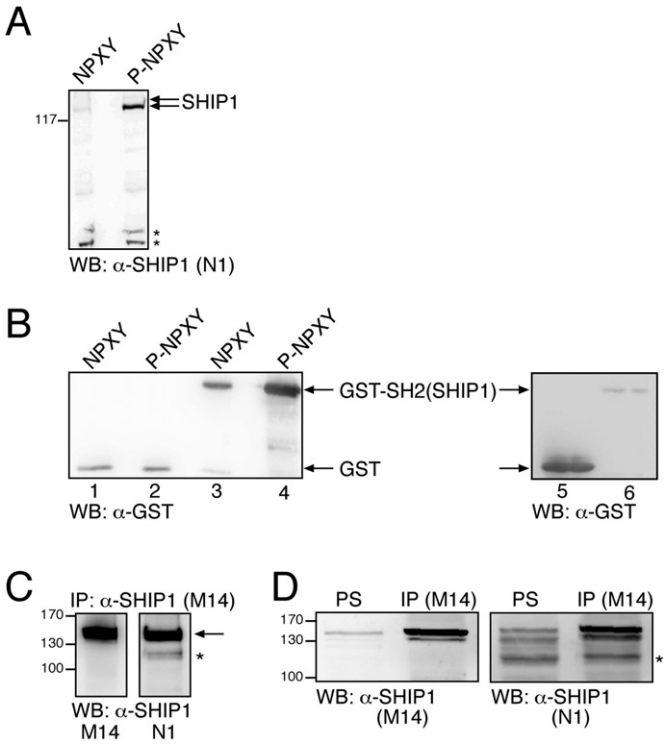

Fig. 6. The SHIP1 SH2-domain binds to phosphorylated Y^{1020} in the SHIP1 C-terminus. A. PS of J558L cells were subjected to pull-down with NPXY or p-NPXY peptides corresponding to the amino acid sequence around SHIP1's Y^{1020}. Precipitates were analyzed by anti-SHIP1 (N1) immunoblotting. SHIP1 is indicated by arrows. Cross-reacting bands confirming comparable loading are marked by asterisks. B. GST (lanes 1 and 2) or GST-SH2(SHIP1) (lanes 3 and 4) were subjected to pull-down with NPXY or p-NPXY SHIP1-peptides. Precipitates were analyzed by anti-GST Western blotting (left panel). The right panel shows the amount of GST (lane 5) or GST-SH2(SHIP1) proteins (lane 6) used in this experiment. C. J558L cells were lysed and PS were subjected to anti-SHIP1 (M14) immunopurification. Precipitates were analyzed by anti-SHIP1 (M14; left panel) and anti-SHIP1 (N1; right panel) immunoblotting to visualize full-length SHIP1 and copurified smaller SHIP1 forms, respectively. Full-length SHIP1 is marked by an arrow and a shorter form of SHIP1 with an asterisk. D. BMMCs were lysed and PS were subjected to anti-SHIP1 (M14) immunoprecipitation. Precipitates (IP) as well as PS were analyzed by anti-SHIP1 (M14; left panel) and anti-SHIP1 (N1; right panel) immunoblotting. Full-length SHIP1 is marked by an arrow and a shorter form of SHIP1, detected by N1, with an asterisk.

4. Discussion

The BCR regulates B cell differentiation, proliferation, and activation [18] and depending on the developmental step it can do so in an antigen-dependent as well as -independent fashion [39]. Therein, the Ig-α/Ig-β heterodimer plays a central role by coupling the BCR to the intracellular signaling machinery via its cytoplasmic ITAM-containing tails. By far, most of the interactions between BCR and intracellular signaling molecules are dependent on Ig-α/Ig-β tyrosine phosphorylation. The respective intracellular signaling proteins contain one or more SH2-domains enabling them to bind to the various phosphorylated tyrosine(s) of Ig-α/Ig-β, thus contributing to the activation of different signaling pathways.

Since there is a so-called functional dichotomy between ITAM and ITIM sequences, it was even more intriguing when two groups reported on a negative signaling role for Ig-α in immature B cells [20,21]. Immature B cells from mice which carry a C-terminal truncation of Ig-α (Ig-$\alpha^{\Delta c/\Delta c}$ mice) respond to BCR engagement with an exaggerated and prolonged calcium response and induction of protein tyrosine phosphorylation. In keeping with the activating nature of the ITAM one could assume that Ig-α couples indirectly, via its interaction with an activating element (e.g. Syk or Lyn), to a downstream-located negative regulatory element, which then establishes a negative feedback loop. Otherwise, Ig-α might be capable of directly interacting with one or more negative regulator(s). The PTK Lyn might be a promising candidate since it is able to initiate a well-studied negative feedback via the co-receptor CD22 and the PTP SHP-1 with strong impact on BCR-mediated calcium mobilization and tyrosine phosphorylation [40,41]. However, with respect to its binding to the BCR, Lyn seems not to discriminate strongly between Ig-α and Ig-β [42] and thus the pronounced effect of the truncation of the Ig-α C-terminus cannot be explained.

We have studied the effect of a C-terminal truncation of Ig-α on PV-induced substrate tyrosine phosphorylation and identified the inositol 5′-phosphatase SHIP1, the tyrosine phosphorylation of which is strongly dependent on the Ig-α tail. Using biochemical as well as cell biological *in vitro* and *in vivo* systems, we demonstrated direct interaction between the SH2-domain of SHIP1 and tyrosine-phosphorylated Ig-α. The interaction between full-length SHIP1 and Ig-α, however, does not have affinity high enough for being observed in detergent lysates. Interestingly, different ITAMs seem to possess varying affinities for SHIP1, since SHIP1 can be pulled-down from cellular lysates by FcεR1 β-chain ITAM phosphopeptides, but not by FcεR1 γ-chain ITAM phosphopeptides [12]. Furthermore, the phosphorylated ITAM of the FcγRIIa has been demonstrated to strongly bind to the SH2-domain of SHIP1 *in vitro* [43]. Moreover, Pesesse et al. have shown binding of SHIP1 and SHIP2 via their SH2-domains to a tyrosine-phosphorylated peptide mimicking an immunoreceptor signaling motif (combining activating and inhibitory properties) [44]. Since sequence differences between Ig-α and Ig-β with respect to the N-terminal ITAM tyrosine appear, on first sight, non-dramatic (ENL

YEGL NLDD vs. DHT YEGL NIDQ), the SHIP1 SH2-domain seems to be very selective (it only binds to Ig-α and not to Ig-β) and thus binding of it to a so far non-studied ITAM has to be newly analyzed by biochemical means.

Several features of SHIP1 make it a likely candidate to transduce some of Ig-α's negative signals and thus to contribute to the phenotype of the Ig-$\alpha^{\Delta c/\Delta c}$ mice: i) SHIP1 is known in the context of several activating receptor systems (FcεR1, BCR, c-kit) to be a negative regulator of calcium mobilization [7,10,45]; ii) SHIP1 is involved in the attenuation of BCR-mediated MAPK and PKB signaling [46]; and iii) the SH2-domain of SHIP1 strongly discriminates between Ig-α and Ig-β (this study).

Our finding that full-length SHIP1 in comparison to shorter forms of this molecule is unable to bind to Ig-α was unexpected in the beginning. A possible explanation was obtained by the finding that the SH2-domain of SHIP1 can interact with the phosphorylated Y^{1020} in SHIP1's C-terminus allowing for inter- and most probably intramolecular interactions. Though intramolecular interaction was not formally proven, its presence seems very likely based on the published crystal structure of the 5′-phosphatase synaptojanin [47]. Like in SHIP1, the 5′-phosphatase domain of synaptojanin is centrally located in the molecule and the N- and C-terminal extensions enter and leave, respectively, the catalytic domain at the same side of its globular structure [47]. In the case of SHIP1 such a structure would strongly promote the interaction of the SH2-domain with the C-terminus of the same molecule. Irrespective of the question whether the SH2-domain of SHIP1 binds inter- or intramolecularly to the SHIP1 C-terminus, in full-length SHIP1 the SH2-domain seems to be blocked and thus not available for binding to the Ig-α ITAM. This suggests that primarily shorter forms of SHIP1, which are not tyrosine-phosphorylated, bind to Ig-α. Otherwise, release of the SH2-domain of full-length SHIP1 from the inter-/intra-molecular interaction would allow for Ig-α binding of full-length SHIP1. Y^{1020} is part of an NPxY motif known to bind, in its phosphorylated form, to PTB-domain-containing proteins like Shc or Dok-1 [5,48]. Thus, binding of Shc/Dok-1 to the C-terminus of SHIP1 would allow for the release of SHIP1's SH2-domain and its interaction with another binding partner, such as tyrosine-phosphorylated Ig-α.

There is still another problem to be discussed. The SH2-domain of SHIP1, even when released from competing interactions, has to compete against the tyrosine kinase Syk, which via its tandem-SH2 domains can tightly bind to the phosphorylated ITAM of Ig-α [49]. In our experiments analyzing membrane recruitment of a GST-SH2(SHIP1) protein by the BCR, the potential of the SHIP1 SH2-domain to compete against Syk binding is clearly shown. Interestingly, recent data on Syk function in B cells sheds light on a possible scenario [50]. Syk has been demonstrated to act as a dual-specificity kinase phosphorylating Ser197 within the Ig-α cytoplasmic tail. Ser197 is located close to the C-terminal tyrosine of the Ig-α ITAM (Tyr193) and its phosphorylation results in release of Syk from Ig-α [50]. Since SHIP1 via its SH2-domain most likely binds to the N-terminal ITAM tyrosine, as suggested by our peptide-pulldown data, the Ig-α ITAM phosphorylated on the ITAM tyrosines as well as Ser197 would be well accessible for SHIP1. Thus, an exchange of an activating signaling protein (Syk) against an inhibitory molecule (SHIP1) would be realized.

Since SHIP1 is such a powerful negative regulator, hemopoietic cells have to possess several means to control SHIP1's activity and/or localization. However, the regulation of the regulator itself is only slowly becoming evident. In this respect, our data offer a model in which SHIP1-receptor interactions via SHIP1's SH2-domain are regulated via affinity differences for SHIP1's SH2-domain between the respective receptor and SHIP1's C-terminus. Thus, high-affinity phosphotyrosines, like the P-ITIM of FcγRIIB, are able to release full-length SHIP1 from their inter- or intramolecular N-terminus-to-C-terminus interaction. In contrast, low-affinity phosphotyrosines, like the P-ITAM of the BCR/Ig-α, are unable to do so. Therefore, these receptors can only – or specifically – interact with shorter forms of SHIP1. The different properties of full-length vs. short forms of SHIP1 are not known yet, however, most probably differences in respective signaling complexes will occur. Interestingly, Garrison and coworkers have demonstrated recently that a SHIP1 molecule lacking its C-terminus (truncation mutant 1–822) is activated about 8–10 fold compared to full-length SHIP1 [51]. This suggests that SHIP1's C-terminus not only controls interactions of SHIP1 with respective binding partners, but also the catalytic activity of SHIP1. These two functions are very likely coupled. In this regard, truncation of the homologous SHIP2 molecule by removal of the C-terminal region (truncation mutant 1–822 as well) has also been shown to activate the enzyme [52]. In analogy, this might suggest that the SH2-domain of SHIP2 can bind to the single NPxY sequence within the molecule's C-terminus as well. In this respect, interaction between SHIP2 and SHIP1 has been demonstrated to be dependent on the SH2-domain of SHIP2 [53]. However, no interacting phosphorylated tyrosine residue in SHIP1 was identified. Moreover, SH2-domain-dependent SHIP2–SHIP2 interaction has been shown with the E3-ligase c-Cbl discussed as a potential bridging protein [54].

In conclusion, a major future task would be to unravel the proper regulation of SHIP1 and SHIP2 with respect to intra-/inter-molecular interactions and catalytic function. Given SHIP1's and SHIP2's regulatory roles in inflammation, allergy, osteoporosis, and diabetes [55–58], this upcoming information will certainly be of pharmacological interest.

Supplementary materials related to this article can be found online at doi:10.1016/j.bbamcr.2011.11.019.

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