

# Protein Interactions of Src Homology 2 (SH2) Domain-Containing Inositol Phosphatase (SHIP): Association with Shc Displaces SHIP from Fc $\gamma$ RIIb in B Cells<sup>1</sup>

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Our recent studies revealed that the inositol phosphatase Src homology 2 (SH2) domain-containing inositol phosphatase (SHIP) is phosphorylated and associated with Shc exclusively under negative signaling conditions in B cells, which is due to recruitment of the SHIP SH2 domain to the Fc $\gamma$ RIIb. In addition, we reported that SHIP-Shc interaction involves both SHIP SH2 and Shc phosphotyrosine binding domains. These findings reveal a paradox in which the single SH2 domain of SHIP is simultaneously engaged to two different proteins: Shc and Fc $\gamma$ RIIb. To resolve this paradox, we examined the protein interactions of SHIP. Our results demonstrated that isolated Fc $\gamma$ RIIb contains SHIP but not Shc; likewise, Shc isolates contain SHIP but not Fc $\gamma$ RIIb. In contrast, SHIP isolates contain both proteins, revealing two separate pools of SHIP: one bound to Fc $\gamma$ RIIb and one bound to Shc. Kinetic studies reveal rapid SHIP association with Fc $\gamma$ RIIb but slower and more transient association with Shc. Affinity measurements using a recombinant SHIP SH2 domain and phosphopeptides derived from Fc $\gamma$ RIIb (corresponding to Y273) and Shc (corresponding to Y317) revealed an approximately equal rate of binding but a 10-fold faster dissociation rate for Fc $\gamma$ RIIb compared with Shc phosphopeptide and yielding in an affinity of 2.1  $\mu$ M for Fc $\gamma$ RIIb and 0.26  $\mu$ M for Shc. These findings are consistent with a model in which SHIP transiently associates with Fc $\gamma$ RIIb to promote SHIP phosphorylation, whereupon SHIP binds to Shc and dissociates from Fc $\gamma$ RIIb. *The Journal of Immunology*, 1999, 162: 1408–1414.

The positive signal transduction process triggered by the B cell Ag receptor, surface Ig (sIg),<sup>4</sup> features three independent biochemical pathways including activation of the  $\gamma$  isoform of phospholipase C, stimulation of phosphatidylinositol (PtdIns) 3-kinase, and induction of the Ras pathway (1). The Ras pathway is presumably important for B cell proliferation, although there is no direct information in B cells in this regard.

Induction of the Ras pathway by sIg, similar to other receptors, involves phosphorylation of sIg-associated proteins Ig $\alpha$  and Ig $\beta$  on tyrosine residues within the conserved immunoreceptor tyrosine-based activation motif (ITAM) (reviewed in Refs. 2 and 3). ITAM phosphorylation leads to recruitment of the adapter protein Shc via its Src homology 2 (SH2) domain that in turn promotes Shc phosphorylation at tyrosine residues 239, 240 (4), and 317 (5, 6). Phosphorylation at these residues creates an optimal binding site for the SH2 domain of the

adapter protein Grb2 (6) and the SH2 domain-containing inositol phosphatase (SHIP) (7, 8). Recent reports of B cell signal transduction indicated that, concomitant with the above events, Grb2 association with the Ras exchange factor Sos is also enhanced (9). Translocation of Sos to the sIg signaling complex at the plasma membrane through these protein interactions appears to catalyze GTP binding to Ras (10) and is rate-limiting for Ras activation.

SH2 domains act as independent protein interaction modules and bind to a tyrosine phosphorylated residue within a larger target protein (reviewed in Refs. 11–13). The SH2-phosphotyrosine interaction is modified and specified by residues in the +1 to +3 position, C terminal to the phosphorylated tyrosine residue of the interaction target. Thus, while the phosphorylated ITAM motif presents an optimal binding site for the SH2 domain of Shc (14), the same phosphorylated ITAM is incapable of binding to the SH2 domain of SHIP (15).

Co-cross-linking the B cell IgG receptor, Fc $\gamma$ RIIb, to sIg blocks B cell activation in a process termed “negative” signaling and opposed to “positive” signaling of sIg cross-linking alone. Our recent studies indicated that negative but not positive signaling conditions promoted tyrosine phosphorylation of SHIP and its interaction with Shc (16). To date, SHIP and Fc $\gamma$ RIIb are the only proteins known to be tyrosine phosphorylated exclusively under conditions of negative signaling. Further studies revealed an essential role for Fc $\gamma$ RIIb expression in the induction of SHIP phosphorylation under negative signaling conditions (15, 17). Thus, these experiments indicated that SHIP was recruited to the phosphorylated ITAM-like immunoreceptor tyrosine-based inhibitory motif (ITIM; ITYSLL; reviewed in Refs. 2 and 3) through its N-terminal SH2 domain. We further observed that ITIM recruitment of SHIP was necessary for SHIP phosphorylation on tyrosine and its interaction with Shc (15).

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<sup>4</sup> Abbreviations used in this paper: sIg, surface immunoglobulin; GST, glutathione S-transferase; ITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibitory motif; PTB, phosphotyrosine-binding; PtdIns, phosphatidylinositol; SH2, Src homology 2; SHIP, SH2 domain-containing inositol phosphatase; SHP-2, SH2 domain-containing phosphotyrosine phosphatase-2.

At the same time, SHIP interacts with the adapter protein Shc and this interaction is limited by SHIP but not Shc phosphorylation. We (8) and others (7) have demonstrated that SHIP-Shc interaction is bidentate, such that the phosphotyrosine-binding domain (PTB) of Shc interacted with either of two NPxY motifs within SHIP (including SHIP residues Y931 and Y1035, respectively) and the SH2 domain of SHIP bound phosphorylated Shc at Tyr<sup>317</sup> and doubly phosphorylated tyrosines 239/240. We have further proposed (18, 19) that SHIP SH2 engagement by phosphorylated Shc competes with and ultimately precludes an interaction of Grb2 SH2 with phosphorylated Shc, thereby accounting for the observed inhibition of the Ras pathway in B cells stimulated under negative signaling conditions (18–20).

Together, these findings from several laboratories reveal a paradox in which the single SH2 domain of SHIP is simultaneously engaged to two different proteins: Fc $\gamma$ R2b and Shc. To address this issue, we have analyzed protein interactions of SHIP and Shc in B cells stimulated under negative signaling conditions of sIg-Fc $\gamma$ R2b co-cross-linking. Unlike mast cells responding to IL-3 (21), we did not detect SHP-2 associated with SHIP; rather, SHIP was associated with two phosphoproteins: Shc and Fc $\gamma$ R2b. However, Shc precipitates do not contain Fc $\gamma$ R2b; likewise, Fc $\gamma$ R2b precipitates do not display Shc. Experiments on the association kinetics indicated that SHIP binds Fc $\gamma$ R2b very early, within 30 s of stimulation, and this association is maintained for several minutes. In contrast, SHIP association with Shc appeared later and was shorter-lived. The data argue for two separate and distinct pools of SHIP, one bound to Fc $\gamma$ R2b and another bound to Shc. Affinity measurements using the recombinant SH2 domain of SHIP indicated transient and lower affinity binding to a phosphopeptide corresponding to Y273 of murine Fc $\gamma$ R2b but stable and higher affinity binding to a phosphorylation site of Shc. We propose a model in which SHIP transiently associates with Fc $\gamma$ R2b to promote SHIP phosphorylation, whereupon it disengages Fc $\gamma$ R2b and stably associates with phosphorylated Shc. These novel findings regarding the affinity of the SH2 domain of SHIP and Shc for their interaction partners and the kinetics of their association account for several unusual features of negative signaling. In addition, the rank order of affinities among Fc $\gamma$ R2b, SHIP, Shc and Grb2 reveal a system optimally designed to turn on and off B cell sIg-mediated signal transduction.

## Materials and Methods

### *Abs, cells, and reagents*

F(ab')<sub>2</sub> fragments and whole molecule of rabbit anti-mouse IgG Ab were obtained from Cappel Research Products (Durham, NC); other immunoprecipitating and immunoblotting Abs were from Upstate Biotechnology (Lake Placid, NY). Anti-SHIP Ab was generated using a glutathione S-transferase (GST) fusion protein of SHIP residues 874–941, obtained by PCR amplification of SHIP cDNA, as described earlier (15). The purified protein was injected into rabbits and tested by immunoblot and immunoprecipitation. Protein G-agarose was purchased from Life Technologies/BRL (Gaithersburg, MD); glutathione-agarose was from Sigma (St. Louis, MO); and the enhanced chemiluminescence kit was from Kirkegaard & Perry (Gaithersburg, MD). A20 murine B cells were obtained from the American Type Culture Collection (Manassas, VA). The Fc $\gamma$ R-deficient cell line was a gift from I. Mellman (Yale University, New Haven, CT). GST-Grb2 fusion protein was obtained from Dr. Andreas Kazlauskas (National Jewish Hospital, Denver, CO). Prevanadate was generated by mixing 3 mM Na<sub>3</sub>VO<sub>4</sub> with 1.5% H<sub>2</sub>O<sub>2</sub>; 10 × 10<sup>6</sup> cells in 100  $\mu$ l were stimulated with 10  $\mu$ l of the mixture.

### *Lysis, immunoprecipitation, and immunoblotting*

Cell lysis, immunoprecipitation, and immunoblotting were performed as previously described (16). Briefly, B cells were stimulated with 10  $\mu$ g/ml F(ab')<sub>2</sub> fragment or whole molecule of rabbit anti-mouse IgG for the indicated times at 37°C and lysed with TN1 buffer (50 mM Tris (pH 8.0), 10

mM EDTA, 10 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 10 mM NaF, 1% Nonidet P-40, 125 mM NaCl, 10 mM Na<sub>3</sub>VO<sub>4</sub>, and 10  $\mu$ g/ml each aprotinin and leupeptin). Post-nuclear extracts were incubated overnight with the Ab of interest followed by protein G-agarose. Samples were washed with lysis buffer and resuspended in SDS sample buffer (60 mM Tris (pH 6.8), 2.3% SDS, 10% glycerol, 0.01% bromphenol blue). Precipitated proteins were separated by SDS-PAGE, transferred to nitrocellulose filters, probed with the Ab of interest, and developed by enhanced chemiluminescence. In some cases, filters were stripped of primary Ab as described earlier (8, 15), washed, and reprobed.

### *GST in vitro binding assay*

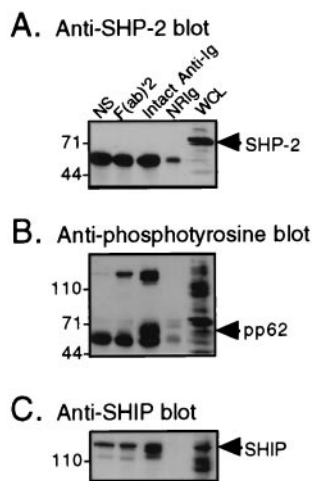
SHIP was immunoprecipitated from resting and activated A20 lysates with anti-SHIP antisera, collected with protein G-agarose and the sample was divided into two aliquots. One half was reprecipitated with 100 nM GST-Grb2 fusion protein to obtain SHIP-associated, phosphorylated Shc. The protein complexes were collected as previously described (8, 15) with glutathione-agarose beads, washed three times with lysis buffer, resuspended in SDS sample buffer, and separated by 10% SDS-PAGE. The separated proteins were transferred to nitrocellulose and analyzed by immunoblotting with anti-Shc. The anti-Shc Ab is a rabbit polyclonal sera made against a GST-Shc fusion protein and thus is immunoreactive with GST-Grb2. The other half was resolved by 10% SDS-PAGE and immunoblotted with antiphosphotyrosine to detect SHIP-associated p62 Fc $\gamma$ R2b.

### *Deglycosylation of Fc $\gamma$ R*

Fc $\gamma$ R from activated A20 cells was captured with human IgG-Sepharose using human IgG covalently attached to Sepharose at 10 mg/ml packed beads or in anti-SHIP immunoprecipitates. The bound protein was eluted from the beads with 20  $\mu$ l of 0.7% SDS. The eluate was split in half, and one half was treated with endoglycosidase F (Boehringer Mannheim, Indianapolis, IN; 20 U/100  $\mu$ l); the other half was mock-treated with enzyme diluent. The reaction mixtures were incubated overnight at 37°C, and 5 volumes of cold acetone was added to each sample for 15 min at –20°C to precipitate protein. The samples were spun at 14,000 rpm for 10 min and precipitated proteins were redissolved in SDS sample buffer, run on 10% SDS-PAGE, transferred to nitrocellulose membrane, and probed with antiphosphotyrosine Ab.

### *Affinity measurements of the SHIP SH2 domain for phosphopeptides*

Synthetic N-terminally biotinylated phosphopeptides corresponding to Y273 of the murine Fc $\gamma$ R2b (containing the ITIM motif) and to Y317 of human Shc were purchased from Quality Controlled Biochemicals (Torrance, CA) and were generated as earlier described (8). Both of these peptides were shown to directly bind the SHIP SH2 domain and to compete with the binding of the endogenous proteins to SHIP (8). GST fusion protein encoding the SH2 domain of SHIP was generated as previously described (15). Peptides were immobilized in the two sensor cells of a planar biotin cuvette for use on an IAsys evanescent-wave optical biosensor according to the manufacturers protocol (IAsys; Affinity Sensors, Cambridge, U.K.). Briefly, the sensor surfaces bearing biotin were covered with a 2-min application of 5 mg/ml neutravidin (Pierce, Rockford, IL). The excess neutravidin was washed out with 10 mM PBS (pH 7.4) with 0.05% Tween 20 (binding buffer). Free biotin-binding sites were saturated with an excess of biotinylated peptide in each cell, giving signals of 16 and 30 arcs of immobilized Shc and Fc $\gamma$ R2b peptides, respectively. Biotin cuvettes were completely regenerated by a 1-min exposure to 12 M KOH followed by copious buffer washing; surfaces were then reloaded with the same amount of neutravidin and biotinylated peptide for further analysis. Kinetic experiments were performed using the pseudo-first order approach outlined in the IAsys user documentation. Measurements were made at 22°C, with the stirrer speed set at 100% of maximum and data collection at 0.3 s per data point. For successive binding cycles, varying amounts of stock GST-SHIP SH2 domain were diluted into binding buffer to initiate binding (arrow 1 in Fig. 5). Real-time rate data were collected for 3–5 min, then the cuvette cells were washed twice with 100- $\mu$ l aliquots of fresh running buffer to commence collection of dissociation rate data (arrow 2 in Fig. 5). At the end of the 3- to 5-min dissociation period, the surface was regenerated with 3 M MgCl<sub>2</sub>. Preliminary controls were performed to establish that GST-SHIP SH2 domain did not bind at all to the neutravidin platform lacking peptide (Fig. 5A, curve C). Initial (first 20–40 s) binding rates for each peptide at each GST-SHIP SH2 domain concentration were fitted using FASTfit software (Affinity Sensors) by nonlinear regression analysis to a simple monophasic binding equation:  $R = R_{eq} [1 - \exp(-k_{on}t)]$ , where



**FIGURE 1.** SHIP is not stably associated with SHP-2 in B cells. A20 B cells ( $10 \times 10^6$ ) were stimulated with F(ab')<sub>2</sub> or intact anti-Ig or left unstimulated (NS). Cells were lysed with TN1 lysis buffer, and the cell lysates were incubated with anti-SHIP Ab overnight at 4°C. Immune complexes were collected with protein G-agarose, separated on 10% SDS-PAGE, and transferred to nitrocellulose membrane. The membrane was probed with anti-SHP2 (A), antiphosphotyrosine (B), and anti-SHIP (C) Abs. Whole cell lysate (WCL) was used as a positive control for immunoblotting.

the amount of GST-SHIP SH2 domain bound ( $R$ ) at time ( $t$ ) is some fraction of the final level bound at equilibrium ( $R_{eq}$ ) as a function of the observed binding rate ( $k_{on}$ ) at the concentration of GST-SHIP SH2 domain present after injection. Second-order association rate constants ( $k_{ass}$ ) and dissociation rate constants ( $k_{diss}$ ) were inferred from the slope and  $y$ -intercept of those plots, respectively. Dissociation rates were also measured by direct fitting of dissociation data and found to be comparable to  $y$ -intercepts-derived values to within error. Initial kinetics were used to simplify biosensor data sets that revealed secondary, later-occurring kinetic behavior that was attributed to self-association of GST domains. Efficient thrombin cleavage of GST domains was not possible.

## Results

Our earlier experiments revealed an unidentified 62-kDa tyrosine-phosphorylated protein that coimmunoprecipitated with tyrosine-phosphorylated SHIP and was additional to phospho-Shc (15). Other studies of SHIP protein interactions in IL-3-stimulated cells revealed that the p70 phosphotyrosine phosphatase SHP-2 associated with phospho-SHIP through the SHIP SH2 domain (21). To investigate the possibility that the SHIP-associated p62 band of B lymphocytes was SHP-2, we probed SHIP immunoprecipitates with Abs to SHP-2. In contrast to findings reported in cytokine-stimulated cells, SHIP immunoprecipitates from B lymphocytes did not display a band immunoreactive with Abs to SHP-2 (Fig. 1A), although p62 was observed in antiphosphotyrosine immunoblots (Fig. 1B) and SHP-2 was expressed in these cells, as shown in whole cell lysates (Fig. 1A). These data indicate that p62 is associated with SHIP upon SHIP tyrosine phosphorylation induced by negative signaling and that p62 is not SHP-2. In similar experiments, we found no association of SHP-1 with SHIP in stimulated B cells, although SHP-1 is expressed in these cells (data not shown).

SHIP associates with tyrosine-phosphorylated 52 Shc (7, 8, 16) and to the ITIM motif of Fc $\gamma$ RIIb (15, 22); both interactions involve the SHIP SH2 domain. Although the protein core of Fc $\gamma$ RIIb is  $\sim$ 40 kDa, the mature protein migrates with an apparent molecular mass of  $\sim$ 45 kDa (22) to 60 kDa (23, 24). The variability in apparent m.w. is likely due to differential glycosylation in different

cells, although Fc $\gamma$ RIIb from the A20 murine B cell lymphoma line used in these experiments has been reported as a 45-kDa (25) to a 60-kDa (23) protein. To assess whether the SHIP-associated p62 protein was either phospho-Shc or Fc $\gamma$ RIIb, B cells were stimulated with pervanadate and immunoprecipitated with Abs to Shc, SHIP (rabbit polyclonal antisera) or Fc $\gamma$ RIIb (2.4G2 mouse mAb) and the resulting samples were immunoblotted with antiphosphotyrosine. The results, shown in Fig. 2A, revealed that p62 was present in SHIP but not Shc immunoprecipitates and comigrated with a 62-kDa tyrosine-phosphorylated protein present in 2.4G2 anti-Fc $\gamma$ RIIb immunoprecipitates. The same three tyrosine-phosphorylated proteins (p145SHIP, p62, and p52Shc) were found in SHIP immunoprecipitates of pervanadate-stimulated A20 lymphoma and primary splenic B cells (Fig. 2B). These findings are consistent with the notion that the SHIP-associated p62 protein represents B cell Fc $\gamma$ RIIb which is tyrosine phosphorylated by pervanadate treatment (Fig. 2) or by anti-Ig reagents (15).

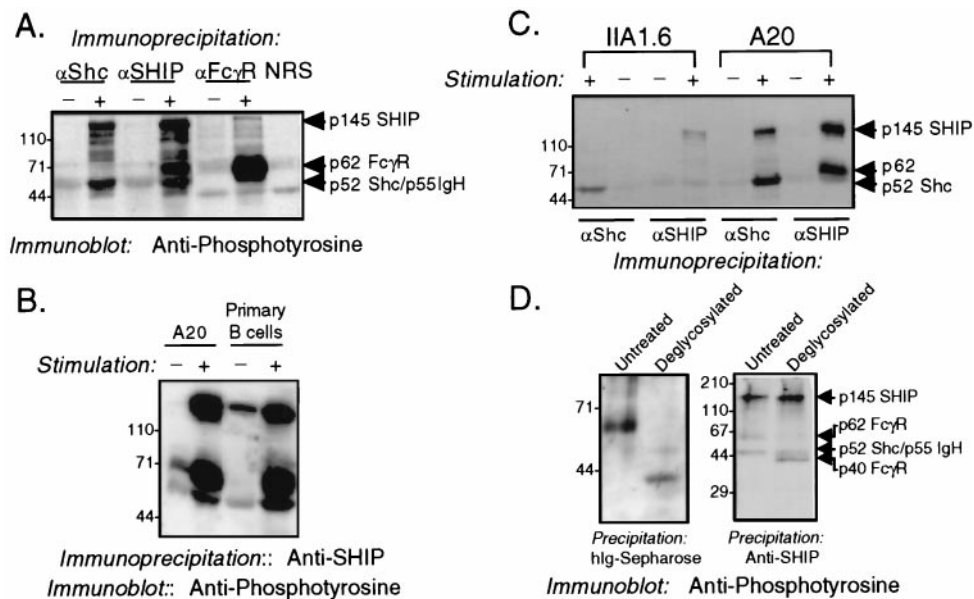
To confirm that p62 represents the IgG receptor Fc $\gamma$ RIIb, the B cell line A20 or the Fc $\gamma$ RIIb-deficient A20 derivative IIA1.6 were stimulated with intact anti-Ig to induce co-cross-linking of sIg and Fc $\gamma$ RIIb. The cells were lysed in detergent and the lysates were immunoprecipitated with Abs to Shc or SHIP. The immunoblot results shown in Fig. 2C revealed a tyrosine-phosphorylated p62 protein associated with SHIP in A20 B cells that was absent in Fc $\gamma$ RIIb-deficient IIA1.6 B cells. As above, p62 was apparent in SHIP but not Shc immunoprecipitates.

Because there is no immunoblotting reagent that specifically detects the core protein of murine Fc $\gamma$ RII, we used two additional approaches to test the possibility that p62 represents murine Fc $\gamma$ RII. First, we affinity-adsorbed all IgG receptors from detergent lysates of pervanadate-stimulated A20 B cells using normal human IgG-coated Sepharose. Second, we obtained p62 associated with SHIP by subjecting A20 B cells stimulated with intact anti-Ig with Abs to SHIP. The bound material from both samples was deglycosylated with endoglycosidase F, separated by SDS-PAGE, and probed with antiphosphotyrosine Abs after transfer to filters. The results (Fig. 2D) demonstrated that IgG-Sepharose or anti-SHIP coprecipitates a tyrosine-phosphorylated p62 protein that, upon deglycosylation, migrates at  $\sim$ 40 kDa, similar to the reported core protein of Fc $\gamma$ RIIb (26, 27). Together, these findings strongly support the hypothesis that p62 represents a highly glycosylated form of Fc $\gamma$ RIIb in A20 B cells.

Results in these experiments revealed both Shc and p62 Fc $\gamma$ RIIb in SHIP immunoprecipitates (Fig. 2A, lane 4, and Fig. 2B, lane 8). Likewise, SHIP was found in immunoprecipitates of Shc, indicating these two proteins inducibly associate, as earlier reported (7, 8, 16). In contrast, we did not observe the presence of p62 Fc $\gamma$ RIIb in Shc immunoprecipitates (Fig. 2A, lane 2, and Fig. 2C, lane 6), suggesting that Fc $\gamma$ RIIb does not associate with Shc, either directly through the SH2 domain of Shc or indirectly through SHIP. If so, this observation implies that SHIP dissociates from the ITIM motif of Fc $\gamma$ RIIb upon binding to Shc, although it is not clear how or why dissociation would occur.

To more rigorously test the possibility that Shc is excluded from tyrosine-phosphorylated Fc $\gamma$ RIIb, immunoprecipitates of Shc or Fc $\gamma$ RIIb from resting or pervanadate-stimulated A20 B cells were probed with Abs to phosphotyrosine or to Shc. The antiphosphotyrosine blots (Fig. 3, top) revealed tyrosine-phosphorylated p52 and p46 Shc in the anti-Shc but not the Fc $\gamma$ RIIb immunoprecipitates. Probing the same samples with anti-Shc Abs demonstrated that Shc was not detected in Fc $\gamma$ RIIb immunoprecipitates, consistent with data in Fig. 2A showing that Fc $\gamma$ RIIb was absent from





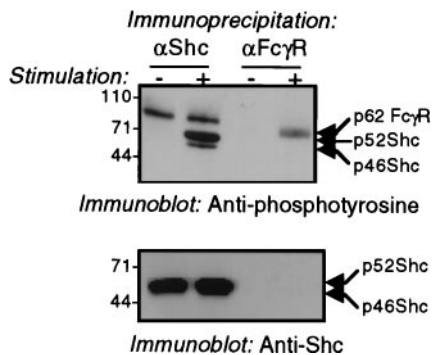
**FIGURE 2.** The SHIP-associated p62 phosphoprotein is Fc $\gamma$ RIIb. **A**, A20 B cells ( $10 \times 10^6$ ) were stimulated with pervanadate (+) or left unstimulated (-). Cells were lysed and immunoprecipitated as indicated with anti-Shc, anti-SHIP, or with biotinylated anti-Fc $\gamma$ RIIb Ab (2.4G2) for 5 h at 4°C. Immune complexes were collected with protein G-agarose or with streptavidin beads. Bound proteins were resolved on 10% SDS-PAGE and transferred to nitrocellulose and probed with antiphosphotyrosine Ab. Immunoprecipitation with normal rabbit serum (NRS) was used as a negative control. **B**, A20 or primary splenic B cells ( $10 \times 10^6$ ) were stimulated with intact anti-Ig (+) or left unstimulated (-). The cells were lysed and immunoprecipitated with anti-SHIP Ab overnight at 4°C. Bound proteins were resolved on SDS-PAGE, transferred to nitrocellulose, and blotted for tyrosine-phosphorylated proteins. **C**, IIA1.6 or A20 B cells ( $10 \times 10^6$ ) were stimulated with intact anti-Ig (+) or left unstimulated (-). Cells were lysed and immunoprecipitated with anti-Shc or anti-SHIP Ab overnight at 4°C. The immunoprecipitates were resolved on SDS-PAGE, transferred to nitrocellulose, and blotted with antiphosphotyrosine Ab. **D**, The p62 Fc receptor was captured with hlg-Sepharose (*left panel*) or with anti-SHIP Abs (*right panel*) from  $10 \times 10^6$  A20 B cells stimulated with intact anti-Ig. The precipitated proteins were divided into two; one half was treated with endoglycosidase F and the other half mock-treated with the enzyme diluent. The reactions were run on SDS-PAGE and immunoblotted with antiphosphotyrosine Ab to detect p62.

Shc immunoprecipitates. Thus, we observed the activation-induced formation of two distinct complexes, one containing SHIP-Shc and another containing SHIP-Fc $\gamma$ RIIb but not one that includes Shc-Fc $\gamma$ RIIb. These findings then specifically exclude a heterotrimeric complex that would include all three proteins.

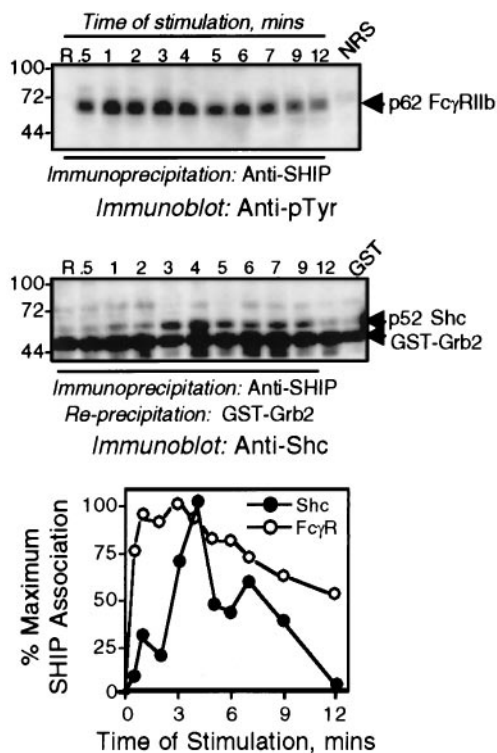
One interpretation of these data is that SHIP associates with Fc $\gamma$ RIIb, becomes phosphorylated and dissociates from Fc $\gamma$ RIIb upon binding to Shc. To test this possibility, we measured the kinetics of association of SHIP to Fc $\gamma$ RIIb and of SHIP to Shc. For these experiments, A20 B cells were stimulated under negative

signaling conditions with intact rabbit anti-mouse Ig at different times, then lysed and subjected to immunoprecipitation with anti-SHIP. The immune complexes were isolated and proteins were eluted with SDS sample buffer. One half of the eluate was then diluted in lysis buffer and reprecipitated with GST-Grb2 to pull down phosphorylated Shc, as we earlier described (18) and to prevent the interference of the Ig heavy chain. These samples were immunoblotted with anti-Shc Ab while the other half was probed with antiphosphotyrosine to detect p62 Fc $\gamma$ RIIb. The antiphosphotyrosine blots, shown in Fig. 4, *top*, revealed that SHIP associated with p62 Fc $\gamma$ RIIb within 30 s of stimulation, was maximal within 60 s, and that the association was maintained over the entire stimulation period of 12 min. In contrast, SHIP association with Shc (Fig. 4, *middle*) did not appear until 3 min of stimulation, was maximal at 4 min, and declined to background levels by 12 min. These distinct kinetics of SHIP association by Fc $\gamma$ RIIb and Shc are consistent with the hypothesis mentioned above that Shc binding to phospho-SHIP induces SHIP dissociation from Fc $\gamma$ RIIb, although the kinetic results likewise do not suggest an immediately obvious mechanism for this to occur.

Our earlier experiments (8) and those of others (7) indicated that the Shc PTB domain associated with either of the two NPxY motifs present in SHIP and was likely the first of two interactions between these two proteins, since the SHIP SH2 domain is bound to Fc $\gamma$ RIIb and is essential for phosphorylation of the NPxY motifs (15). The apparent lack of a stable trimeric complex between Fc $\gamma$ RIIb-SHIP-Shc indicates that SHIP disengages Fc $\gamma$ RIIb upon Shc PTB domain binding. A possible explanation for SHIP disengagement and re-engagement of Fc $\gamma$ RIIb and Shc, respectively, is that the intrinsic affinity of the SH2 domain of SHIP is greater for

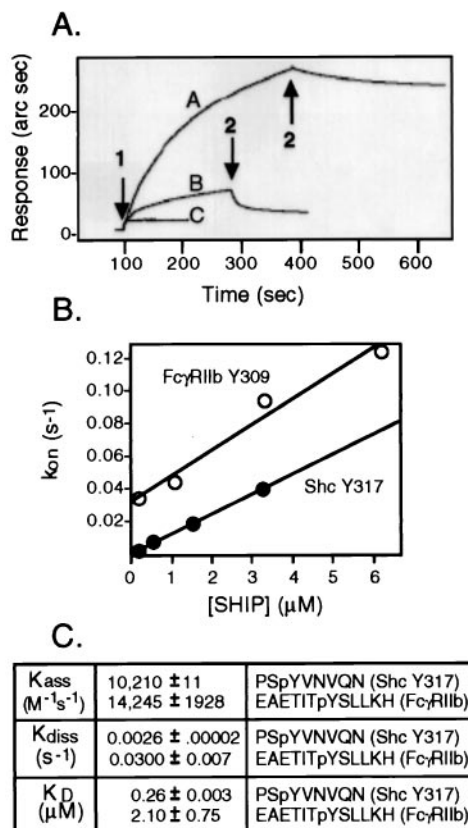


**FIGURE 3.** p52 Shc is excluded from Fc $\gamma$ RIIb under negative signaling conditions. A20 B cells ( $10 \times 10^6$ ) were stimulated with intact anti-Ig (+) or left unstimulated (-). Cells were lysed and immunoprecipitated with anti-Shc or biotinylated anti-Fc $\gamma$ RIIb Abs. The immune complexes were collected with either anti-rabbit IgG agarose or streptavidin beads. Precipitated proteins were resolved by SDS-PAGE, transferred to nitrocellulose, and the filter was probed with antiphosphotyrosine (*upper panel*), stripped, and re-probed with anti-Shc Ab (*lower panel*).



**FIGURE 4.** Kinetics of SHIP-Fc $\gamma$ RIIb and SHIP-Shc associations. Ly-sates from  $10 \times 10^6$  resting (R) or intact anti-Ig stimulated A20 cells were first immunoprecipitated with anti-SHIP Ab or normal rabbit serum (NRS). The immuno-isolated protein complexes were divided and one half was separated on SDS-PAGE and probed with antiphosphotyrosine Ab (*upper panel*) to reveal p62 Fc $\gamma$ RIIb. The other half was re-precipitated with GST-Grb2 or GST alone (*last lane*) and immunoblotted with anti-Shc Ab (*middle panel*). The intensity of the bands representing p62 in the *upper panel* and Shc in the *lower panel* was quantitated by laser densitometry and plotted against time of stimulation (*lower panel*).

phospho-Shc than for phospho-Fc $\gamma$ RIIb. According to this possibility, upon Shc PTB engagement of SHIP, the SHIP SH2 domain releases Fc $\gamma$ RIIb and engages phospho-Shc. To examine this issue, we applied the SH2 domain of SHIP to biotinylated, immobilized phosphopeptides corresponding to the ITIM motif of Fc $\gamma$ RIIb (pITIM) or of Y317 in Shc (pYVNV); both of these peptides were earlier shown to engage the SH2 domain of SHIP (8, 15), whereas the Shc Y317 phosphopeptide bound with high affinity to the SH2 domain of Grb2 (7). Kinetic properties of the interaction between biotinyl-pITIM and biotinyl-pYVNV peptides were determined using an evanescent-wave optical biosensor (IAsys Auto+Advantage, Affinity Sensors). In this assay, the biotin linked to the sensor surfaces was coated with tetrameric neutravidin, which in turn captured comparable quantities of biotinylated peptides. The recombinant GST-SHIP SH2 domain was added at varying concentrations to the 80  $\mu$ l cuvette cells. Resulting SHIP binding and dissociation profiles are compared for a single SHIP SH2 domain concentration in Fig. 5A. We observed slightly faster SH2 domain association (arrow 1) but a considerably slower dissociation rate (arrow 2) for pYVNV (curve A), compared with pITIM (curve B) peptides. Kinetic analysis of the initial binding period of several GST-SHIP SH2 domain concentrations with each peptide was performed as described earlier (28). Dissociation rates were determined by direct fitting and as the  $y$ -intercept of the  $k_{on}$  vs concentration of GST-SHIP SH2 domain; both methods that derive dissociation rate constants were in agreement. Data describing the initial  $k_{on}$  vs GST-SHIP SH2 domain concentration for comparable quantities of peptide (Fig. 5B) revealed nearly parallel



**FIGURE 5.** Affinity measurements of the SH2 domain of SHIP to Fc $\gamma$ RIIb and to Shc. *A*, Real-time binding and dissociation of 3.12  $\mu M$  SHIP binding to biotinylated pYVNV (curve A), biotinylated pITIM (curve B), and bare neutravidin-coated biotin cuvette sensor surface (curve C). Bulk refractive index shifts from protein injection were excluded from analysis of kinetic rate by excluding the first data point of each curve (0.3 s). Binding was initiated at arrow 1 and stopped at arrow 2. *B*, Observed association rates for the SH2 domain of SHIP binding to each phosphopeptide are plotted as a function of the concentration of the SHIP SH2 domain. The slope of each plot was taken as  $k_{ass}$  and the  $y$ -intercept was taken as  $k_{diss}$ . *C*, Comparative kinetic and equilibrium constants for SHIP binding pYVNV (Shc Y317) and pITIM (Fc $\gamma$ RIIb Y273).

slopes for the two peptides, indicating the peptides bind the SH2 domain of SHIP with nearly equal second-order rate constants. In contrast, the significantly different  $y$ -intercepts (reflecting the dissociation rates) reveal a 10-fold greater dissociation rate for phospho-Fc $\gamma$ RIIb compared with phospho-Shc and represents the essential difference between the two affinities. Thus, although GST-SHIP SH2 domain binds to both peptides at approximately the same rate, it dissociates  $\sim 10$ -fold more rapidly from the Fc $\gamma$ RIIb pITIM than from Shc pYVNV, resulting in an  $\sim 10$ -fold greater overall affinity of Shc pYVNV (2.1  $\mu M$ ) than pITIM of Fc $\gamma$ RIIb (0.26  $\mu M$ ) in binding the SH2 domain of SHIP. The kinetic and equilibrium constants determined for the SH2 domain of SHIP binding to both phosphopeptides and derived from this analysis is shown in Fig. 5C.

## Discussion

The recently cloned inositol 5-phosphatase SHIP has been shown to be a negative regulator in different cell types, including B cells (16, 19), mast cells (22) developing *Xenopus* oocytes (29) as well as cells responding to granulocyte CSF (30) and macrophage CSF (31). Recent studies revealed that the biochemical events during B cell negative signaling, such as reduced Ras activation (18, 19) and attenuated calcium influx (24, 32), are outcomes of the protein

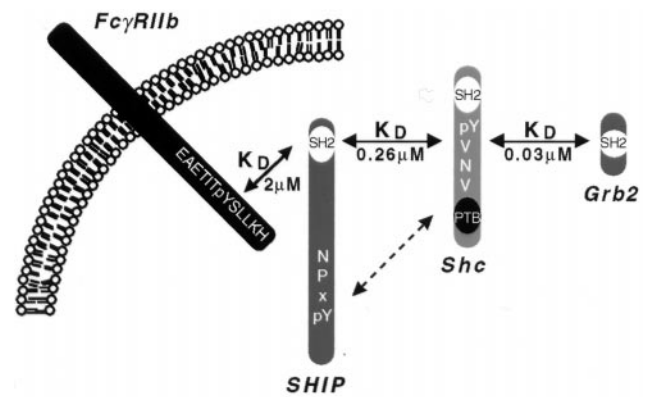
interactions of SHIP and/or its enzymatic activity. Here, we have investigated the protein interactions of SHIP to gain further insight into its function during negative signaling. In addition to the B cell paradigm, other reports suggest that protein interactions of SHIP serve to negatively regulate growth factor-mediated signaling. Thus, studies indicated that SHIP binds to SHP-2, in addition to Shc, in response to IL-3 stimulation (21). Since SHP-2 is known to be involved in positive signaling as an adapter protein for the Ras pathway (33–35), it is possible that SHIP contributes to negative signaling by sequestering SHP-2 and precluding its involvement in Ras induction, much like SHIP competes with Grb2 for binding to phosphorylated Shc (19). Other studies (36) demonstrated that SHIP inducibly associates with p72 Syk, in addition to Shc, in B cells (16) but the functional consequences of this interaction are unknown. In antiphosphotyrosine immunoblots of SHIP immunoprecipitates, we detected only p52 Shc and p62 Fc $\gamma$ RIIb and there are no detectable amounts of either SHP-2 or SHP-1 in these immunoprecipitates. Likewise, we failed to detect a 72-kDa tyrosine-phosphorylated Syk candidate protein in SHIP immunoprecipitates, although we have not specifically examined such samples for the presence of the Syk tyrosine kinase.

We had earlier observed a 62-kDa tyrosine-phosphorylated protein associated with SHIP (15), whereas we (15) and others (22) have described association of SHIP to the B cell IgG receptor, Fc $\gamma$ RIIb. The precise molecular mass of Fc $\gamma$ RIIb has been reported with varying molecular masses, partly due to differences in glycosylation from cell to cell but also due to the lack of a definitive immunoblotting reagent. Findings described here establish that the SHIP-associated p62 represents Fc $\gamma$ RIIb from A20 because p62 is absent from Fc $\gamma$ RIIb-deficient B cells (Fig. 2C), because p62 comigrates with phosphorylated Fc $\gamma$ RIIb obtained using the established immunoprecipitating monoclonal anti-Fc $\gamma$ RIIb Ab, 2.4G2 (Fig. 2A), and because deglycosylated p62 migrates with a core protein size of  $\sim$ 40 kDa, the predicted molecular mass of Fc $\gamma$ RIIb (Fig. 2B).

In the course of identifying p62, we observed that p62 Fc $\gamma$ RIIb was absent in Shc immunoprecipitates and that Shc was absent in Fc $\gamma$ RIIb immunoprecipitates, whereas SHIP immunoprecipitates contained both Shc and Fc $\gamma$ RIIb. Thus, the three proteins do not form a stable, heterotrimeric complex of p62-SHIP-Shc, but do form heterodimeric complexes of p62 Fc $\gamma$ RIIb-SHIP and SHIP-p52 Shc, and these complexes are stable because they survive co-immunoprecipitation. The two pools of SHIP are therefore exclusive, but may represent transitional forms such that one is converted to another during the course of stimulation.

SHIP association with Fc $\gamma$ RIIb is consistent with earlier reports that the SH2 domain of SHIP associates with the phosphorylated ITIM motif of Fc $\gamma$ RIIb (15). Likewise, SHIP association with Shc is consistent with observations that the Shc PTB domain interacts with phosphorylated SHIP and that the SHIP SH2 domain binds phosphorylated Shc (7, 8), although alternative views regarding the role of SHIP SH2 domain in its interaction with Shc have been reported (37). Nevertheless, observations invoking a role for the SHIP SH2 domain in SHIP-Shc interaction raise a paradox wherein the single SH2 domain of SHIP is concomitantly bound to two different proteins: Fc $\gamma$ RIIb and Shc.

One possible explanation for this paradox is that the SH2 domain of SHIP has greater affinity for phospho-Shc than for phospho-Fc $\gamma$ RIIb. According to this model, SHIP weakly engages phosphorylated Fc $\gamma$ RIIb and acquires phosphorylated tyrosine residues. The Shc PTB domain is then engaged, bringing phosphotyrosines of Shc within range of the SH2 domain of SHIP. Because of the intrinsically higher affinity, the SH2 domain of SHIP releases Fc $\gamma$ RIIb and binds to phospho-Shc. This hypothesis ac-



**FIGURE 6.** Model of SHIP protein interactions. The  $K_d$  of the SH2 domain of SHIP for the ITIM peptide of Fc $\gamma$ RIIb and for Shc Y317 are derived from this study and are in agreement with other measurements of Shc Y317 for the SH2 domain of SHIP (7). The  $K_d$  for the SH2 domain of Grb2 for Shc Y317 is derived from the earlier study (7).

counts for the existence of two mutually exclusive pools of SHIP, one pool transiently bound to Fc $\gamma$ RIIb and one pool stably bound to Shc. In addition, this hypothesis accounts for our findings regarding SHIP-protein interaction kinetics, shown in Fig. 4, in which SHIP engages p62 Fc $\gamma$ RIIb early during negative signaling and later binds p52 Shc.

Direct measurements of the intrinsic affinity of the SHIP SH2 domain revealed a 10-fold difference in the overall affinity between phospho-ITIM of Fc $\gamma$ RIIb and phospho-Shc. However, the lower affinity of the ITIM motif was due not to a reduced rate of association but rather to an accelerated rate of dissociation, relative to phospho-Shc. This intriguing observation indicates that phosphorylated Fc $\gamma$ RIIb rapidly associates with the SH2 domain of SHIP to promote its tyrosine phosphorylation and rapidly dissociates to permit its interaction with Shc.

Based on the observations reported here, we have formulated a model shown in Fig. 6. Early in negative signaling, the SH2 domain of SHIP is engaged to the phospho-ITIM motif of Fc $\gamma$ RIIb, necessary for SHIP tyrosine phosphorylation as well as for the subsequent interaction with Shc. Upon SHIP tyrosine phosphorylation, the Shc PTB engages either or both of the NPxPY motifs within SHIP. The interaction with the Shc PTB domain may raise the local concentration of the phosphorylated tyrosine residues within Shc, with its intrinsically higher affinity, thereby promoting the SH2 domain of SHIP to release Fc $\gamma$ RIIb and bind Shc. This new model accounts for several earlier and unusual observations regarding SHIP in B lymphocytes. First, the tyrosine phosphorylation of SHIP is much greater under conditions of negative signaling (16). This fact is likely due to the relatively weak affinity of the SH2 domain of SHIP for phospho-ITIM of Fc $\gamma$ RIIb, exclusively phosphorylated under negative signaling conditions due to coclustering with sIg-associated protein tyrosine kinases. Phospho-ITIM recruitment of SHIP as a prerequisite for its phosphorylation is consistent with our earlier genetic studies (15). Second, SHIP associates with Shc only under negative signaling conditions; i.e., there is minimal interaction between these two proteins under positive signaling conditions, despite the fact that Shc is highly phosphorylated and the SH2 domain of SHIP displays affinity for phospho-Shc (7, 8, 16). This observation is likely due to the relatively higher affinity of the Grb2 SH2 domain for phospho-Shc, as compared with that of the SHIP SH2 domain (7). Thus, Shc phosphorylation in the absence of SHIP phosphorylation favors Shc-Grb2 interaction rather than Shc-SHIP interaction. Third, Shc and Grb2



do not form a stable complex under negative signaling conditions, despite the relatively higher affinity of Grb2 for phospho-Shc (18). However, SHIP-Shc complexes are readily apparent under negative but not positive signaling (16, 18). These earlier findings in conjunction with the affinity and kinetic data reported here indicate that the formation of a stable SHIP-Shc interaction complex requires SHIP tyrosine phosphorylation and interaction through the PTB domain of Shc. The added contribution of the PTB domain of Shc binding to phospho-SHIP, along with the SH2 domain of SHIP engaging phospho-Shc, generates a stable, bidentate complex and one in which the SH2 domain of SHIP can successfully compete, despite its lower affinity, with that of Grb2 for binding to Shc (19) to block the Ras pathway. The system, with its hierarchy of affinities between the various interacting partners, dynamically modulated by phosphorylation of the Fc $\gamma$ RIIb ITIM, is thus optimally designed to inhibit B cell activation during negative but not positive signaling.

While these findings are useful in understanding the physiology of SHIP-mediated inhibition of lymphocyte activation, they do not address nor exclude a role for SHIP enzymatic activity. In this regard, recent studies have revealed an association between SHIP phosphorylation and cellular levels of the PtdIns-3 kinase product, PtdIns-3,4,5 trisphosphate (38, 39). These studies also reported a reduction in Bruton's tyrosine kinase, a PtdIns-3 kinase-dependent enzyme (40). However, both positive and negative signaling proceed through a variety of protein interactions that include SH2, SH3, and pleckstrin homology domains associating with transient components formed during the signaling process. Indeed, protein interactions involving SH2 domain-containing proteins engaging phosphorylated cytoplasmic tyrosines of the receptor are rate-limiting for many signaling pathways. Thus, understanding these interactions is important in dissecting signaling pathways and the ultimate changes in cell physiology.

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