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Recruitment and Phosphorylation of SH2-Containing Inositol Phosphatase and Shc to the B-Cell Fcγ Immunoreceptor Tyrosine-Based Inhibition Motif Peptide Motif

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Recently, we and others have demonstrated that negative signaling in B cells selectively induces the tyrosine phosphorylation of a novel inositol polyphosphate phosphatase, p145SHIP. In this study, we present data indicating that p145SHIP binds directly a phosphorylated motif, immunoreceptor tyrosine-based inhibition motif (ITIM), present in the cytoplasmic domain of FcγRIIB1. Using recombinant SH2 domains, we show that binding is mediated via the Src homology region 2 (SH2)-containing inositol phosphatase (SHIP) SH2 domain. SHIP also bound to a phosphopeptide derived from CD22, raising the possibility that SHIP contributes to negative signaling by this receptor as well as FcγRIIB1. The association of SHIP with the ITIM phosphopeptide was activation independent, while coassociation with Shc was activation dependent. Furthermore, experiments with FcγRIIB1-deficient B cells demonstrated a genetic requirement for expression of FcγRIIB1 in the induction of SHIP phosphorylation and its interaction with Shc. Based on these results, we propose a model of negative signaling in which co-cross-linking of surface immunoglobulin and FcγRIIB1 results in sequential tyrosine phosphorylation of the ITIM, recruitment and phosphorylation of p145SHIP, and subsequent binding of Shc.

Antigen-mediated clustering of the B-cell antigen receptor, surface immunoglobulin (sIg), induces a transient increase in membrane-localized tyrosine kinase activity that activates several biochemical pathways and culminates in the induction of proliferation and antibody secretion (14). One of the several substrates for these tyrosine kinases is an immunoreceptor tyrosine-based activation motif (ITAM) contained in the Ig-α and Ig-β subunits associated with sIg (4). The phosphorylated ITAM acts as a high-affinity ligand for signaling molecules containing Src homology region 2 (SH2) domains and is thus able to recruit a number of enzymes to the membrane that assemble through SH2-dependent associations (7, 24). Thus tyrosine phosphorylation provides a mechanism by which the intracellular regions of sIg, ordinarily devoid of endogenous catalytic activity, are able to transduce a signal generated by ligand binding.

B cells express a low-affinity Fc receptor for IgG (FcγRIIB1), an integral membrane protein that contains the ITAM-related immunoreceptor tyrosine-based inhibitory motif (ITIM) (2). Co-cross-linking of sIg and FcγRIIB1 abrogates the proliferative signal induced by stimulation of sIg alone in a process termed negative signaling (3, 9, 26, 27). While the mechanism(s) by which Fc receptors exert their inhibitory effect on sIg-mediated growth induction is unclear, experiments with mutated Fc receptors reveal a requirement for a functional ITIM (2, 6, 9, 20, 28). Both ITAM and ITIM contain a YxxI/L sequence but impart opposing signaling effects on B cells: phosphorylation of the ITAM is associated with B-cell activation while phosphorylation of the ITIM is associated with inhibition of B-cell activation. The molecular basis for the difference in signal transduction between ITAM and ITIM is not clear.

Results of previous studies indicate a role for the tyrosine phosphatases SHP-1 and SHP-2 in the negative signaling process (10, 23). Interestingly, co-cross-linking of sIg and FcγRIIB1 does not appear to significantly reduce the induction of tyrosine kinase activity or reduce the overall number of tyrosine phosphorylated proteins (30), suggesting that the action of tyrosine phosphatases is likely restricted to a few specific targets.

Recently, we (5) and others (22) have demonstrated that the SH2-containing inositol polyphosphate 5-phosphatase (SHIP) (12, 15, 17) is phosphorylated on tyrosine residues and is associated with the adapter protein Shc upon co-cross-linking of sIg and FcγRIIB1. Consequently, we postulated that the phosphorylation of SHIP on tyrosine residues and its association with Shc could contribute to negative signaling through effects on phosphatidylinositol and inositol phosphate metabolism. Other studies demonstrated coimmunoprecipitation of SHIP with the phosphorylated ITIM of FcγRIIB1 (22), although the mechanism of binding was not clear.

Here, we have further analyzed the role of SHIP in negative signaling. In confirmation of previous findings (22), we observed that SHIP specifically bound the ITIM phosphorylated on the tyrosine residue (pITIM) of FcγRIIB. We extend these previous data by demonstrating that the pITIM-SHIP interaction is direct and is mediated by the SHIP SH2 domain. SHIP associated with the pITIM derived from CD22 and corresponding to murine CD22 residues 809 to 818. Furthermore, while SHIP derived from resting B-cell lysates associated with pITIM of FcγRIIB1, Shc coassociation to pITIM required prior B-cell activation and was observed only under negative signaling conditions. These data suggest a model in which phosphorylation of the ITIM within FcγRIIB creates a binding

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site for the SH2 domain of SHIP, thereby recruiting it into an sIg complex where it is phosphorylated and subsequently associates with Shc. We tested this model with FcγRIIB1-deficient B-cell line IIA1.6. Results indicated a genetic requirement for expression of the FcγRIIB1 receptor, consistent with the recruitment model mentioned above.

MATERIALS AND METHODS

Antibodies, cells, and reagents. $F(ab')_2$ fragments and whole molecules of rabbit anti-mouse IgG antibody were obtained from Cappel Research Products, Durham, N.C.; other immunoprecipitating and immunoblotting antibodies were from Upstate Biotechnology, Inc. (Lake Placid, N.Y.). FcγRIIB1-deficient IIA1.6 cells and IIA1.6 cells stably transfected with the murine gene encoding FcγRIIB1 were a gift from I. Mellman (Yale University, New Haven, Conn.). Anti-SHIP antibody was generated by using a glutathione *S*-transferase (GST) fusion protein of SHIP residues 874 to 941, obtained by PCR amplification of SHIP cDNA. The purified protein was injected into rabbits and tested by immunoblotting and immunoprecipitation and compared with authentic anti-SHIP sera (obtained from G. Krystal, University of British Columbia). Protein G-agarose was purchased from Gibco/BRL, Gaithersburg, Md.; glutathione-agarose was purchased from Sigma; the enhanced chemiluminescence kit was purchased from Kirkegaard and Perry, Gaithersburg, Md. Phosphopeptides derived from CD22 (TVTpYSVIQKR and TVTFSVIQKR [murine CD22 residues 809 to 818]; SIHpYSELVQF and SIHFSELVQF [murine CD22 residues 834 to 844]) or from FcγRIIB1 (EAETITpYSLLKH [murine FcγRIIB1 residues 303 to 314]) were purchased from Quality Controlled Biochemicals, Hopkinton, Mass.; other phosphopeptides were purchased from Bachem Bioscience, Torrance, Calif. A20 murine B cells were obtained from the American Type Culture Collection.

Lysis, precipitation, and immunoblotting. Cell lysis, immunoprecipitation, and immunoblotting were performed as previously described (5). Briefly, B cells were stimulated with 10 μg of $F(ab')_2$ fragment or whole molecules of rabbit anti-mouse IgG per ml for the indicated times at 37°C and lysed with TN1 buffer (50 mM Tris [pH 8.0], 10 mM EDTA, 10 mM $Na_4P_2O_7$, 10 mM NaF, 1% Nonidet P-40, 125 mM NaCl, 10 mM Na_3VO_4, 10 μg of aprotinin and leupeptin per ml). Postnuclear extracts were incubated overnight with the antibody of interest or with the indicated amounts of biotinylated phosphopeptides. This incubation was followed by the addition of protein G-agarose or neutravidin-agarose (Pierce; Rockford, Ill.). Samples were washed five times with 1 ml of lysis buffer, resuspended in sodium dodecyl sulfate (SDS) sample buffer (60 mM Tris [pH 6.8], 2.3% SDS, 10% glycerol, 0.01% bromphenol blue) and boiled at 95°C for 5 min. Precipitated proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose filters, probed with the antibody of interest, and developed by enhanced chemiluminescence. In some cases, filters were stripped of primary antibody with 2% SDS–0.1 M 2-mercaptoethanol in 0.1 M Tris (pH 6.8), washed, and reprobed. Total cell lysates were prepared by lysing 2×10^6 cells in 50 μl of lysis buffer. SDS sample buffer was added to postnuclear extracts, and samples were boiled at 95°C for 5 min.

Far-Western blots. A total of 10^{-9} mol of GST-SHIP SH2 (34 μg), GST-Shc SH2 (65 μg), or GST (26 μg) was separated by SDS-PAGE. SHIP, Shc, or normal rabbit Ig (NRIg) immunoprecipitates from 15×10^6 A20 B cells were separated on the same gel. The proteins were transferred to nitrocellulose, and the filters were probed with 1 μM pITIM peptide overnight at 4°C, washed, and probed with 0.2 μg of horseradish peroxidase (HRP)-conjugated streptavidin (Pierce) per ml before development by chemiluminescence. Filters were stripped as described above and reprobed with affinity-purified rabbit polyclonal anti-GST antibodies followed by HRP–goat anti-rabbit Ig to ensure equal loading of GST fusion proteins. A duplicate gel containing a portion of the immunoprecipitates was simultaneously probed with the IgG fraction of anti-SHIP (rabbit polyclonal antisera; described above) to detect the presence of the target molecules. This filter was likewise blotted with HRP–goat anti-rabbit Ig and developed by chemiluminescence. Bands were quantitated by laser densitometry, and results are indicated in the text or in the figures.

Generation of GST-SHIP and GST-Shc SH2 domains. SHIP cDNA was amplified with primers corresponding to amino acid residues 1 to 105. The sequences of the primers were as follows: 5′-ATGCCTGCCATGGTCCCTGG-3′ and 5′-TACTCCTCCAGGGGCACGGG-3′, with *Eco*RI restriction sequences at the 5′ ends of both primers. The SH2 domain spans SHIP amino acid residues 5 to 105 (12). Shc SH2 was amplified from cDNA of Jurkat human T cells with the following primers: 5′-GGCGGGGATCCGGACAC-3′ and 5′-TCACAGTT TCCGCTCCACAG-3′. The PCR product includes Shc amino acid residues 154 to 473; the SH2 domain spans Shc amino acid residues 377 to 473 (25). The PCR products were inserted into pGEX-3X at the *Eco*RI site, and DH5α-transformed *Escherichia coli* cells were screened for the insert; positive clones were sequenced. The protein was induced with IPTG (isopropyl-β-D-thiogalactopyranoside), purified with glutathione-agarose (Sigma), and analyzed by SDS-PAGE and anti-GST immunoblotting. The purified GST-SHIP SH2 domain displayed a single major band at ~34 kDa, and the purified GST-Shc SH2 domain displayed a major band at ~65 kDa. These proteins were used in the experiments described below.

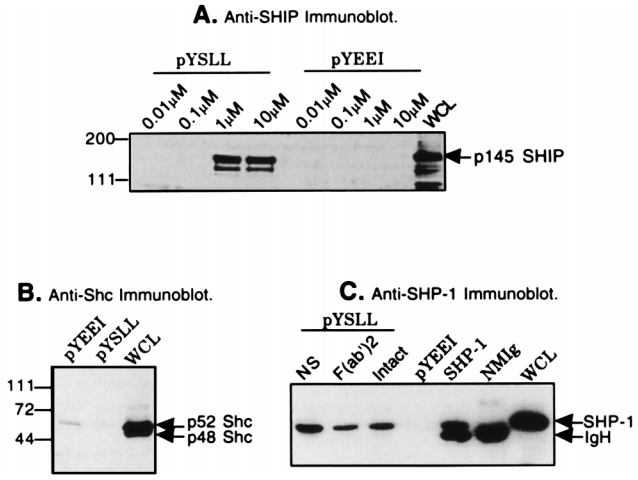

FIG. 1. SHIP binds the phosphorylated ITIM sequence. (A) A total of 10×10^6 A20 B cells were lysed and probed overnight at 4°C with the indicated concentration of pITIM (pYSLL) or pYEEI phosphopeptides. Peptide-bound protein precipitates were collected by incubation for an additional hour with neutravidin beads, washed in lysis buffer, and suspended in SDS sample buffer. Precipitated proteins were analyzed by SDS–10% PAGE and immunoblotted with anti-SHIP antibodies. Whole-cell lysate (WCL) from 2×10^6 A20 cells was used as a positive control. (B) A20 cell lysates were precipitated with pYEEI or pITIM phosphopeptides, as described above. The peptide-bound proteins were separated by SDS-PAGE and immunoblotted with anti-Shc antibodies. (C) A total of 10×10^6 A20 B cells were left untreated (resting) or stimulated for 3 min with 10 μg of intact or $F(ab')_2$ fragments of rabbit anti-mouse Ig antibodies per ml. The cells were lysed and precipitated overnight at 4°C with 1 μM pITIM or pYEEI peptides or with anti-SHP-1 or normal mouse sera (NMIg), as indicated. Precipitates were collected by incubation for an additional hour with neutravidin beads for phosphorylated peptides or with protein A and G beads for antisera, were washed in lysis buffer, and were suspended in SDS sample buffer. Precipitated proteins were analyzed by SDS–10% PAGE and immunoblotted with anti-SHP-1 antibodies. The presence of the Ig heavy chain from the immunoprecipitating sera is indicated (IgH).

RESULTS

Our previous findings (5) demonstrated that SHIP was maximally tyrosine phosphorylated upon sIg-FcγRIIB1 co-cross-linking (negative signaling conditions), induced by intact anti-Ig antibodies. Recent experiments (22) demonstrated that SHIP coprecipitates with B-cell FcγRIIB1 and binds a phosphopeptide corresponding to the pITIM sequence (pYSLL). In confirmation of these findings, we likewise observed that resting A20 B-cell lysates precipitated with biotinylated pITIM peptide displayed the presence of an ~145-kDa band immunoreactive with anti-SHIP antibodies (Fig. 1A). The association of SHIP with pITIM was maximal at 1 μM peptide and did not require B-cell activation, in contrast to FcγRIIB1 coprecipitation (22), indicating that phosphorylation of the ITIM of FcγRIIB1 and not SHIP is essential and rate limiting for SHIP-FcγRIIB1 association. SHIP did not bind a phosphopeptide resembling the C-terminal YxxL motif of Ig-α and Ig-β (pYEEI) at the same dose. However, pYEEI bound to Shc in lysates of resting B cells, showing that the peptide was competent in binding SH2 domains of endogenous proteins (Fig. 1B). SHP-1, previously shown to bind pITIM at a concentration of 1 μM (10), likewise displayed binding in our assay, and this binding did not require B-cell activation (Fig. 1C).

SH2 domains recognize tyrosine-phosphorylated peptides at the phosphotyrosine itself and amino acids at the +1 to +3 positions, C terminal to the phosphotyrosine residue (32, 33). Since SHIP contains an SH2 domain, it is likely that the observed interactions with phosphorylated ITIM peptide and with FcγRIIB1 were mediated by an SH2-phosphotyrosine in-

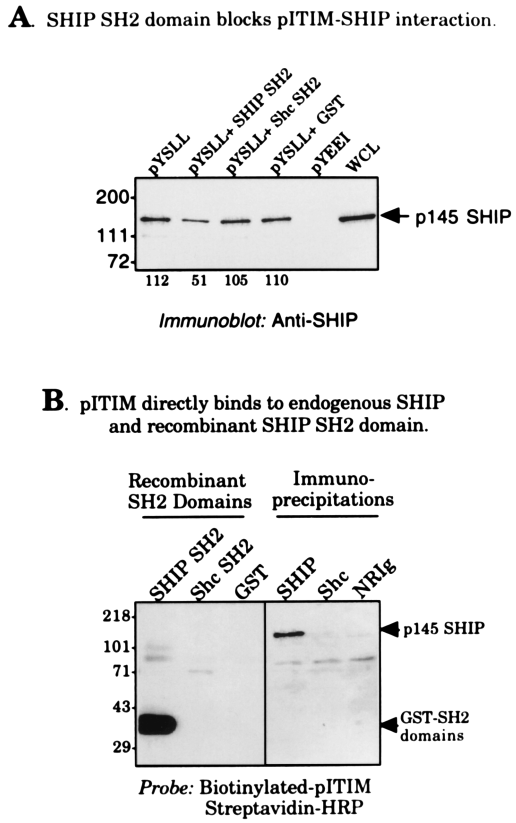

FIG. 2. SHIP binds pITIM via the SHIP SH2 domain. (A) A total of 10×10^6 A20 cells were lysed, and extracts were precipitated with 1 μM pITIM (pYSLL) alone or with 1 μM pYSLL in the presence of 10 μM GST-SHIP SH2, 10 μM GST-Shc SH2, or 10 μM GST. Precipitation with pYEEI or whole-cell lysates (WCL) constituted negative and positive controls, respectively. The peptide-bound material was collected with neutravidin beads, separated by SDS–10% PAGE, and immunoblotted with anti-SHIP antibodies. The SHIP band was scanned by laser densitometry; the integrated area corresponding to SHIP is shown immediately below the lanes. (B) A total of 1 nmol of GST-SHIP SH2, GST-Shc SH2, and GST alone was run on an SDS–10% PAGE gel along with anti-SHIP, anti-Shc, or NRIg immunoprecipitates derived from 15×10^6 A20 B cells. The separated proteins were transferred to nitrocellulose membranes and probed overnight with 1 μM biotinylated pITIM peptide, followed by streptavidin-HRP. This experiment is representative of three others.

teraction. To address this issue, a GST-SHIP SH2 domain fusion protein was generated by PCR amplification of SHIP cDNA and a GST-Shc SH2 domain fusion protein was generated by PCR amplification of Jurkat human T-cell cDNA. The ability of 1 μM pITIM peptide to bind SHIP in resting cell lysates in the presence or absence of 10 μM recombinant GST-SHIP SH2 domain, GST-Shc SH2 domain, or GST as the competitor was examined. Anti-SHIP immunoblots of pITIM peptide-bound material (Fig. 2A) demonstrated that the presence of the competing GST-SHIP SH2 domain but not the GST-Shc SH2 domain or GST alone reduced the association of endogenous SHIP. These findings suggest that pITIM peptide binding of SHIP is likely mediated via the SH2 domain of SHIP. This result, however, does not exclude the possibility that SHIP binds the pITIM peptide via an adapter protein. To address this question, we tested the ability of pITIM to directly bind endogenous SHIP and GST-SHIP SH2 fusion protein on a nitrocellulose membrane in a far-Western analysis (Fig. 2B). For these experiments, filters containing 1 nmol of the recombinant GST-SH2 domains of SHIP or Shc or immunoprecipitates of endogenous SHIP and Shc from 15×10^6 A20 B cells were probed with 1 μM biotinylated pITIM phosphopeptide followed by HRP-conjugated streptavidin to detect the biotinylated phosphopeptide. Results showed that the pITIM peptide directly bound a protein comigrating with endogenous SHIP but not with Shc (Fig. 2B, right three lanes). Likewise, pITIM phosphopeptide directly bound to the recombinant SH2 domain of SHIP but not to the SH2 domain of Shc nor to GST (Fig. 2B, right three lanes). Together, these data indicate that SHIP does not require an adapter protein to bind the phosphorylated ITIM of FcγRIIB1. In control experiments, duplicate filters were simultaneously probed with anti-SHIP, anti-Shc, and anti-GST to ensure the presence of the immunoprecipitated target molecule and an essentially equal loading of fusion protein in all lanes (data not shown).

The preferential binding of SHIP to pYSLL and not pYEEI indicates selective binding of the SHIP SH2 domain to distinct phosphopeptides. To begin to address this specificity, the association of SHIP to several sequence-related phosphopeptides derived from CD22 was examined. For these experiments, resting lysates of A20 B cells were precipitated with biotinylated peptides or phosphopeptides and the associated proteins were immunoblotted with anti-SHIP antibodies. Results (Fig. 3) indicated SHIP association to FcγRIIB1-derived pYSLL (Fig. 3, lane pYSLL) but not pYEEI (Fig. 3, lane pYEEI), as shown above. Furthermore, SHIP associated with CD22-derived phosphopeptide pYSVI, albeit with a lower affinity (Fig. 3, lane pYSVI) but did not associate with a related phosphopeptide, pYSEL (Fig. 3, lane pYSEL). SHIP did not bind to any unphosphorylated peptide. These findings extend SHIP SH2 domain interactions to peptides derived from CD22, a B-cell receptor functionally related to FcγRIIB1 in the promotion of negative signals (31) and raise the possibility that SHIP binding is causally related to the function of both receptors.

Previous experiments revealed the association of Shc with SHIP upon the activation of cells via cytokines (11, 18) or antireceptor antibodies (5, 8). To investigate conditions of Shc association to SHIP, lysates of resting or intact anti-Ig-activated A20 B cells were precipitated with biotinylated, phosphorylated ITIM peptides and the bound material was immunoblotted with either antiphosphotyrosine (Fig. 4, top panel), anti-SHIP (Fig. 4, middle panel), or anti-Shc antibodies (Fig. 4, bottom panel). Tyrosine-phosphorylated SHIP was detected within 30 s of receptor triggering. However, SHIP phosphorylation was not essential for pITIM association since SHIP was observed in pITIM precipitates from resting cells under conditions in which SHIP was not tyrosine phosphorylated. In contrast, Shc coprecipitated with pITIM peptides only in activated lysates, i.e., under conditions that lead to SHIP tyrosine

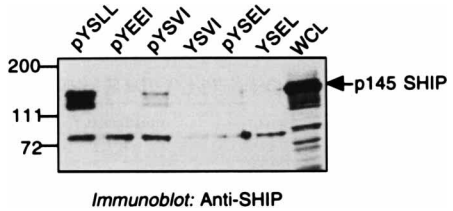

FIG. 3. Differential binding of SHIP to phosphopeptides. A total of 10×10^6 A20 cells were lysed and precipitated with various phosphorylated and nonphosphorylated, biotinylated peptides as indicated in the figure. Peptide-bound proteins were collected with neutravidin beads, separated by SDS–10% PAGE and immunoblotted with anti-SHIP antibodies. A20 whole-cell lysate from 2×10^6 cells (WCL) was used as a positive control for immunoblotting. This experiment is representative of three others.

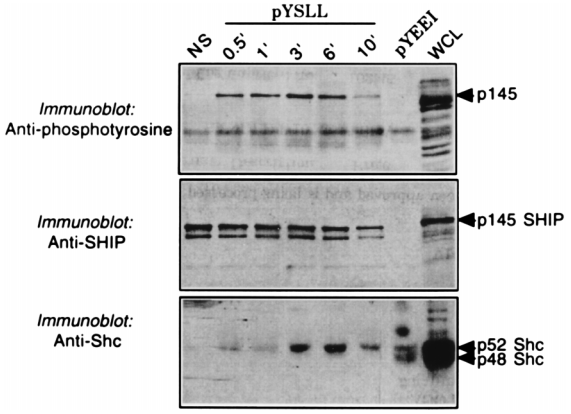

FIG. 4. Association of SHIP and Shc to phosphorylated ITIM. A total of 10×10^6 A20 cells were left unstimulated (NS) or were stimulated with 10 μg of intact rabbit anti-mouse Ig antibodies per ml for different time periods (in minutes) as indicated in the figure. The cells were lysed, and lysates were precipitated with 1 μM pYSLL or pYEEI peptides. The peptide-bound material was collected with neutravidin beads, separated by SDS–10% PAGE and immunoblotted with anti-phosphotyrosine antibodies (top panel). The filter was stripped and reprobed with anti-SHIP antibodies (middle panel) followed by anti-Shc antibodies (bottom panel). These results are representative of five other similar experiments.

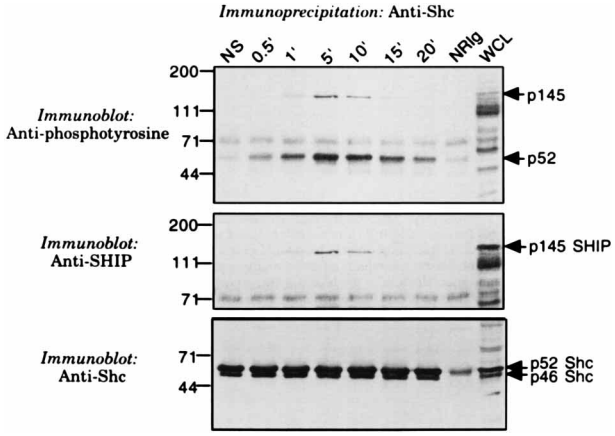

FIG. 6. Kinetics of SHIP-Shc association in activated B cells. A total of 10×10^6 A20 B cells were left unstimulated (NS) or were stimulated for the times indicated (in minutes) in the figure, lysed, and subjected to immunoprecipitation with anti-Shc antibodies overnight at 4°C. The immunoprecipitated proteins were separated by SDS–10% PAGE, transferred to nitrocellulose membranes, and probed with antiphosphotyrosine antibodies (top panel). The membrane was stripped and reprobed with anti-SHIP antibodies (middle panel), and anti-Shc antibodies (bottom panel). Similar results were observed in two separate experiments.

phosphorylation. The extent of Shc association to SHIP appeared to directly correlate with the extent of SHIP tyrosine phosphorylation, such that the SHIP-Shc interaction is highest when SHIP tyrosine phosphorylation is maximal. These findings, together with data in Fig. 1 and 2, demonstrate that unphosphorylated SHIP binds the pITIM phosphopeptide via its SH2 domain. The data suggest that, in contrast to SHIP, Shc binds pITIM indirectly through its association with phosphorylated but not unphosphorylated SHIP.

To test this model, the kinetics of SHIP and Shc tyrosine phosphorylation and SHIP-Shc association were examined. Tyrosine phosphorylation of SHIP was determined by immunoprecipitating SHIP from resting and activated A20 B-cell lysates and immunoblotting the samples with antiphosphotyrosine antibodies. Results revealed an inducible phosphorylation of SHIP at 30 s which peaked at around 5 min and declined by 15 to 20 min (Fig. 5, top panel). To ensure equal amounts of SHIP, the filter was reprobed with anti-SHIP antibodies (Fig. 5, bottom panel). The presence of the Ig heavy chain from the anti-SHIP antisera present on this filter (approximately 15 to 30 μg of Ig) precluded an analysis of Shc coprecipitation. Nevertheless, these findings indicate that SHIP is rapidly tyrosine phosphorylated and therefore capable of interacting with Shc from 30 s to approximately 15 min after stimulation.

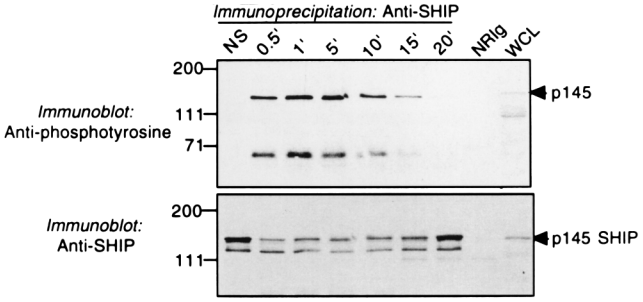

FIG. 5. Kinetics of tyrosine phosphorylation of p145 SHIP upon B-cell activation. A total of 10×10^6 A20 B cells were left unstimulated (NS) or stimulated with 10 μg of intact rabbit anti-mouse Ig antibodies per ml for the indicated time periods (in minutes). Lysates were immunoprecipitated with anti-SHIP antibodies overnight at 4°C. The immunoprecipitated proteins were collected with protein A and G beads, separated by SDS–10% PAGE, transferred to nitrocellulose membranes, and immunoblotted with antiphosphotyrosine antibodies (top panel). The membrane was subsequently stripped and reprobed with anti-SHIP antibodies (bottom panel). A20 whole-cell lysate (WCL) was run as a positive control for immunoblotting. Similar results were observed in two separate experiments.

Tyrosine phosphorylation of Shc was determined by probing anti-Shc immunoprecipitates (1 μg of purified IgG from rabbit polyclonal anti-Shc) with antiphosphotyrosine antibodies (Fig. 6, top panel). Results indicated that phosphorylation of Shc, like that of SHIP, occurs rapidly (detectable within 30 s), peaks at 5 min, and begins to decline by 15 min. A 145-kDa tyrosine-phosphorylated protein that coprecipitated with Shc appeared at 1 min, was maximal at 5 min, and began to decline by 15 min. Reprobing the same membrane with anti-SHIP antibodies revealed that the 145-kDa protein coprecipitating with Shc is indeed SHIP (Fig. 6, middle panel). The membrane was immunoblotted again with anti-Shc antibodies to verify immunoprecipitation of equal amounts of Shc in all lanes (Fig. 6, bottom panel). Some of the signal at ~55 kDa comigrating with p52 and p46 Shc is contributed by the heavy chain of the immunoprecipitating IgG, as seen in samples precipitated with 1 μg of purified NRIg (lane NRIg); however, the contribution by the Ig heavy chain is clearly less than that of p52 and p46 Shc. These results provide evidence of a phosphotyrosine-mediated interaction between SHIP and Shc in B cells that is initiated upon and requires SHIP tyrosine phosphorylation.

The ability of unphosphorylated SHIP to bind the phosphorylated ITIM peptide via the SHIP SH2 domain raises the possibility that FcγRIIB1 plays an adapter role in recruitment and phosphorylation of SHIP. To investigate the role of FcγRIIB1 in SHIP tyrosine phosphorylation, we used the murine B-cell lymphoma A20, IIA1.6 (an FcγRIIB1-deficient derivative of A20), and IIA1.6 transfected with wild-type FcγRIIB1 (IIA1.6WT) (1). We first examined the efficacy of sIg stimulation in these cells by assessing induction of tyrosine-phosphorylated proteins following sIg triggering with intact anti-Ig. For

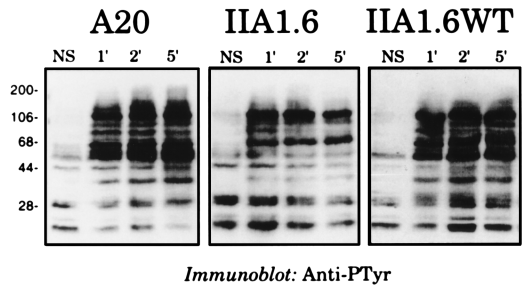

FIG. 7. sIg induction of tyrosine-phosphorylated proteins in FcγRIIB1-expressing or -deficient B cells. A total of 2×10^6 cells per sample were left unstimulated (NS) or were stimulated for the indicated times (in minutes) with 10 μg of intact rabbit anti-mouse Ig per ml before lysis. Whole-cell lysates were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antiphosphotyrosine (Anti-PTyr) antibodies. Similar results were observed in multiple experiments.

these experiments, whole-cell lysates of resting or anti-Ig-stimulated cells were immunoblotted with antiphosphotyrosine. The results, shown in Fig. 7, revealed that all three cell lines respond positively to anti-Ig by this assay with similar kinetics. These observations indicate that each cell line expresses protein tyrosine kinases (PTKs) that are functionally linked to sIg and that are competent to respond to sIg triggering by intact anti-Ig in this respect, regardless of the expression of FcγRIIB1.

To test whether FcγRIIB1 expression is required for induction of SHIP phosphorylation, we examined SHIP phosphorylation in these three A20 derivatives. The cells were stimulated with intact anti-Ig to promote sIg-FcγRIIB1 co-cross-linking, as described above, and the tyrosine phosphorylation of SHIP was measured by antiphosphotyrosine immunoblotting of SHIP immunoprecipitates. The results (Fig. 8A) demonstrated potent SHIP tyrosine phosphorylation in FcγRIIB1-expressing A20 cells (~10-fold above the unstimulated level) and IIA1.6WT cells (~5-fold above the unstimulated level) but not in FcγRIIB1-deficient IIA1.6 cells (<1.5-fold above the unstimulated level). Nevertheless, all three cell lines expressed essentially equivalent levels of the SHIP protein (Fig. 8B).

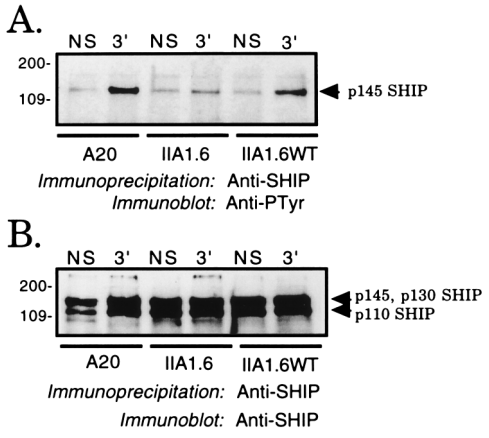

FIG. 8. sIg-induced SHIP tyrosine phosphorylation in FcγRIIB1-expressing or -deficient B cells. Lysates of 10×10^6 cells, resting (NS) or stimulated with 10 μg of intact rabbit anti-mouse Ig per ml for the time (in minutes) indicated were immunoprecipitated with anti-SHIP antisera. Immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose, and probed with anti-phosphotyrosine antibodies (A). The filters were stripped and reprobed with anti-SHIP antibodies (B). This result is representative of three experiments.

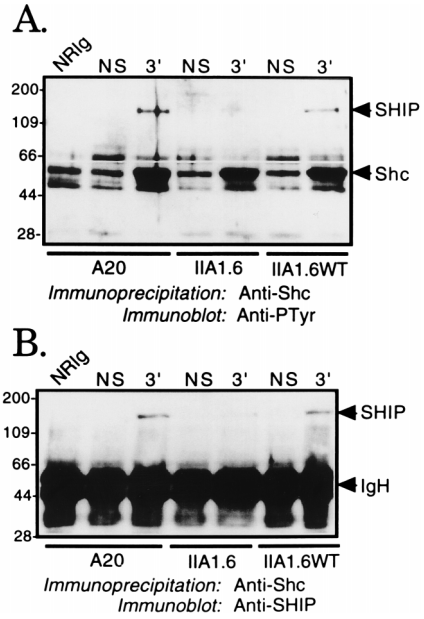

FIG. 9. SHIP phosphorylation and association with Shc in FcγRIIB1-expressing or -deficient B cells. Lysates of 10×10^6 B cells, resting (NS) or stimulated with 10 μg of intact rabbit anti-mouse Ig per ml, were immunoprecipitated with anti-Shc antibodies. Immunoprecipitates were separated by SDS-PAGE and immunoblotted with anti-phosphotyrosine antibody (A). The filters were stripped and reprobed with anti-SHIP antibodies (B). The result is representative of three experiments.

These findings demonstrate that SHIP is minimally phosphorylated in the absence of FcγRIIB1 expression and support the hypothesis that SHIP association to the phosphorylated ITIM of FcγRIIB1 is required for SHIP phosphorylation.

The studies described above suggested that the SHIP-Shc interaction is dependent on phosphorylation of SHIP and therefore should be reduced or absent in the FcγRIIB1-deficient IIA1.6 B-cell line that does not display SHIP tyrosine phosphorylation. To more rigorously test this hypothesis, cell lysates of the three B-cell lines were immunoprecipitated with anti-Shc antibodies after 3 min of stimulation, followed by antiphosphotyrosine immunoblotting to detect the association and extent of phosphorylated proteins. The results (Fig. 9A) revealed a 145-kDa tyrosine-phosphorylated protein coimmunoprecipitating with Shc in FcγRIIB1-expressing cell lines A20 and IIA1.6WT; this band was greatly reduced with the FcγRIIB1-deficient derivative IIA1.6. Reprobing the same filter with anti-SHIP antibodies identified the 145-kDa tyrosine-phosphorylated band as SHIP, present in Shc immunoprecipitates from FcγRIIB1-expressing A20 and IIA1.6WT cells and reduced in FcγRIIB1-deficient IIA1.6 cells. Thus, like SHIP tyrosine phosphorylation, the interaction of SHIP with Shc is greatest in cells expressing FcγRIIB1.

The kinetics and stability of Shc phosphorylation and SHIP-Shc association in A20 and IIA1.6 cells were investigated. The results (Fig. 10) reveal phosphorylation of a 52-kDa band that comigrates with Shc and that was maximally phosphorylated after approximately 3 min of stimulation. In A20 but not in IIA1.6 cells, a 145-kDa tyrosine-phosphorylated band that comigrated with SHIP was observed in Shc immunoprecipitates. These findings indicate that, like SHIP phosphorylation, the interaction of SHIP with Shc is enhanced by expression of FcγRIIB1. However, Shc phosphorylation did not display a

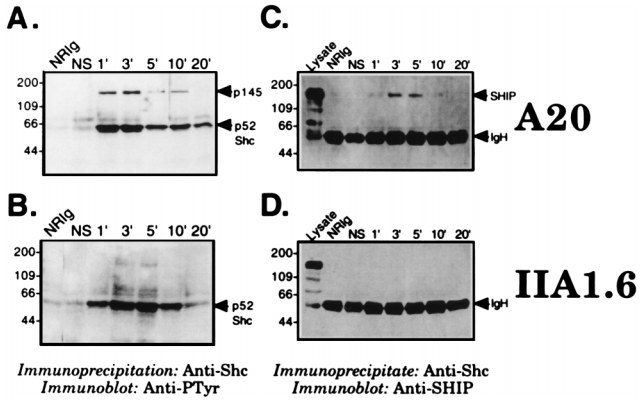

FIG. 10. Kinetics of SHIP phosphorylation and association with Shc in FcγRIIB1-expressing or -deficient B cells. Lysates of 10×10^6 B cells, resting (NS) or stimulated with 10 μg of intact rabbit anti-mouse Ig per ml, were immunoprecipitated with anti-Shc antibodies. Immunoprecipitates were separated by SDS-PAGE and immunoblotted with anti-phosphotyrosine antibodies (A and B). The filters were stripped and reprobed with anti-SHIP antibodies (C and D). The result is representative of three experiments.

similar requirement, and Shc was equivalently phosphorylated in both FcγRIIB1-expressing and -deficient B-cell lines.

DISCUSSION

An earlier report from our laboratory (5) showed that SHIP was maximally tyrosine phosphorylated under negative signaling conditions of sIg-FcγRIIB1 co-cross-linking. Other experiments established that these same conditions lead to ITIM tyrosine phosphorylation within FcγRIIB1 (20). Here we show, in confirmation of earlier findings (22), that SHIP binds phosphorylated ITIM. We have extended these data to show that association of SHIP to the phosphorylated ITIM peptide is direct, that binding is mediated by the SH2 domain of SHIP, that binding to phosphorylated ITIM is activation independent, and that SHIP does not bind a structurally related (4) pYEEI motif. Furthermore, we identified a genetic requirement for FcγRIIB1 expression in the induction of SHIP phosphorylation and SHIP-Shc interaction, while Shc phosphorylation did not display such a requirement.

Based on these and earlier findings (5, 34, 35), we propose a model in which the phosphorylation of tyrosine 309 within the ITIM of FcγRIIB1 promotes the binding of unphosphorylated SHIP via its SH2 domain. SHIP binding to phosphorylated ITIM brings the protein into contact with the Src family and Syk PTKs, and one or more of these kinases phosphorylate SHIP. Phosphorylated SHIP then binds Shc, an interaction that appears to involve minimally the binding of the Shc phosphotyrosine binding domain (PTB) to a SHIP NPxY motif(s), as suggested earlier (15, 17). Other interaction modules of the two proteins, such as the SH2 domains, may also be involved in the interaction. The model implies that phosphorylated ITIM is essential for the induction of SHIP tyrosine phosphorylation in B cells and that SHIP tyrosine phosphorylation is required for Shc association. The model thus accounts for our earlier findings that SHIP is maximally tyrosine phosphorylated and associated with Shc under conditions of negative but not positive signaling; that is, conditions which lead to tyrosine phosphorylation of ITIM within FcγRIIB1.

Although we observed SHIP tyrosine phosphorylation under negative growth conditions in B cells, others have observed SHIP tyrosine phosphorylation under positive growth conditions in cytokine-stimulated cells (18). An ITIM has not been reported in either of these cell activation models. SHIP may be recruited to receptor PTKs in order to be phosphorylated, as we have proposed here, under negative signaling conditions with SHIP adapting to the phosphorylated ITIM of FcγR. The human proto-oncogene c-*kit* (38) encodes a YSNL sequence at residues 936 to 939 which resembles the YSLL within the ITIM and the YSVI within CD22 and may be sufficient for SHIP SH2 domain recognition. Likewise, the CD3-ε (YSGL), -γ (YSHL), and -δ (YSRL) chains contain a YSxL sequence as part of their ITAMs, in contrast to the ITAMs of Ig-α and Ig-β, which contain YEGL or YEDI (24). TCR-ζ contains a single YSEI motif out of its six ITAM YxxI/L SH2 domain binding sites (13). If we consider only the residues from the +1 to +3 position following the phosphotyrosine residue, as described earlier (32), the SH2 domain of SHIP appears to prefer the presence of the neutral serine residue in the +1 position of the ITIM (pY**S**LL) and/or the absence of the acidic residues aspartic and/or glutamic acid, which are present in the +1 or +2 position of the tyrosine within an ITAM of B-cell Ig-α or Ig-β (pY**E**D/GI/L) or of the CD22 YS**E**L. The residues in the +1 to +3 positions may represent a critical feature(s) distinguishing an ITIM from an ITAM by permitting association of inhibitory proteins such as SHP-1 and SHIP and by excluding activating proteins such as Shc. The binding of such inhibitory phosphatases as SHP-1 and SHIP may account for the blockade in cell signaling observed under negative signaling conditions and likely contributes to the termination of signaling biochemistry in cytokine receptors like the c-*kit* product.

The PTK that phosphorylates SHIP is not known. Based on the model proposed here, SHIP phosphorylation could potentially be mediated by any of the PTKs present in the activated sIg complex, which includes Src family PTKs Lyn, Fyn, and Blk and p72Syk (4, 14). Studies examining PTKs that phosphorylate Shc using Lyn- or Syk-deficient B cells (21) identified a tyrosine-phosphorylated ~140-kDa protein that coprecipitated with Shc; the 140-kDa protein may be SHIP. Other experiments using the PTK-deficient B-cell lines demonstrated phosphorylation of pp140 in Syk-deficient but not Lyn-deficient B cells, while Shc was not phosphorylated in either cell line. If pp140 represents SHIP, the results would suggest that Lyn (or Src family PTK members) is of greater importance in SHIP phosphorylation than is Syk. On the other hand, the results may indicate that Lyn more efficiently phosphorylates the ITIM within FcγRIIB1 than does Syk, thereby promoting SHIP phosphorylation by either kinase through the binding of SHIP to the phosphorylated ITIM.

The mechanism by which SHIP associates with Shc is of considerable interest, given the role of the Shc-Grb2-SOS complex in Ras activation. Recent experiments from our laboratory (27a) and those of others (18, 19) indicate a bidentate interaction in which the Shc PTB domain binds phosphorylated NPxY motifs of SHIP; concomitantly, the SHIP SH2 domain binds phosphorylated Shc at tyrosine 317 and doubly phosphorylated tyrosines 239 and 240. The latter tyrosine residues are precisely those which interact with the Grb2 SH2 domain (29, 37); therefore, this suggests that SHIP and Grb2 compete for binding to limited amounts of phosphorylated Shc. Such competition can potentially influence the activation of Ras. However, studies of SHIP-Shc interaction in a transfected COS cell model (16) indicated no role for the SH2 domain of SHIP in its interaction with Shc. If so, SHIP and Grb2 would not compete for binding of phospho-Shc.

In support of the notion that SHIP and Grb2 compete for binding to phospho-Shc, we have previously demonstrated (34, 35) inhibition of the Ras signaling pathway and defective Shc-Grb2 association in B cells under conditions promoting SHIP-

Shc interaction. Based on these findings, we hypothesized (35) that the SHIP-Shc interaction involves the SH2 domain of SHIP and prevents formation of the Ras-activating complex of Shc-Grb2-SOS by SH2 domain competition between Grb2 and SHIP. However, in view of the studies of COS cells mentioned above, the precise mechanism of SHIP-Shc association is at present unclear and potentially complex: both SHIP and Shc contain an SH2 domain, and both proteins become tyrosine phosphorylated. Additionally, Shc contains a PTB domain, known to bind NPxpY motifs (36, 39), and SHIP encodes two such NPxY motifs C terminal to the putative catalytic domain (12). While the phosphorylation site(s) of SHIP has not yet been identified, it is known that Shc is phosphorylated at Y239, Y240 (37), and Y317 (29, 37). Further study of the mode of SHIP-Shc interaction and its relationship to Ras induction is necessary.

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