p135 Src Homology 2 Domain-containing Inositol 5'-Phosphatase (SHIPβ) Isoform Can Substitute for p145 SHIP in FcγRIIB1-mediated Inhibitory Signaling in B Cells*

Michael E. March‡, David M. Lucas‡, M. Javad Aman‡, and Kodimangalam S. Ravichandran‡¶

From the ‡Beirne B. Carter Center for Immunology Research and the Department of Microbiology, University of Virginia, Charlottesville, Virginia 22908 and ¶Ohio State University, Department of Internal Medicine, Columbus, Ohio 43210

The inositol 5'-phosphatase, SHIP (also referred to as SHIP-1 or SHIPα), is expressed in all cells of the hematopoietic lineage. Depending on the cell type being investigated and the state of differentiation, SHIP isoforms of several different molecular masses (170, 160, 145, 135, 125, and 110 kDa) have been seen in immunoblots. However, the function of the individual isoforms and the effect of expressing multiple isoforms simultaneously are not understood. Some of these SHIP isoforms have recently been characterized at the level of primary sequence. In this report, we investigated the function of the recently characterized 135-kDa SHIP isoform (SHIPβ), which appears to possess the catalytic domain but lacks some of the protein-protein interaction motifs at the C terminus. By reconstituting SHIP-deficient DT40 B cells with either SHIPβ or the better-characterized p145 SHIPα, we addressed the function of SHIPβ in the complete absence of SHIPα. We observed that SHIPβ had enzymatic activity comparable with SHIPα and that SHIPβ was able to reconstitute FcγRIIB1-mediated inhibition of B cell receptor-induced signaling events such as calcium flux and Akt and mitogen-activated protein kinase. SHIPβ was readily phosphorylated in response to B cell receptor cross-linking with the inhibitory receptor FcγRIIB1 and SHIPβ also interacted with the adapter protein Shc. During these studies we also observed that the SHIPα or SHIPβ interaction with Grb2 is not required for FcγRIIB1-mediated inhibition of calcium flux. These data suggest that SHIPβ, which is normally expressed in B cells along with SHIPα, functions comparably with SHIPα and that these two isoforms are not likely to be antagonistic in their function in vivo.

The SH2 domain-containing inositol 5'-phosphatase, SHIP,1 is a negative regulator of signaling events regulating cell proliferation, cell survival, and protein synthesis in various receptor systems. The major murine isoform of SHIP, p145 SHIP, contains 1190 amino acids and is composed of an N-terminal Src homology (SH) 2 domain, a central inositol 5'-phosphatase domain, and a C-terminal region consisting of at least two tyrosine phosphorylation sites and several proline-rich regions (1–6).

The different regions of SHIP have been ascribed specific roles in mediating SHIP function as a negative regulator. The SH2 domain has been shown to bind to tyrosine-phosphorylated receptors such as FcγRIIB1 (7–10), the erythropoietin receptor (11), and CDw150(12). The inositol phosphatase domain (recently defined to exist between amino acids 400 and 866 of the murine sequence (13)) dephosphorylates both of the known substrates of SHIP in vitro, the membrane lipid phosphatidylinositol-3,4,5-trisphosphate (PIP3) and the soluble phosphoinositide inositol-1,3,4,5-tetrakisphosphate (1–4, 14). SHIP-mediated dephosphorylation of PIP3, a key signaling lipid involved in many cellular processes, has been correlated with negative regulation through SHIP (8, 15, 16). Within the C terminus of SHIP, the two tyrosines at positions 917 and 1020 have been shown to provide binding sites, when phosphorylated, for the phosphotyrosine binding (PTB) domain of Shc (17). Other PTB domain-containing proteins, Dok-1, Dok-3, and Dab-1, have also been shown to bind to these two tyrosines via their PTB domains (18–20). The polyproline motifs within the C terminus also interact with proteins that contain Src homology 3 (SH3) domains. One such protein, the adaptor protein Grb2, is believed to bind to one or more of the proline-rich stretches of SHIP through one or both of its SH3 domains (1, 3, 14, 21). The precise role of Grb2 binding in SHIP function is currently unknown.

Studies in various cells of hematopoietic origin have revealed SHIP isoforms of multiple molecular weights. Depending on the cell type being studied, SHIP species of 139, 125, and 110 kDa can be seen (3, 22, 23). Some of these isoforms can be derived by proteolytic cleavage of the 145-kDa isoform (24). Others have been shown to be the result of alternative splicing of the SHIP mRNA (3, 25). Recently, Lucas and Rohrschneider demonstrated that in murine myeloid cells the p135 isoform results from an alternatively spliced mRNA using cryptic splice donor and acceptor sites contained within the p145 coding sequence (22). This splice results in the deletion of 183 bases from the mRNA and 61 amino acids from the protein. The deletion occurs immediately after tyrosine 917 and results in the loss of the putative phosphatidylinositol 3-kinase (PI3K) binding site and one of the proline-rich regions. Another splice

* This work was supported by National Institutes of Health Grants AI-43425 and GM-55761 (to K. S. R.) and by National Research Service Award F32 DK09774-01 (to D. M. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Carter Immunology Center, Bldg. MR4, Rm. 4012P, HSC, University of Virginia, Charlottesville, VA 22908. Tel.: 804-243-6093; Fax: 804-924-1221; E-mail: kr4h@virginia.edu.

‡ The abbreviations used are: SHIP, the SH2 domain-containing 5'-inositol phosphatase; SHIPα, 145-kDa isoform of SHIP; SHIPβ, the 135-kDa Δ183 isoform of SHIP; SH2, Src homology 2; SH3, Src homology 3; PIP3, phosphatidylinositol-3,4,5-trisphosphate; PTB, phosphotyrosine binding; BCR, B cell receptor; MAPK, mitogen-activating protein kinase; mAb, monoclonal antibody; PAGF, polyacrylamide gel electrophoresis; PI3K, phosphatidylinositol 3-kinase; FAK, FAK-related non-kinase focal adhesion kinase.

29960 This paper is available on line at http://www.jbc.org
variant has recently been described in which 167 nucleotides are spliced out of the message, resulting in a deletion followed by a frameshift mutation (26). The resulting protein product has a calculated molecular mass of 108 kDa, providing a possible identification of the approximately 110-kDa isoform. In addition to the shorter isoforms, FDC-P1 cells contain immunoreactive species of 165 kDa, and murine bone marrow cells express another isoform of approximately 175 kDa (23). These larger molecular mass isoforms have not been characterized at the DNA level.

Many of the shorter isoforms of SHIP have been identified to contain alterations in the C terminus of SHIP (22, 24). We recently demonstrated that in addition to the catalytic activity of SHIP, the C terminus of SHIP is required for efficient SHIP function in B cells (13). These data suggest a requirement for as yet undefined regions in the C terminus of SHIP and may be indicative of a function for one or more of the polyproline regions in the SHIP C terminus. Since the differences in SHIP isoforms are predominantly in the C terminus, it is possible that these naturally occurring SHIP variants would have varying abilities to function as negative regulators due to the perturbations of the C terminus. If this were the case, an interesting possibility is that the function of p145 SHIP could be regulated through expression of other isoforms.

Analysis of the function of individual isoforms of SHIP is complicated by the fact that more than one isoform of SHIP is expressed in any given cell line (1, 3, 4, 22, 23, 25). A system is therefore desired where the isoform to be studied is the only one expressed. Toward this goal, we have chosen to express individual isoforms of SHIP in a SHIP-deficient B cell line (27). Studies from a number of laboratories have defined a critical role for SHIP in negative regulation of B cell receptor (BCR)-initiated signals (28). Cell signaling pathways initiated by BCR stimulation include calcium flux and activation of many kinases, including the serine threonine kinases Akt/protein kinase B and MAPK/extracellular signal-regulated kinase (29–32). In the later stages of an immune response, when a B cell binds an antigen through its BCR, the antibodies already bound to the antigen can simultaneously engage another receptor, FcγRIIB1, and the BCR. The phosphorylation of an inhibitory motif in the cytoplasmic tail of FcγRIIB1 and the subsequent recruitment of SHIP (through its SH2 domain) leads to a potent negative signal, resulting in inhibition of BCR-induced proliferation and Ig synthesis (8, 9, 27). SHIP-mediated dephosphorylation of PIP_2 has been linked to inhibition of calcium flux (15, 16, 33–35), Akt activation (30, 32, 36), and MAPK activation (37, 38). Studies in cell lines and mice rendered SHIP-deficient through targeted gene disruption have shown that SHIP is absolutely required for the FcγRIIB1-mediated inhibition of these signaling events (27, 37, 39).

In this report, we have addressed the function of the 135-kDa isoform of the murine SHIP (referred to as SHIPβ as per Rohrschneider et al. (26)) in FcγRIIB1-mediated inhibitory signaling and compared it to the better-characterized 145-kDa isoform of SHIP (referred to as SHIPα). Since SHIPβ has been seen in many cell lines, we reconstituted the SHIP-deficient DT40 B cell line with either SHIPα or SHIPβ and tested the function of each in the absence of the other isoform. We demonstrate that SHIPβ can fully substitute for SHIPα in DT40 B cells, suggesting that these two isoforms of SHIP are functionally interchangeable and that concurrent expression of both isoforms in B cells is not likely to be antagonistic in this system.

**Experimental Procedures**

**Cells and Reagents**—The murine A20 cell line was cultured in RPMI 1640 media supplemented with 10% fetal calf serum, penicillin, streptomycin, 2 mM L-glutamine, and 20 μM β-mercaptoethanol at 37 °C and 5% CO_2. The chicken B cell line DT40 was cultured in the same media as above, supplemented with 1% chicken serum (Sigma) and 50 μM β-mercaptoethanol. The DT40 cell lines with the disruptions of the SHIP and Grb2 genes have been previously described (27, 40). Murine monoclonal anti-chicken IgM (M4) was obtained from Southern Biological Associates (Biology Associates, AL). Rabbit anti-monoclonal IgM (Fab′) and intact IgM were obtained from Zymed Laboratories Inc. (South San Francisco, CA). Anti-FcγRIIB1 antibody 2.4.2G2 was purchased from Pharmingen (San Diego, CA). Phosphospecific anti-Akt antibody (recognizing phosphorylated threonine 308) and the phosphospecific pp44/pp42 MAPK antibody were purchased from New England Biolabs. The sheep polyclonal anti-Akt antibody used for immunoprecipitation and immunoblotting was purchased from Upstate Biotechnology Inc (Lake Placid, NY). The anti pp44/pp42 MAPK antibody used for immunoblotting was purchased from New England Biolabs. The rabbit polyclonal anti-Shc antibody used for immunoprecipitations was purchased from Transduction Laboratories (Lexington, KY). The mouse monoclonal anti-Shc antibody (PG-797) used for immunoblotting was acquired from Santa Cruz Biotechnology (Santa Cruz, CA). The P1C1 anti-SHIP mAb was acquired from Santa Cruz Biotechnology (Santa Cruz, CA). The P1D7 hybridoma was derived from a BALB/c mouse injected with a histidine-tagged protein representing amino acids 866–1020. The P2A8 hybridoma was generated by injecting mice with a synthetic peptide (EMINNYIANRGPFCR, made by United Biochemical Research, Seattle WA) conjugated to keyhole limpet hemocyanin (Pierce) (22). Hybridoma supernatants were used in immunoblotting experiments at dilutions of 1:100 (P1D7) or 1:200 (P2A8).

**SHIP cDNAs**—SHIP cDNA with a specific 183-nucleotide deletion (A183) has been described previously (22). The second spliced SHIP cDNA, lacking 167 nucleotides, has recently been reported (26). When translated, this A167 cDNA encodes a SHIP isoform of 108 kDa. These cDNAs, in the expression vector pBK-CMV (Stratagene, La Jolla, CA), were transfected into 293T epithelial cells by calcium phosphate precipitation (13). Lysates from either 293T cells, transfected with various SHIP constructs or mock-transfected without DNA, or untreated FDC-P1 cells were subjected to SDS-PAGE and immunoblot analysis as described previously (22). Protein sizes were estimated by comparison to Perfect Protein Markers (Novagen, Madison, WI).

**Generation of Stably Transfected DT40 Cells**—SHIP-deficient DT40 cells expressing the murine FcγRIIB1 have been previously described (27). 10^5 cells were resuspended in 0.5 ml of phosphate-buffered saline and transfected with 30 μg of linearized plasmid DNA at 250 volts and 960 microfarads in a Gene Pulser II electroporator (Bio-Rad). The plasmids used were pPuro-FL-SHIP and pPuro-SHIPβ (which uses the I. Puro promoter to drive expression of an hemagglutinin-tagged full-length SHIPα or the untagged SHIPβ, respectively). Grb2-deficient DT40 cells have been previously described (40). 10^5 of these cells were transfected with 30 μg of linearized pPuro-FcγRIIB1 plasmid DNA as described above. Stable transfecants were selected in 0.5 μg/ml puromycine (Sigma). Expression of SHIPα and SHIPβ proteins was determined by immunoblotting whole cell lysates with the anti-SHIP antibody P1C1. Surface expression of FcγRIIB1 was determined by flow cytometry (13).

**Determination of Calcium Mobilization**—Indo-1-labeled DT40 cells were stimulated by cross-linking the BCR alone or BCR plus FcγRIIB1 as described previously. The ratio of indo-1 fluorescence at wavelengths of 395 and 480 nm after antibody addition was recorded using a Hitachi model 2050 fluorescence spectrophotometer and was plotted (13).

**Detection of MAP Kinase Activation**—2 × 10^5 cells/ml of SHIPα- or SHIPβ-expressing cells were stimulated and lysed in a lysis buffer containing 50 mM Tris (pH 7.6), 150 mM NaCl, 10 mM sodium pyrophosphate, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 10 μg/ml each peptatin, leupeptin, aprotinin, and 4-(2-aminoethyl)benzenesulfonfyl fluoride, and 1% Nonidet P-40. After the cellular debris was cleared by centrifugation, 2× SDS-PAGE sample dye was added to the lysates, and the total cellular lysates were separated on an 8% SDS-PAGE gel, transferred to nitrocellulose, and immunoblotted with the phosphospecific anti-MAPK antibody (New England Biolabs). The membrane was washed and subsequently immunoblotted with the rabbit anti-MAPK antibody (New England Biolabs). All blots were developed using the Western BLOT Pico enhanced chemiluminescence reagent (Pierce).

**Detection of Activated Akt**—BCR-induced phosphorylation of Akt and the inhibition by BCR plus FcγRIIB1 cross-linking was monitored by immunoblotting anti-Akt immunoprecipitations after 10 min of stimulation using the anti-phospho-threonine308 antibody (New England Biolabs) as described previously (41).

**Immunoprecipitation of Shc**—SHIPα- and SHIPβ-expressing DT40...
cells were stimulated via BCR plus FcγRIIB1, and Shc immunoprecipitations were performed as described previously (13). The immunoprecipitations were separated on an 8% SDS-PAGE gel, transferred to nitrocellulose, and blotted with anti-phosphotyrosine (Transduction Labs), anti-Shc (Santa Cruz Biotech), or anti-SHIP antibodies.

**Inositol Phosphatase Assays—**SHIP immunoprecipitations were performed out of 10^6 DT40 cells expressing either SHIPα or SHIPβ using 10 μg of mAb P1C1. 20% of the immunoprecipitations were used for each time point in the phosphatase assay. Another 20% was separated on an 8% SDS-PAGE gel and analyzed by anti-SHIP immunoblotting. SHIP immunoprecipitations were assayed for inositol phosphatase activity using [γ-32P]ATP and p110* for 30 min at room temperature. The reaction was stopped by the addition of EDTA, and the lipids were extracted with chloroform/methanol (1:1) and 1 M HCl. The reaction was dried, and the lipids were resuspended in 50 mm Tris (pH 7.5) and 0.125% Nonidet P-40 by sonication and used as a substrate for the phosphatase assay. Beads from the SHIP immunoprecipitations were incubated with this substrate at 37 °C for 5, 15, or 35 min. The lipids were extracted, separated on a TLC plate, and visualized by autoradiography.

**RESULTS**

**Generation of SHIPα and SHIPβ-specific Antibodies and Characterization of SHIPβ Expression—**To clarify references to the isoforms of SHIP at the protein level, we use the term SHIPα to describe p145 SHIP and SHIPβ for the p135 isoform (Fig. 1A). It has been previously shown that the SHIPβ protein is the product of the Δ183 SHIP mRNA (22). To further demonstrate the identity of SHIPβ and to distinguish this band from an approximately 120–130 kDa band seen in some SHIP immunoblots, we generated a mAb using as an immunogen the sixteen amino acids spanning the predicted splice junction (see "Experimental Procedures"). We were successful in generating a mAb, P2A8, that specifically recognizes SHIPβ and has no reactivity with either SHIPα or the translated product of Δ167 cDNA expressed in 293T cells (Fig. 1B). When tested on the interleukin 3-dependent murine myeloid cell line, FDC-P1 (Fig. 1C), the P2A8 mAb detected a band at 135 kDa, whereas the previously described mAb P1D7 recognized SHIPα but not SHIPβ. Before examining SHIPβ function in B cells, we wished to determine if this isoform was normally expressed in B cells. Total lysates from the murine B cell line A20 were resolved on an 8% SDS-PAGE gel and immunoblotted with the SHIPβ-specific antibody P2A8. We observed that A20 cells express the SHIPβ isoform in addition to the major SHIPα form (Fig. 1D).

**Generation of DT40 Cells Stably Expressing SHIPα or SHIPβ—**To study the function of SHIPβ, we reconstituted the SHIP-deficient DT40 cell line using plasmids encoding either SHIPα or SHIPβ. The advantage of such a system is that SHIPβ can be expressed in the absence of the SHIPα isoform. As shown in Fig. 2A, we obtained several DT40 clones stably expressing SHIPα or SHIPβ. The data presented in this manuscript were obtained from multiple clones, and the data shown are representative of multiple experiments from more than one clone. Transfection of the SHIPα-encoding plasmid led to expression of the expected 145-kDa protein. There was no detectable expression of smaller SHIP proteins, including SHIPβ. It is possible that the mRNA-splicing events that lead to SHIPβ expression require an unprocessed mRNA and cannot take place in transcripts from a cDNA. As expected, SHIPβ-expressing cells did not show expression of the larger 145-kDa isoform.

**SHIPβ Possesses Inositol Phosphatase Activity Comparable with That of SHIPα—**We have recently defined that the bound-
arys of the inositol phosphatase domain of SHIP lie between amino acids 400 and 866 (13). The deletion in the SHIPβ isoform does not include any portion of the catalytic domain. However, it is possible that the region contained within this deletion possesses some ability to modify the enzymatic activity of SHIP. To test this possibility, cells stably expressing either SHIPα or SHIPβ were lysed, and anti-SHIP immunoprecipitations were performed using the mAb P1C1, which recognizes both isoforms. The immunoprecipitates were analyzed in an in vitro inositol phosphatase assay using radiolabeled PIP_3 as a substrate. As seen in the autoradiogram in Fig. 2B, SHIPα and SHIPβ immunoprecipitates led to comparable dephosphorylation of PIP_3. This was confirmed by counting the radioactivity in the spots and plotting the percent conversion of PIP_3 to phosphatidylinositol-(3,4)-bisphosphate and PIP_2 (PIP_2) were cut out of the TLC plate, and the radioactivity present was determined by counting in a scintillation counter. The percent conversion was determined by dividing the amount of radioactivity in the phosphatidylinositol-3,4-bisphosphate spot by the sum of the radioactivities of the phosphatidylinositol-3,4,bisphosphate and PIP_2 spots (graph, bottom panel). The data are representative of two independent experiments.

SHIPβ Is Able to Reconstitute FcγRIIB1-mediated inhibition of calcium flux. Indo-1-loaded DT40 cells expressing the full-length SHIPα or SHIPβ proteins as well as the SHIP-deficient parental cell line were stimulated through the BCR alone or the BCR plus FcγRIIB1 (see “Experimental Procedures”). Stimulation of all the clones with BCR alone resulted in calcium flux, although expression of SHIPα or SHIPβ caused a reduction in both the initial calcium spike and sustained flux compared with SHIP-deficient cells. This was shown previously for SHIPα, and it suggests that SHIPβ is functioning in this system. As demonstrated previously, cross-linking of the BCR and FcγRIIB1 in the SHIP-deficient cells did not inhibit calcium flux, whereas restoration of SHIPα expression resulted in inhibition. BCR plus FcγRIIB1 cross-linking in the SHIPβ-expressing clones caused inhibition of calcium flux similar to that seen in the SHIPα-expressing cells (Fig. 3). These results demonstrate that the sequences contained within the Δ183 deletion are not required for FcγRIIB1/SHIP-mediated inhibition of calcium flux.

**Fig. 2.** SHIPα and SHIPβ possess comparable inositol phosphatase activities. A, expression of SHIPα and SHIPβ in the SHIP-deficient DT40 cell line. Equal numbers of cells were lysed, and total lysates were analyzed for expression of SHIP proteins by immunoblotting. B, inositol phosphatase activities of SHIPα and SHIPβ. SHIPα and SHIPβ were immunoprecipitated (IP) from DT40 cellular lysates. One-fifth of the immunoprecipitations was analyzed by immunoblotting to determine relative protein levels (upper panel). The remainder of the immunoprecipitations was assayed for phosphatase activity toward PIP_3 (labeled with 32P at the 3' position using purified PI3K) in a time course of 5, 15, and 35 min and analyzed by thin layer chromatography followed by autoradiography (middle panel). Immunoprecipitations from DT40 cells expressing either SHIPα or SHIPβ using non-immune rabbit Ig (Control Ig) were performed to determine nonspecific conversion. The spots representing PIP_3 and phosphatidylinositol-3,4-bisphosphate (PIP_2) were cut out of the TLC plate, and the radioactivity present was determined by counting in a scintillation counter. The percent conversion was determined by dividing the amount of radioactivity in the phosphatidylinositol-3,4-bisphosphate spot by the sum of the radioactivities of the phosphatidylinositol-3,4-bisphosphate and PIP_2 spots (graph, bottom panel). The data are representative of two independent experiments.
and MAPK, suggesting that despite the loss of the 61 amino acids due to the Δ183 internal deletion, SHIPβ is fully capable of inhibiting these downstream signaling pathways.

**FIG. 3.** SHIPβ is able to reconstitute FcγRIIB1-mediated inhibition of BCR-induced calcium flux. SHIP-deficient DT40 cells and stable transfectants expressing SHIPα or SHIPβ were loaded with indo-1 and analyzed for calcium flux (as described under “Experimental Procedures”) after cross-linking of BCR alone (thick line) or BCR plus FcγRIIB1 (thin line). Stimulating antibodies were added 20 s after the beginning of data recording. The data are representative of at least three independent experiments.

**FIG. 4.** SHIPβ reconstitutes FcγRIIB1-mediated inhibition of MAPK and Akt inhibition. A, SHIP-deficient DT40 cells or clones expressing SHIPα or SHIPβ were left unstimulated (φ) or stimulated through BCR alone (B) or BCR plus FcγRIIB1 (B+F) for 10 min, as described under “Experimental Procedures.” Total cellular lysates were analyzed by immunoblotting. The extent of MAPK activation was determined using the phosphospecific anti-MAPK antibody (top panel). MAPK protein levels were determined with the anti-MAPK antibody (bottom panel). B, cells were stimulated as in A and lysed. Anti-Akt immunoprecipitations were performed, and Akt activation was analyzed by immunoblotting with anti-phospho-Akt Thr308 antibody. Akt protein levels were determined by anti-Akt immunoblotting.

Grb2 interacts through its SH3 domain(s) with proline-rich regions of SHIP. We wished to examine the effect of the deletion in SHIPβ on the interaction with Shc in BCR plus FcγRIIB1-stimulated B cells. DT40 cells expressing either SHIPα or SHIPβ were left unstimulated or stimulated through the BCR plus FcγRIIB1 for 5 min. The cells were lysed, and Shc immunoprecipitations were performed from the lysates followed by anti-phosphotyrosine immunoblotting (Fig. 5). Tyrosine-phosphorylated proteins of the correct apparent molecular masses of 145 and 135 kDa were seen in the Shc immunoprecipitates from stimulated cells, suggesting that both SHIPα and SHIPβ proteins co-immunoprecipitated with
SHIPβ forms a complex with Shc upon BCR plus FγRIIB1 cross-linking. DT40 cells expressing either SHIPα or SHIPβ were left unstimulated (φ) or stimulated through BCR plus FγRIIB1 (+). Anti-Shc immunoprecipitations (IP) were performed and analyzed by anti-phosphotyrosine (Anti-P-Tyr) immunoblotting. The same blot was stripped and reprobed with anti-SHIP or anti-Shc antibodies (data not shown), and the bands corresponding to Shc, SHIPα, and SHIPβ are denoted by the arrows.

Shc. This was confirmed by subsequent stripping of this membrane and blotting with anti-SHIP antibodies (data not shown). This suggested that both SHIPα and SHIPβ can interact with Shc upon BCR plus FγRIIB1 cross-linking.

Although Grb2 could reliably interact with SHIPβ upon over-expression in COS cells (data not shown), we had difficulties in detecting an interaction between Grb2 and SHIPα or SHIPβ in DT40 cells. However, Harmer and DeFranco (21) clearly demonstrated a role for Grb2 in the formation of the Shc-SHIP complex. Thus, to investigate the role of Grb2 and its interaction with proline-rich regions of SHIP, we determined if FγRIIB1-mediated inhibition of calcium flux occurs in Grb2-deficient DT40 cells. Our working hypothesis was that if Grb2 binding to the SHIP proline-rich regions is required for efficient SHIP function, then Grb2-deficient cells will not be able to inhibit calcium flux when the BCR and FγRIIB1 are coligated. The murine FγRIIB1 was stably transfected into Grb2-deficient DT40 cells, and surface expression of FγRIIB1 was determined by flow cytometry (Fig. 6A). FγRIIB1-expressing clones were then stimulated through BCR alone or BCR plus FγRIIB1. As seen in Fig. 6B, Grb2-deficient cells are able to mobilize calcium in response to BCR stimulation, and this calcium flux is inhibited when FγRIIB1 is coligated with the BCR. These data suggest that the interaction of SHIPα and SHIPβ with Grb2 is not required for FγRIIB1-mediated inhibition of BCR-induced calcium flux in DT40 cells.

DISCUSSION

In this report we have demonstrated that SHIPβ, a naturally occurring isoform of the 5'-inositol phosphatase SHIP, functions very comparably to the better-characterized SHIPiso isoform. We have shown that SHIPβ possesses inositol phosphatase activity toward PIP3 in vitro and that this activity is similar to the activity of full-length SHIP. We have used SHIP-deficient DT40 cells reconstituted with SHIPβ to demonstrate that SHIPβ retains the ability to function in FγRIIB1-mediated inhibition of B cell receptor-induced calcium flux, Akt activation, and MAPK activation. SHIPβ has also retained the ability to interact with Shc, a well characterized interaction partner of SHIP. These data demonstrate that at least in BCR plus FγRIIB1 signaling, these two isoforms are functionally comparable, suggesting that they may not act in an antagonistic manner when expressed in vivo.

We have recently defined a requirement for the C terminus of SHIPα in FγRIIB1-mediated inhibition of BCR-induced calcium flux (13). The 61-amino acid deletion found in the SHIPβ isoform provided the opportunity to investigate the functional requirement for several of the specific protein interaction regions found in the C terminus of SHIP. In particular, the SHIPβ isoform helped to directly address the role for SHIP interaction with PI3K; the p85 subunit of PI3K has been shown to bind to SHIP following coligation of the BCR and FγRIIB1. Murine SHIP contains a canonical p85 SH2 domain binding site that follows tyrosine 917. The deletion in the SHIPβ isoform results in the destruction of this putative p85 binding site. Despite this, it has been previously shown that p85 is still able to bind to SHIPβ, although the interaction is very weak in comparison with the interaction of p85 with SHIPα (22). The data presented in this report show that SHIPβ is able to fully substitute for SHIP in the FγRIIB1 system. This suggests that the full affinity interaction between SHIP and PI3K is not required for SHIP function in this system. Also of note, the PI3K binding site is not conserved in the human p145 SHIP. However, SHIP is involved in signaling initiated by many receptors, including several cytokine receptors. The interaction between SHIP and PI3K may play a role in these other cases.

Although the deletion in SHIPβ alters the sequence that follows the NXY motif surrounding tyrosine 917, it does not alter the sequences N-terminal to the NXY. Since residues N-terminal to the phosphorylated tyrosines are more critical for PTB domain binding than residues C-terminal to the tyrosine, the interactions of PTB domain-containing proteins with the SHIPα C terminus would be expected to be intact in the SHIPβ isoform. Consistent with this hypothesis, we find that SHIPβ is able to interact with Shc. Recently, two other PTB-containing proteins, p62 Dok and Dok-3, have been shown to interact with phosphorylated SHIP. p62 Dok interacts with the Ras GTPase-activating protein RasGAP and has been implicated in FγRIIB1 inhibition of MAPK (18). Dok-3 has been demonstrated to have negative regulatory effects on BCR-induced NF-AT activity, suggesting that it regulates BCR-induced calcium flux (19). The fact that SHIPβ retains the ability to inhibit both calcium flux and MAPK suggests that it also retains the ability to interact with these two PTB domain-containing proteins.

It has previously been shown that SHIP, Shc, and Grb2 form a ternary complex following stimulation of B cells with BCR and FγRIIB1 (21). Our data with Grb2-deficient DT40 cells suggests that the interaction between SHIP and Grb2 is not important for SHIP function in this system. However, this may not rule out a function for this interaction in other receptor systems or cell types. The interaction between Grb2 and SHIP occurs between one or both of the Grb2 SH3 domains and one or more of the polyproline-rich stretches in the C terminus of SHIP. The fact that SHIPβ still interacts with Grb2 (22) indicates that the polyproline-rich stretch contained within the 61-amino acid deletion is not essential. SHIPβ still contains three other proline-rich regions, one or more of which may be important for the interaction with Grb2. This is currently under investigation.

Although SHIP was originally identified as a 145-kDa protein, it quickly became apparent that related proteins of multiple molecular masses exist. Depending on the cell type being considered, protein species of 167, 135, 125, and 110 kDa can be recognized by antisera raised against the 145-kDa form of SHIP. Two mechanisms have been proposed for generation of the smaller forms of SHIP. Damen et al. (24) demonstrate that smaller forms of SHIP can be generated from the 145-kDa form by proteolytic cleavage in the C terminus of SHIP. The N
terminus and the central region containing the 5′-inositol phosphatase domain are left intact under these conditions. A human 110-kDa isoform was shown to result from an alternatively spliced mRNA that lacks the first 214 amino acids of SHIP, including the entire SH2 domain (3). As mentioned earlier, SHIPβ arises from an alternatively spliced form of the p145 SHIP mRNA (22). The existence of so many isoforms of SHIP that are missing various putative protein interaction domains suggests that these multiple species may have different functions within the cell. This hypothesis is supported by the observation that different isoforms of SHIP are expressed at different stages of bone marrow cell differentiation (23). Regulation of the function of one isoform of a protein by a second isoform is known in other systems. For example, the function of the focal adhesion kinase FAK can be negatively regulated by the autonomous expression of the non-catalytic C terminus of the protein (referred to as the FAK-related non-kinase, or FRNK). Expression of FRNK inhibits FAK functions in cell spreading, cell mobility, and cell cycle progression by competing for binding to other proteins and ultimately excluding FAK from focal adhesions (43–45). Such regulation could be envisioned for the different isoforms of SHIP. One important difference between the FAK/FRNK system and the SHIP isoforms is that all the known SHIP isoforms retain the catalytic domain, making a model of direct dominant negative function less likely. Given the recent findings that the C terminus is critical for SHIP signaling in inhibitory signaling (13), it is possible that the various isoforms of SHIP could have differing interaction partners that may affect their function. The data presented here show that SHIPα and SHIPβ have similar functions in the BCR and FcγRIIB1 signaling pathways. Thus the concurrent expression of SHIPβ in B cells may not affect SHIPα function in this system. However, SHIP has been demonstrated to play a role in regulating signaling from other receptors such as the interleukin-3 receptor (46–48), the granulocyte-macrophage colony-stimulating factor receptor (48), and the Fcε receptor of mast cells (49). Further studies are necessary to determine if SHIPβ can also reconstitute SHIPα function in these systems. Our recent observations suggest that SHIP proteins themselves may form dimers or oligomers. 2 An interesting possibility is that one SHIP isoform may form heterodimers with other SHIP isoforms. It could then be hypothesized that the function of SHIPα could be regulated through expression of and heterodimerization with other isoforms. The ultimate result of SHIP function in a given cell type could then be both a result of competition between SHIP isoforms and the consequence of dimerization between isoforms. This possibility is currently under investigation.

Acknowledgments—We thank Dr. Larry Rohrschneider for generously providing SHIPβ reagents and Scott Walk and other members of the Ravichandran laboratory for technical assistance and helpful discussion. We thank Dr. Elizabeth Wayner and the staff of the Fred Hutchinson Biologics Production Facility for the generation of the P2A8 hybridoma.

REFERENCES
1. Damen, J. E., Liu, L., Rosten, P., Humphries, R. K., Jefferson, A. B., Majerus, P. W., and Krystal, G. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 1689–1693
2. Drayer, A. L., Pesesse, X., De Smedt, F., Woscholski, R., Parker, K., and Erneux, C. (1996) Biochem. Biophys. Res. Commun. 225, 243–249
3. Kavanagh, W. M., Pot, D. A., Chin, S. M., Deuter-Reinhard, M., Jefferson, A. B., Norris, F. A., Masiarz, F. R., Cousins, L. S., Majerus, P. W., and Williams, L. T. (1996) Curr. Biol. 6, 438–445
4. Lioubin, M. N., Algate, P. A., Tsai, S., Carlborg, K., Aebersold, A., and Rohrschneider, L. H. (1996) Genes Dev. 10, 1084–1095
5. Osborne, M. A., Zenner, G., Lubinus, M., Zhang, X., Songyang, Z., Cantley, L. C., Majerus, P., Burn, P., and Kochan, J. P. (1996) J. Biol. Chem. 271, 29273–29278
6. Ware, M. D., Rosten, P., Damen, J. E., Liu, L., Humphries, R. K., and Krystal, G. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 1689–1693
7. T. D. Lankin, M. E. March, and K. S. Ravichandran, manuscript in preparation.
