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ARTICLES

# Activation-Induced Bi-dentate Interaction of SHIP and Shc in B Lymphocytes

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**Abstract** SHIP is a SH2 domain-containing inositol polyphosphatase that is selectively tyrosine phosphorylated and associated with the adapter protein Shc in B lymphocytes upon co-crosslinking surface immunoglobulin and FcγRIIB1. We previously observed that this stimulation condition is associated with a reduction in the interaction of Grb2 with phosphorylated Shc, an enhanced interaction of Shc with SHIP, and a block in the Ras signaling pathway. We proposed that the SH2 domain of SHIP competes with Grb2 in binding to phospho-Shc, resulting in a block in Ras signaling. To test this model, we examined the mode of SHIP–Shc interaction. Using recombinant Shc and SHIP interaction domains and purified Shc and SHIP phosphopeptides, we show that the interaction is bi-dentate such that the SH2 domain of SHIP recognizes phosphorylated Y317 and doubly-phosphorylated Y239/Y240 of Shc and the Shc PTB domain recognizes phosphorylated NPxpY motifs within SHIP. We observed no role for the Shc SH2 domain in the interaction. These findings are consistent with our earlier model that SHIP and Grb2 compete for binding to phospho-Shc and support the notion that, in addition to the hydrolysis of inositol phosphates and phospholipids, SHIP contributes to anti-proliferative biochemistry by blocking protein–protein interactions. J. Cell. Biochem. 67:32–42, 1997. 

**Key words:** SHIP; Ras signaling; Shc; bi-dentate interaction

## INTRODUCTION

B-cell activation is induced by antigen occupancy of surface immunoglobulin (sIg), the B-cell antigen receptor. F(ab′)2 fragments of antibodies directed against sIg can serve as a surrogate antigen, triggering identical biochemical and biological activation events in B-cells. Early events elicited by sIg include activation of Src and Syk protein tyrosine kinases, stimulation of phosphatidylinositol-3 kinase (PtdIns-3 kinase) and phospholipase Cγ(PLCγ) activity, and induction of the Ras pathway [reviewed in Gold and DeFranco, 1994]. Triggering of these biochemical events culminates in B-cell proliferation and antibody secretion.

Activation of Ras in B-lymphocytes is a complex process, initiated by tyrosine phosphorylation of sIg-associated Ig-α/β within their immunoreceptor tyrosine-based activation motif (ITAM). ITAM phosphorylation promotes ITAM association of the adapter protein Shc [Cambier and Johnson, 1995; D'Ambrosio et al., 1996] via the Shc SH2 domain. Association of Shc brings the molecule within contact of the available protein tyrosine kinases, thereby promoting Shc phosphorylation at tyrosine residues 239, 240 [van der Geer et al., 1996b], and 317 [Salcini et al., 1994; van der Geer et al., 1996b]. The Grb2-SOS complex of proteins then binds the doubly-phosphorylated Y239/Y240 and Y317 of Shc through the SH2 domain of Grb2, bringing the Ras-activating protein complex in contact with membrane-associated Ras.

While clustering sIg by antigen or F(ab′)2 fragments of anti-Ig induces B-cell activation, co-clustering sIg and the B-cell IgG receptor, FcγRIIB1, promotes a dominant-negative signal that blocks subsequent B-cell proliferation [Sarkar et al., 1996]. Co-crosslinking these receptors is thought to arise during a normal immune response following the generation of antigen-specific IgG and represents a feedback-suppression mechanism to reduce the continued synthesis of soluble IgG by negative signaling [Sinclair and Panoskaltsis, 1989]. The

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nature and mechanism of action of the dominant-negative signal is not understood.

Biochemical analysis of the negative signaling induced by sIg-FcγRIIB1 co-crosslinking indicated tyrosine phosphorylation of an immunoreceptor tyrosine-based inhibitory motif (ITIM) within FcγRIIB1 [Muta et al., 1994]. ITIM phosphorylation was shown to promote the association of the tyrosine phosphatase SHP-1 [D'Ambrosio et al., 1995] and the inositol polyphosphatase, SHIP [Chacko et al., 1996; Ono et al., 1996; Tridandapani et al., 1997c]. SHIP is a multifaceted protein that can contribute to negative signaling in a variety of ways. First, SHIP enzymatic activity hydrolyzes the 5-phosphate on the inositol ring of 3-phosphorylated phosphoinositide lipids [Damen et al., 1996; Kavanaugh et al., 1996; Lioubin et al., 1996]. Thus, SHIP consumes products of the PtdIns-3 kinase pathway and may halt or reverse events downstream of this enzyme by hydrolyzing lipid mediators of signal transduction. Second, SHIP hydrolyzes inositol 1,3,4,5-tetrakisphosphate [Damen et al., 1996; Kavanaugh et al., 1996; Lioubin et al., 1996], reported to open a plasma membrane calcium channel [Luckhoff and Clapham, 1992]. Thus, SHIP-catalyzed hydrolysis of inositol 1,3,4,5-tetrakisphosphate may block entry of extracellular calcium; indeed, inhibition of the entry of extracellular calcium is a phenomenon associated with negative signaling in B-cells [Choquet et al., 1993]. Third, SHIP contains an SH2 domain and, upon SHIP tyrosine phosphorylation, presents an optimal ligand, defined by ψNPxpY [van der Geer et al., 1996a; Zhou et al., 1995], for the Shc phosphotyrosine-binding (PTB) domain. Thus SHIP may compete with other proteins that are involved in proliferation through either or both of these interaction modules to prevent signal transduction.

We have earlier described conditions that selectively lead to tyrosine phosphorylation of SHIP. Specifically, co-crosslinking sIg and FcγRIIB1 with intact antimouse Ig (i.e., negative signaling conditions) led to SHIP tyrosine phosphorylation in B-cells, while crosslinking sIg alone with F(ab′)2 fragments of the same antimouse Ig (i.e., positive signaling conditions) did not [Chacko et al., 1996; Tridandapani et al., 1997a]. Additionally, we earlier demonstrated that stimulation of ex vivo B-cells with F(ab′)2 fragments of anti-mouse Ig promoted Grb2 association with Shc and the induction of the Ras pathway while stimulation with intact anti-mouse Ig blocked these events [Tridandapani et al., 1997a]. Based on these findings, we presented the hypothesis that SHIP and Grb2 compete for binding to phospho-Shc and that SHIP-Shc interaction prevents subsequent activation of the Ras pathway [Tridandapani et al., 1997a,b]. This hypothesis requires that the SHIP SH2 domain recognizes phosphorylated Shc, a notion in contrast to recent findings in T cells [Lamkin et al., 1997] that indicated no role for the SHIP SH2 domain in SHIP-Shc interaction but consistent with other findings in IL3-responsive lymphocytes [Liu et al., 1997]. Furthermore, our earlier data [Chacko et al., 1996; Tridandapani et al., 1997a,c] demonstrated that SHIP–Shc interaction was largely dependent on SHIP, and not Shc tyrosine phosphorylation, implicating an interaction involving phospho-SHIP and the Shc PTB domain and not phospho-Shc associating with the SHIP SH2 domain.

The B-cell paradigm may be more revealing in the analysis of SHIP and Shc interaction than the other systems mentioned above. First, high stoichiometry SHIP phosphorylation and SHIP–Shc interaction can be induced or not by slight variations in the nature of the stimulating reagents; i.e., intact versus F(ab′)2 fragments of anti-Ig [Chacko et al., 1996; Tridandapani et al., 1997a]. Second, the B-cell system is the only one which presents demonstrated and defined consequences regarding SHIP-Shc interaction; i.e., exclusion of Grb2 to phospho-Shc and inhibition of the Ras signaling pathway [Tridandapani et al., 1997a,b], although the mechanism leading to the inhibition is not clear. The role of SHIP in signaling via the IL3 or T-cell antigen receptor is unclear or has not been investigated.

Here, we have addressed SHIP-Shc interaction in the B-cell model using recombinant SH2 domains of SHIP and Shc, the recombinant PTB domain of Shc, and phosphopeptides corresponding to known phosphorylation sites within Shc and potential phosphorylation sites within SHIP. Our findings suggest that the interaction of SHIP and Shc involves both the Shc PTB domain association with both Y931 and Y1035 of p130 SHIP as well as the SH2 domain of SHIP recognizing Y239/Y240 and Y317 of phosphorylated Shc. We propose a bi-dentate interaction model in which SHIP is tyrosine phosphorylated by association with the ITIM of

FcγRIIB1 at Y931 and/or Y1035, thereby providing a docking site for the PTB domain of Shc. This initial interaction is followed by the SH2 domain of SHIP binding to Y317 and/or Y239/Y240 of Shc, which is promoted by ITIM dephosphorylation or by the proximity of the phosphorylated Shc tyrosine residues.

## MATERIALS AND METHODS

### Cells, Antibodies, and Reagents

Murine A20 cells were grown in supplemented RPMI under standard conditions [Sarkar et al., 1996; Tridandapani et al., 1997a]. F(ab′)2 fragments and whole molecule of rabbit antimouse IgG antibody were purchased from Cappel Research Products, NC; other immunoprecipitating and immunoblotting antibodies were from Upstate Biotechnology (UBI, Lake Placid, NY). Polyclonal antisera against murine SHIP was generated as described [Tridandapani et al., 1997c]. Glutathione-agarose beads were purchased from Sigma Chemical Co. (St. Louis, MO). Chemiluminescent substrates were from Kierkegaard and Perry (Gaitherburg, MD). Cell culture reagents were from Gibco-BRL (Bethesda, MD). Biotinylated phosphopeptides were purchased from Quality Controlled Biochemicals (Torrance, CA).

### Peptides

All peptides used were N-terminally biotinylated, followed by a 6-carbon spacer and C-terminally amidylated and contained a single or double phosphorylated tyrosine residue, designated pY. Shc pY317 is PSpYVNVQN; Shc pY239 is DHQpYYNDFPGKE; Shc pY240 DHQYpYNDFPGKE; and doubly-phosphorylated pY239/pY240 is DHQpYpYNDFPGKE. The SHIP NPxpY phosphopeptides are LNEMINPNpYIGM, containing tyrosine 931 of p130 SHIP; and EMFENPLpYGSV, containing tyrosine 1035 of p130 SHIP. Control phosphopeptides were EPQpYEEIPIYL, containing an optimal Src SH2-binding motif (pYEEI) and LSNPTpYMDMLPD (pYMDM), containing and optimal motif for the p85 subunit of PtdIns 3-kinase.

### Lysis, Precipitation, and Immunoblotting

Cell lysis, immunopreciption and immunoblotting were performed as described earlier [Tridandapani et al., 1997a]. Briefly, A20 cells were stimulated with 10 μg/ml F(ab′)2 fragment or whole molecule of rabbit antimouse IgG for 3 min. Stimulations were terminated by addition of TN1 lysis buffer (50 mM Tris pH 8.0; 10 mM EDTA; 10 mM $Na_4P_2O_7$; 10 mM NaF; 1% NP40; 125 mM NaCl; 10 mM vanadate, and 10 μg/ml each aprotinin and leupeptin). Postnuclear extracts were incubated overnight with the GST-fusion protein coupled to the glutathione–Sepharose beads in presence or absence of phosphorylated peptides. Sepharose beads were washed five times with 1 ml of lysis buffer, resuspended in SDS sample buffer and boiled at 95°C for 5 min. Eluted proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), transferred to the nitrocellulose filters, immunoblotted with antibody of interest, and developed by enhanced chemiluminescence. Whole cell lysates were prepared by lysing $2 \times 10^6$ cells in 30 μl lysis buffer.

### SHIP-Shc Co-immunoprecipitation Experiments

A20 B-cells were washed, stimulated and lysed as described above. Postnuclear extracts were incubated with anti-Shc antibodies in the presence or absence of either GST-fusion proteins or biotinylated phosphopeptide competitors. The competitor was removed by the addition of glutathione–Sepharose or neutravidin–Sepharose beads. Proteins bound to anti-Shc antibodies were then collected using protein A/G-Sepharose beads. The Sepharose beads were washed five times with lysis buffer, eluted with SDS sample buffer, resolved on SDS–PAGE, transferred to the nitrocellulose membrane and immunoblotted with anti-SHIP antibody. Anti-SHIP antibody [Tridandapani et al., 1997c] was passed over a GST–Sepharose column to deplete anti-GST activity. The nonbinding fraction of this sera was bound and eluted from a GST-SHIP affinity column.

### Generation of GST-SHIP SH2, GST-Shc PTB, and GST-Shc SH2

DNA encoding p130 SHIP was amplified by polymerase chain reaction (PCR) using primers corresponding to p130 SHIP amino acid residues 1–106 (SHIP SH2 domain). The PCR product was inserted into the EcoRI site of the pGEX vector (Pharmacia) and the material confirmed by sequencing. After transformation of *Escherichia coli* strain DH5α, the fusion protein was induced by incubation with 0.1 mM IPTG for 3 h at 37°C and the protein purified as

previously described [Tridandapani et al., 1997c]. A20 cDNA was amplified by PCR using primers corresponding to mouse Shc amino acid residues 381–480 (Shc SH2 domain) and to residues 46–236 (Shc PTB domain). The PCR products were inserted into the *Eco*RI site of pGEX, transformed into DH5α, as above. The proteins were induced by incubation with 0.1 mM and 1 mM IPTG, respectively, for 5 h at 37°C before purification. All fusion proteins displayed a single prominent band at the appropriate size in SDS–PAGE analysis (not shown).

## RESULTS

### SH2 of SHIP Binds Phosphorylated Shc

To assess the contribution of SHIP SH2 domain toward SHIP–Shc interaction, we used the GST–SHIP SH2 domain or control SH2 domain fusion proteins to probe lysates derived from A20 B-cells. For these experiments, lysates of $10 \times 10^6$ B-cells resting or activated under positive or negative signaling conditions were incubated with the fusion proteins at the final concentration of 1 μM. Results, shown in Figure 1, reveal that the recombinant SH2 domain of SHIP and Grb2, but not p85, precipitated p46 and p52 phospho-Shc derived from activated but not resting B-cell lysates. Furthermore, binding to phospho-Shc was apparent in lysates derived from B-cells stimulated under positive (+) and negative (−) signaling conditions. These findings indicate that the SH2 domain of SHIP and Grb2 are capable of recognizing phosphorylation motifs present within phospho-Shc and are consistent with our previous data demonstrating that Shc is equivalently phosphorylated under both positive and negative signaling conditions [Tridandapani et al., 1997a].

### SH2 Domains of SHIP and Grb2 Compete for the Same Binding Sites on Phospho-Shc

Y239, Y240, and Y317 have been reported to be the major phosphorylation sites on Shc. Of these residues, Grb2 was reported to bind pY317 and doubly-phosphorylated pY239/pY240 [van der Geer et al., 1996b]. Our competition model [Tridandapani et al., 1997b] proposes that SHIP competes with Grb2 for binding phospho-Shc. To examine whether SHIP and Grb2 bind Shc at the same sites, biotinylated peptides corresponding to Shc sequences surrounding pY317, pY239, pY240, and a doubly-phosphorylated peptide at pY239 and pY240 were co-incubated with recombinant SH2 domain of SHIP and with lysates of resting or activated B-cells. The bound material was then probed with anti-Shc antibodies. The results (Fig. 2) indicated that the interaction between SHIP SH2 fusion protein and phospho-Shc was completely blocked by inclusion of the Shc phosphopeptides surrounding pY317 and doubly-phosphorylated peptide pY239/pY240. Other Shc peptides phosphorylated at a single tyrosine residue pY239 or pY240 failed to inhibit the interaction between the SHIP SH2 domain and phospho-Shc, indicating that the SHIP SH2 domain does not stably interact with Shc at these phosphorylated residues. Likewise, control peptides corresponding to those optimal for p85 (pYMDM) or for Src SH2 domain (pYEEI) [Songyang et al., 1993] did not reduce the interaction of phospho-

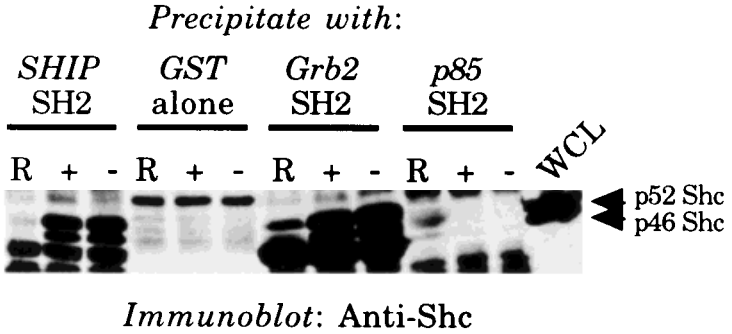


**Fig. 1.** SHIP SH2 domain binds Shc derived from B-cells activated under both positive and negative signaling conditions. $10 \times 10^6$ A20 B-cells were stimulated or not (R) under positive (+) or negative (−) signaling conditions with F(ab')2 or intact antimouse Ig, respectively. Cells were lysed and incubated with 1 μM (approximately 35 μg protein per ml) of the indicated GST-fusion proteins or GST alone. Bound material was collected by the addition of glutathione–Sepharose. Proteins were eluted with SDS sample buffer and analyzed by anti-Shc immunoblot. A whole cell lysate (WCL) was used as a positive control for the Shc immunoblot. These results are representative of four experiments.

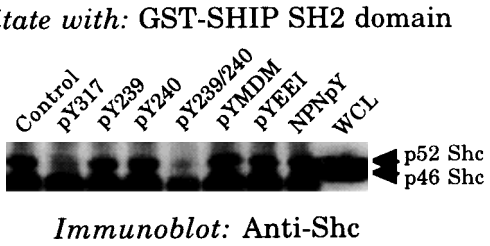


**Fig. 2.** SHIP SH2 domain binding to phospho-Shc is blocked by Shc phosphopeptides. Lysates of $10 \times 10^6$ A20 B-cells, stimulated with intact anti-Ig, were probed with 1 μM GST—SHIP SH2 domain as described in the legend of Figure 1 in the presence of 10 μM of the indicated Shc-derived phosphopeptides. Control lane represents no phosphopeptide addition; WCL represents a whole cell lysate. The results are representative of three identical experiments.

Shc with the SH2 domain of SHIP. These findings indicate that the SH2 domains of both Grb2 and SHIP compete for binding to phospho-Shc at the same phosphorylated residues, pY317 and doubly-phosphorylated pY239/pY240, data consistent with our previous hypothesis suggesting competition between SHIP and Grb2 for binding to Shc [Tridandapani et al., 1997a,b].

### Shc PTB Binds SHIP Involving Both NPNY and NPLY Motifs of Phospho-SHIP

The Shc PTB domain has been reported to recognize phosphorylated SHIP [Kavanaugh et al., 1996; Lamkin et al., 1997; Lioubin et al., 1996]. To confirm this interaction we probed resting or activated B-cell lysates with the recombinant Shc PTB domain as a GST-fusion protein and immunoblotted the associated proteins with anti-phosphotyrosine antibodies. The results (Fig. 3A) revealed higher stoichiometry binding of a 145-kDa tyrosine phosphorylated protein derived from lysates of B-cells stimulated under negative (−) but not resting (R) or positive (+) signaling conditions. The 145-kDa phosphoprotein co-migrated with immunoprecipitated SHIP (SHIP-IP) and did not bind to GST alone. To confirm the identity of the p145 phosphoprotein, the filter was re-probed with anti-GST-depleted, affinity-purified anti-SHIP antibodies. Results (Figure 3B) demonstrated the 145 kDa phosphoprotein was indeed p145 SHIP. These findings show that, as previously described [Kavanaugh et al., 1996; Lamkin et al., 1997; Lioubin et al., 1996], the Shc PTB domain recognizes phosphorylated SHIP. We have extended these previous findings to an analysis of the association of the Shc PTB domain with SHIP derived from B-cells stimulated under positive or negative signaling conditions. Results indicated that the stoichiometry of the interaction is greater under negative than positive signaling conditions, consistent with our earlier findings indicating higher levels of SHIP tyrosine phosphorylation and SHIP–Shc interaction under negative signaling conditions [Chacko et al., 1996; Tridandapani et al., 1997a]. These findings show that the association of phosphorylated SHIP with the Shc PTB domain occurs at elevated levels under negative signaling conditions.

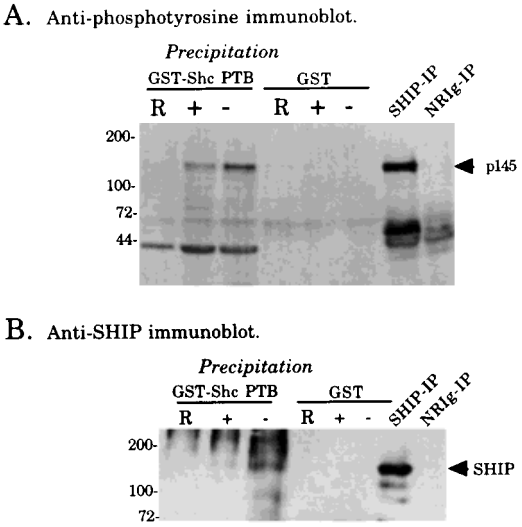


**Fig. 3.** The PTB domain of Shc binds to phosphorylated SHIP. $10 \times 10^6$ A20 B-cells were stimulated or not (R) under positive (+) or negative (−) signaling conditions before lysis. Lysates were incubated with 1 μM of GST alone or GST-Shc PTB domain, as indicated, and bound protein was collected with glutathione–Sepharose. The samples were separated by SDS–PAGE, transferred to nitrocellulose and immunoblotted with antiphosphotyrosine **(A)** or affinity-purified anti-SHIP antibodies **(B).** As controls, immunoprecipitates with anti-SHIP antibodies (SHIP-IP) or normal rabbit Ig (NRIg-IP) are shown on the right two lanes of both panels. This experiment is representative of three others.

While the phosphorylation sites on SHIP have not been identified, the three known forms of SHIP (p145, p130, and p110) each contain two putative binding sites for Shc PTB domain, indicated by an NPxY motif. To examine which NPxY motif(s) are recognized by the Shc PTB domain, we obtained phosphopeptides based on the sequence surrounding SHIP residues NPNpY, encoding tyrosine residue 931 of murine p130 SHIP and NPLpY, encoding tyrosine 1035 of murine p130 SHIP. As above, B-cell lysates were derived from B-cells resting or stimulated with intact anti-Ig and were incu-

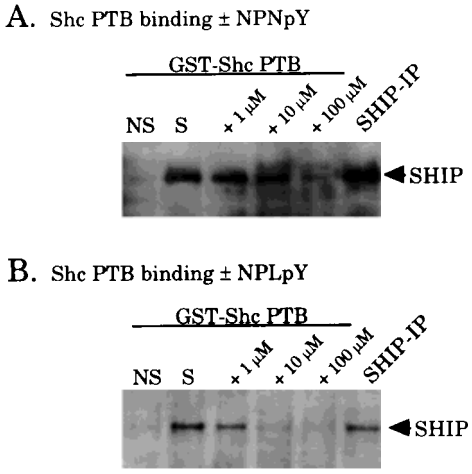


**Fig. 4.** SHIP binding by the Shc PTB domain is blocked by SHIP NPxpY phosphopeptides. $10 \times 10^6$ A20 B-cells were stimulated (S) or not (NS) under negative signaling conditions with intact antimouse Ig before lysis. Lysates were incubated with 1 μM of GST-Shc PTB domain in the presence or absence of the indicated amount of phosphopeptide corresponding to SHIP sequence NPNpY **(A)** or NPLpY **(B).** Bound protein was collected with glutathione–Sepharose. The samples were separated by SDS–PAGE, transferred to nitrocellulose and immunoblotted with affinity-purified anti-SHIP antibodies. These data are representative of four similar experiments.

bated with GST-Shc PTB domain in presence or absence of phosphopeptides containing either NPNpY or NPLpY sequence. The results (Fig. 4A,B) indicated that recognition of SHIP by the Shc PTB domain was significantly reduced by the presence of either NPNpY or NPLpY phosphopeptides. However, the NPLpY phosphopeptide that included tyrosine residue 1035 was slightly more efficient in this analysis as the interaction was effectively eliminated at a concentration of 10 μM phosphopeptide. The interaction was maintained, although reduced, when in the presence of the phosphopeptide containing tyrosine 931 at a concentration of 10 μM but completely eliminated at 100 μM. These findings indicate that the Shc PTB domain recognizes phosphorylated SHIP at either NPxY motif and exhibits a slightly greater affinity for phosphorylated SHIP at the more C-terminal motif containing NPLpY surrounding tyrosine 1035.

### SHIP–Shc Interaction Is Bi-dentate Involving Both SHIP SH2 and Shc PTB

The data described above indicate a bi-dentate interaction between SHIP and Shc. Thus, the SH2 domain of SHIP associates with pY317 and/or doubly-phosphorylated pY239/pY240 of phosphorylated Shc. Additionally, the PTB domain of Shc binds to either of the two NPxY motifs containing tyrosine 931 or 1035 of SHIP. To confirm a bi-dentate mode of interaction in vivo, we examined the presence of SHIP in Shc immunoprecipitates performed in the presence of either the recombinant Shc PTB domain, the recombinant SHIP SH2 domain, or both domains as competitors. The results, shown in Figure 5, revealed that SHIP associated with immunoprecipitated Shc isolated from B-cells stimulated under negative signaling conditions was reduced by about 50% when in the presence of the SHIP SH2 domain or the Shc PTB domain. Inclusion of both the recombinant domains almost completely blocked SHIP-Shc co-immunoprecipitation. These data support the notion that the interaction of SHIP and Shc, when isolated from B-cells undergoing negative signaling, is a bi-dentate one that employs both the Shc PTB domain and the SHIP SH2 domain. In other experiments not shown, we observed that the Shc SH2 domain as a GST-fusion protein was neither able to bind phosphorylated SHIP nor to disrupt SHIP-Shc interaction obtained from stimulated B-cells. However, GST-Shc SH2 was fully competent in binding a number of other tyrosine-phosphorylated proteins in anti-Ig-stimulated B-cells. Thus, there does not appear to be a role for the SH2 domain of Shc in its interaction with SHIP.

To further confirm the bi-dentate nature of the SHIP-Shc interaction in vivo, we assessed co-immunoprecipitation of SHIP in Shc immunoprecipitates when carried out in the presence or absence of the various phosphopeptides used above. Immunoprecipitation of Shc from B-cells stimulated under negative signaling conditions revealed the presence of SHIP, as expected (Fig. 6, lane 1). However, the same immunoprecipitation in the presence of phosphopeptides corresponding to Shc tyrosine residue 317 (lane 2) or doubly-phosphorylated residues 239/240 (lane 5) greatly reduced levels of SHIP, but neither phosphopeptide alone eliminated the co-immunoprecipitation of SHIP. Likewise, inclusion of the PTB recognition sequence corresponding to tyrosine 931 (lane 7) or 1035 (lane 6) reduced but did not eliminate the association of SHIP and Shc. Finally, inclusion of phosphopeptides recognizing both the SHIP SH2 domain and the Shc PTB domain nearly eliminated SHIP co-immunoprecipitation with Shc (lanes 9 and 12). Control phosphopeptide pYEEI had no effect on

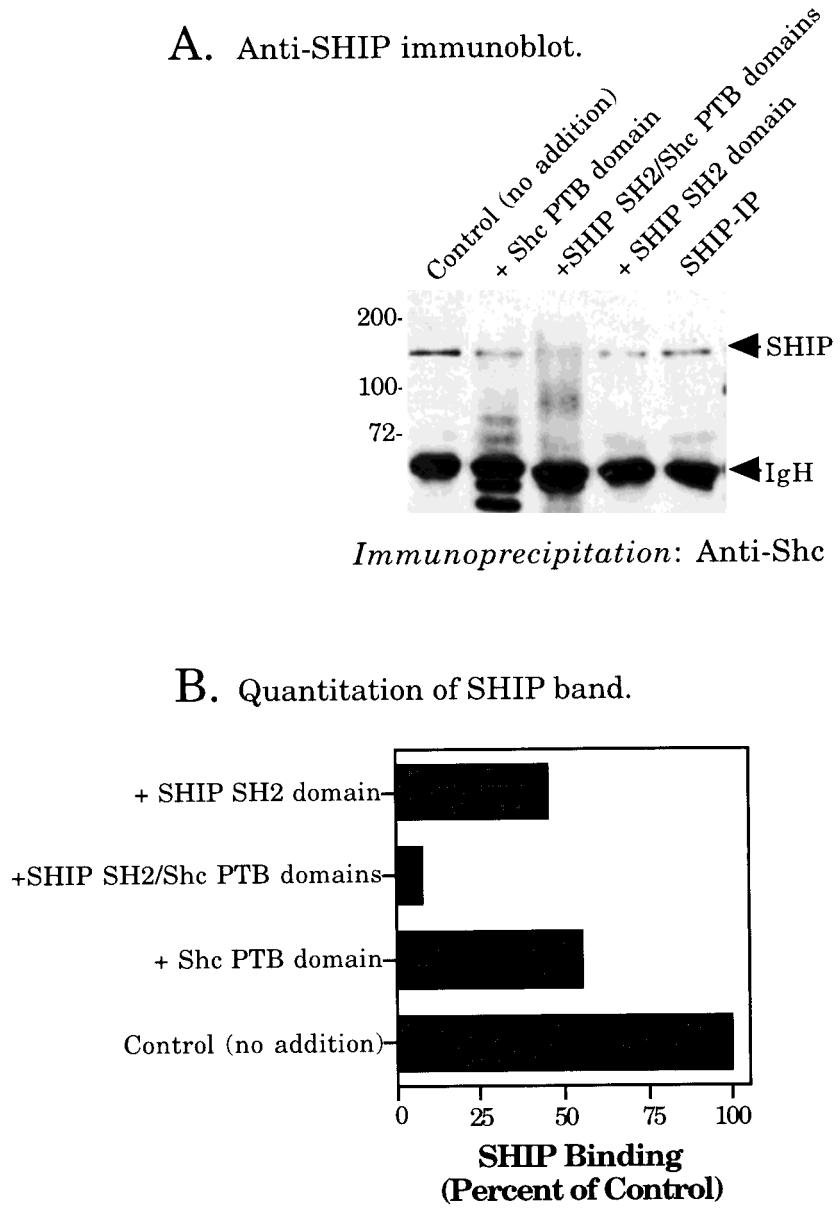


**Fig. 5.** SHIP–Shc co-immunoprecipitation is blocked by the presence of Shc PTB or SHIP SH2. $10 \times 10^6$ A20 B-cells were stimulated under negative signaling conditions with intact anti-mouse Ig before lysis. Lysates were precipitated with anti-Shc antibodies in the presence or absence of 2 μM of the indicated fusion proteins. As a positive control, an anti-SHIP immunoprecipitate (SHIP-IP) is shown in the lane on the far right. Immunoprecipitates were collected with protein A/G–Sepharose. The samples were separated by SDS/PAGE, transferred to nitrocellulose and immunoblotted with affinity-purified anti-SHIP antibodies. The indicated SHIP band was quantitated by laser densitometry; results of this analysis are shown in **B** as a percentage of the amount of SHIP contained in the control sample (no addition; lane 1 in **A**). These data are representative of two similar experiments.

co-immunoprecipitation of SHIP with Shc. These data provide further support for the hypothesis that SHIP–Shc interaction in B-cells undergoing negative signaling is bi-dentate.

## DISCUSSION

We previously hypothesized that phosphorylated SHIP prevails over Grb2 in binding to phosphorylated Shc during the B-cell negative signaling process, resulting in inhibition of the Ras pathway. To confirm this hypothesis, it is necessary to understand the precise mechanism by which SHIP and Shc interact. Two recent studies on this issue reported dissimilar findings: one found a definitive role for the SH2 domain of SHIP in SHIP–Shc interaction; the

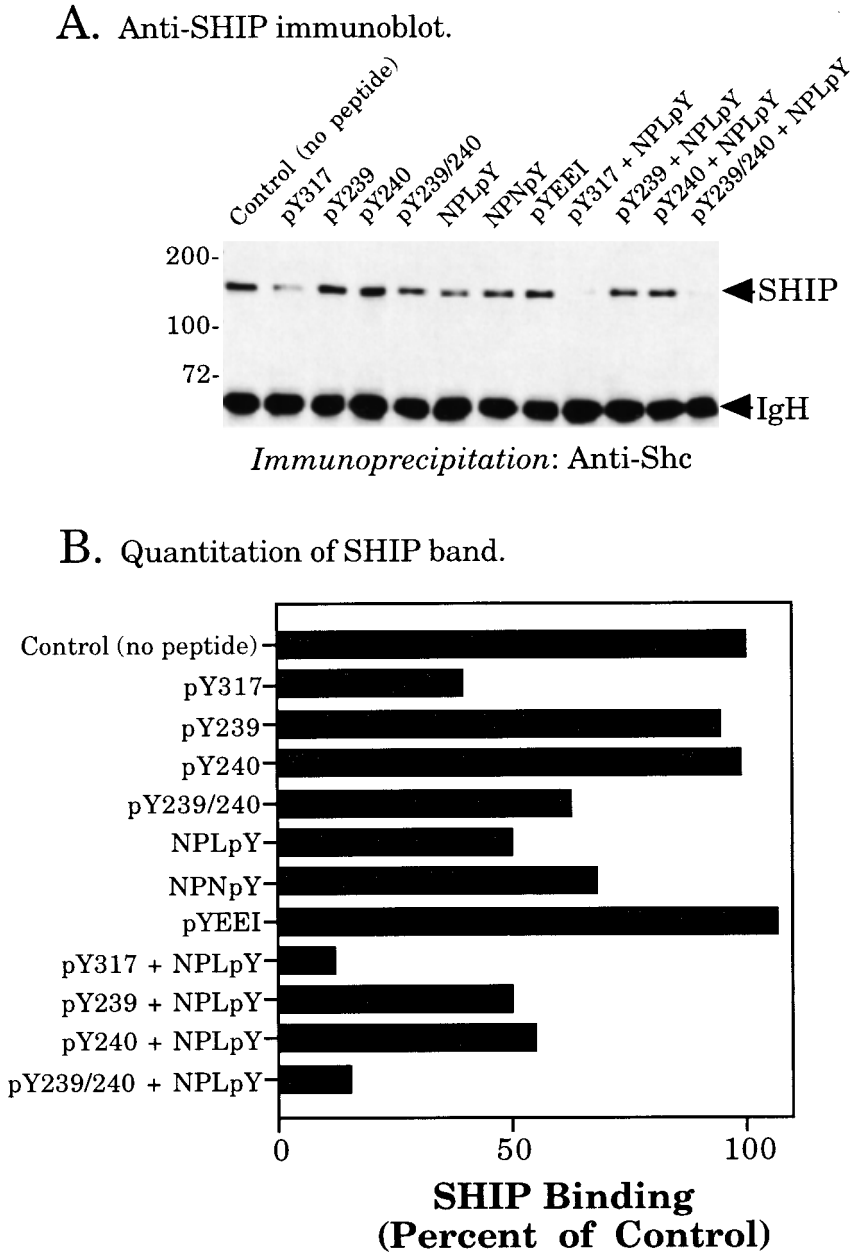


**Fig. 6.** SHIP-Shc interaction is blocked by the presence of peptides corresponding to SHIP or Shc phosphorylation sites. $10 \times 10^6$ A20 B-cells were stimulated under negative signaling conditions with intact antimouse Ig before lysis. Lysates were precipitated with anti-Shc antibodies in the presence or absence of 3 μM of the indicated phosphopeptides. Immunoprecipitates were collected with protein A/G–Sepharose. The samples were separated by SDS–PAGE, transferred to nitrocellulose and immunoblotted with affinity-purified anti-SHIP antibodies. The indicated SHIP band was quantitated by laser densitometry; results of this analysis are shown in **B** as a percentage of the amount of SHIP contained in the control sample (no peptide; lane 1 in **A**). These data are representative of two similar experiments.

other failed to identify a role for the SH2 domain of SHIP but revealed a role for the PTB domain of Shc in the interaction.

We have analyzed the mode of SHIP-Shc interaction in B-lymphocytes, an experimental paradigm that may be more revealing of SHIP function than other cellular models by virtue of the high stoichiometry of SHIP tyrosine phosphorylation and SHIP-Shc interaction, induced by defined and distinct activation conditions, and the recognized downstream effects (negative signaling) associated with SHIP tyrosine phosphorylation. Our findings described here indicated that both the Shc PTB domain and

the SHIP SH2 domain contributed to the interaction while we found no role for the SH2 domain of Shc. Furthermore, we showed that either PTB ligand (NPNpY or NPLpY) within SHIP interact with the PTB domain, although the more C-terminal NPLpY surrounding Y1035 of p130 SHIP appeared to have a slightly higher affinity. Lastly, we found that both pY317 phosphopeptide and doubly-phosphorylated pY239/pY240 phosphopeptide associate with high affinity to the SHIP SH2 domain. Other studies have shown that the SH2 domain of Grb2 is able to bind pY317 and doubly-phosphorylated pY239/pY240 of Shc [van der Geer et al., 1996b] and we reported that conditions leading to SHIP phosphorylation likewise preclude Grb2 binding to phosphorylated Shc [Tridandapani et al., 1997a,b]. Thus, these findings are consistent with our earlier model suggesting a competition between SHIP and Grb2 for binding to phosphorylated Shc and leading to a block in Ras induction during B-cell negative signaling [Tridandapani et al., 1997a,b].

The finding that the SH2 domain of SHIP recognizes phosphorylated Shc suggests that SHIP-Shc interaction would be detectable in any condition leading to Shc phosphorylation. However, SHIP binding to Shc does not occur under "positive" signaling conditions, despite the fact that positive signaling conditions promote Shc but not SHIP tyrosine phosphorylation [Tridandapani et al., 1997a]. Thus, SHIP tyrosine phosphorylation is the limiting feature in SHIP-Shc interaction. This notion suggests that the absolute affinity of the SHIP SH2 for phosphorylated Shc Y317 and/or Y239/Y240 is relatively low compared to that of Grb2. Indeed, recent surface plasmon resonance measurements [Liu et al., 1997] revealed a 10-fold lower affinity of the SH2 domain of SHIP for pY317; however, pY239, pY240 or doubly-phosphorylated pY239/pY240 was not investigated in this regard.

Together, these findings support a model in which SHIP and Shc first interact via the Shc PTB domain recognizing one or both of the NPxY sites of SHIP. This interaction brings the two molecules in close proximity, thereby raising the local concentration of phosphorylated Shc residues with respect to the SH2 domain of SHIP. The higher local concentrations of phosphorylated Shc may then favor binding by the SHIP SH2 domain rather than the Grb2 SH2 domain, preventing the formation of the Ras-activating complex of Shc-Grb2-SOS. This model is consistent with our recent findings that SHIP tyrosine phosphorylation is necessary for subsequent interaction with Shc [Tridandapani et al., 1997c].

Recent experiments examining a role for the SHIP SH2 in interacting with phosphorylated Shc [Lamkin et al., 1997] used a COS cell transfection model in which a PTB domain mutant of Shc was co-transfected with epitope-tagged SHIP and with an activated form of the tyrosine kinase Lck to promote phosphorylation. Although Shc was efficiently phosphorylated in this system, no interaction between SHIP and Shc was observed when using the Shc PTB domain mutant and led to the conclusion that the SH2 domain of SHIP does not contribute to the interaction. This experimental system is similar to our previously described "positive signaling" model, which permits efficient tyrosine phosphorylation of Shc but not SHIP [Tridandapani et al., 1997a]. Hence, in the absence of SHIP tyrosine phosphorylation as in B-cell positive signaling, or the use of a mutant Shc PTB domain as in the COS cell transfection experiments, there is no interaction of SHIP with the Shc PTB domain and no possibility for the SHIP SH2 domain to bind phosphorylated Shc, given its reported lower affinity compared to Grb2 [Liu et al., 1997].

The notion that SHIP-Shc interaction precludes Grb2-Shc interaction and blocks induction of the Ras pathway is consistent with our findings in B-cells [Tridandapani et al., 1997a,b] but inconsistent with data regarding IL3 signal transduction. Triggering of the IL3 receptor promotes Ras activation [Duronio et al., 1992; Satoh et al., 1991] as well as SHIP tyrosine phosphorylation and SHIP-Shc interaction [Cutler et al., 1993; Damen et al., 1996; Liu et al., 1994, 1997]. It may be that the stoichiometry of SHIP tyrosine phosphorylation and hence the level of SHIP-Shc interaction in IL3 signaling is not as high as in the B-cell paradigm. Thus, some free phosphorylated Shc induced by IL3 may be available for binding to Grb2–SOS complex to permit induction of the Ras pathway. Alternatively, these events may be intimately coordinated such that SHIP phosphorylation and SHIP-Shc interaction is quantitative in IL3 signaling, but appears later in the cascade. The SHIP-Shc interaction may then serve to terminate the Ras pathway initiated by IL3.

Lymphocyte antigen receptors appear to induce only proliferative signals, while separate and distinct receptors like FcγRIIB1 in B-cells, CTLA-4 in T-cells [Marengère et al., 1996; Tivol et al., 1995; Waterhouse et al., 1995] and the killer cell inhibitory receptors in NK cells [Binstadt et al., 1996] promote nonactivating, negative signals. In contrast, cytokine receptors appear to concomitantly promote both positive and negative signaling upon ligand binding. For example, the IL-4 receptor encodes four cytoplasmic tyrosine residues; induction of proliferation by a truncated version lacking the three C-terminal tyrosine residues is greatly enhanced over wild-type, implying that one or more of the three C-terminal tyrosine residues participate in a negative signaling pathway [Wang et al., 1996]. Similar findings have been made in the erythropoietin receptor [Klingmuller et al., 1995]. The nature of the signaling output, positive or negative, may ultimately depend on the SH2 domain specificity of the protein that recognizes the various intracellular tyrosine residues on the cytokine receptor. Both SH2 domain-containing proteins SHIP and SHP-1 are associated with negative, antiproliferative biochemistry while many other SH2 domain-containing proteins (e.g., Shc, Srcs, Syk, p85 subunit of PtdIns 3-kinase, PLCγ) are associated with positive, proliferative signaling biochemistry. The influence of these proteins or their enzymatic activity on the ultimate outcome of signaling may then depend on the nature of the protein and the kinetics of its receptor association.

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