The SH2 Domain Containing Tyrosine Phosphatase-1 Down-regulates Activation of Lyn and Lyn-induced Tyrosine Phosphorylation of the CD19 Receptor in B Cells*

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SHP-1 is a cytosolic tyrosine phosphatase implicated in down-regulation of a B cell antigen receptor signaling. SHP-1 effects on the antigen receptor reflect its capacity to dephosphorylate this receptor as well as several inhibitory comodulators. In view of our observation that antigen receptor-induced CD19 tyrosine phosphorylation is constitutively increased in B cells from SHP-1-deficient motheaten mice, we investigated the possibility that CD19, a positive modulator of antigen receptor signaling, represents another substrate for SHP-1. However, analysis of CD19 immunoprecipitates from SHP-1-deficient and wild-type B cells revealed that SHP-1 accounts for only a minor portion of CD19-associated tyrosine phosphatase activity. As CD19 tyrosine phosphorylation is modulated by the Lyn protein-tyrosine kinase, Lyn activity was evaluated in wild-type and motheaten B cells. The results revealed both Lyn as well as CD19-associated Lyn kinase activity to be constitutively and inducibly increased in SHP-1-deficient compared with wild-type B cells. The data also demonstrated SHP-1 to be associated with Lyn in stimulated but not in resting B cells and indicated this interaction to be mediated via Lyn binding to the SHP-1 N-terminal SH2 domain. These findings, together with cyanoen bromide cleavage data revealing that SHP-1 dephosphorylates the Lyn autophosphorylation site, identify Lyn deactivation/dephosphorylation as a likely mechanism whereby SHP-1 exerts its influence on CD19 tyrosine phosphorylation and, by extension, its inhibitory effect on B cell antigen receptor signaling.

B cell responses to antigen stimulation are transduced intracellularly via the B cell antigen receptor (BCR),† a multimeric receptor complex that comprises membrane immunoglobulin and the immunoglobulin α and β chains (1, 2). The signals transmitted consequent to antigen engagement drive B lymphocyte activation via a complex signaling network, which biochemically links the receptor complex to cellular responses such as to proliferation, differentiation, and antibody secretion. Transmission of BCR signals via this intracellular circuitry is further regulated by the integration of accessory signals from BCR comodulators (3) and is highly dependent on reversible protein-tyrosine phosphorylation mediated by the balanced activities of protein-tyrosine kinases (PTKs) and phosphatases (PTPs) (4, 5).

The initial events of BCR signal relay are characterized by the activation of several PTKs, including Lyn, Fyn, Blk, Syk, and Btk, and the subsequent recruitment of secondary signaling molecules, including phosphatidylinositol 3-kinase (PI3K), Shc, BLNK/SLP-65, Vav, SOS1, and phospholipase C (6–12). These initial interactions induce Ras activation, phosphoinositide turnover, increases in intracellular free calcium, and other intermediary events, which ultimately transduce the BCR-evoked signal to the nucleus and consequent proliferation, apoptosis, maturation, or other physiological responses. The mechanisms whereby BCR ligation can induce such a wide diversity of biological outcomes are not well understood but are likely to involve modulation of the BCR signaling pathway by a spectrum of transmembrane and cytosolic signaling effectors that qualitatively and/or quantitatively alter the relay and downstream interpretation of BCR signal (13).

Among the myriad of proteins implicated in the regulation of BCR signaling, the cytosolic protein-tyrosine phosphatase (PTP) SHP-1 is distinguished by its predominant role as an inhibitor of BCR-driven activation events (5). The inhibitory effect of SHP-1 on BCR signaling was initially revealed by the demonstration that BCR-evoked proliferation of mature B cells and clonal deletion of self-reactive B cell precursors are aberrantly increased in the context of SHP-1 deficiency (14, 15). These latter studies involved analysis of B cells from motheaten (me/me) and viable motheaten (me+/me) mice, animals in which expression of no SHP-1 or a catalytically inactive form of SHP-1 protein, respectively, is associated with increased cell receptor for IgG Fc region; GST, glutathione S-transferase; me/me, motheaten; me+/me, viable motheaten; PAGE, polyacrylamide gel electrophoresis; PI3K, phosphatidylinositol 3-kinase; PTK, protein-tyrosine kinase; PTP, protein-tyrosine phosphatase; SH, Src homology domain; Tricine, N-tris(hydroxymethyl)methylglycine; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; PBS, phosphate-buffered saline; ITIM, immunoreceptor tyrosine-based inhibitory motif.
levels of serum immunoglobulins, high autoantibody titer, and a marked expansion of CD5+ B-1 cells in the periphery (16, 17). At present, the biochemical basis whereby SHP-1 exerts its inhibitory effects on BCR-evoked responses is not entirely defined. This PTP has, however, been shown to interact with the BCR complex in resting B cells and likely acts in this context to maintain the receptor in a tyrosine-dephosphorylated state (14). Following BCR ligation, SHP-1 no longer associates with the BCR, but instead interacts with a number of BCR-inducible tyrosine-phosphorylated transmembrane coreceptors (18–20). These coreceptors, which include CD22, PIR-B, and CD72, have all been implicated in the down-regulation of BCR signaling (21–23) and have been shown to interact with the SHP-1 SH2 domains via phosphorylated tyrosine residues embedded within immunoreceptor tyrosine-based inhibitory motifs (ITIMs) (18–20). The inhibitory effects of these receptors depend on their binding to SHP-1 and appear to be realized via SHP-1-mediated dephosphorylation of tyrosine residues within the receptor cytosolic domains and/or other intracellular signaling effectors recruited to these receptors following BCR engagement.

In contrast to the ITIM-containing coreceptor molecules, a number of B cell transmembrane coreceptors modulate BCR signaling so as to amplify the signal and promote its downstream propagation. Among these positive modulatory receptors, the B lineage-specific CD19 molecule appears to play a central role in enhancing BCR coupling to a spectrum of cellular behaviors. CD19, which is expressed as a component of a multimeric complex on the B cell surface (24), becomes rapidly tyrosine-phosphorylated following BCR engagement (25) and consequently interacts with SH2 domain-containing signaling effectors, such as Lyn, Fyn, Syk, Vav, and PI3K, which play integral roles in BCR signal delivery (25–28). Recent data suggest that CD19 effects on BCR signaling reflect its capacity to not only interact with Src-family PTKs but also to amplify the activities of these enzymes (29). As is consistent with the positive role for CD19 in regulation of BCR signaling, mice, which overexpress CD19 consequent to the expression of a CD19 transgene, manifest augmented B cell proliferative responses to BCR cross-linking and show markedly increased serum immunoglobulin levels (30). These animals also display a dramatic increase in the numbers of B-1 lineage cells and a proportionate decrease in the numbers of conventional B cells within the periphery (31). These observations, therefore, reveal the phenotype engendered by CD19 overexpression to be very similar to the B cell phenotype conferred by SHP-1 deficiency, a finding that suggests that the influence of these respective proteins on BCR signaling thresholds reflects the modulation of a common signaling element or cascade. This hypothesis is further supported by our previous data revealing BCR-evoked CD19 phosphorylation to be markedly reduced in cells lacking both the CD45 and SHP-1 PTPs (32) and thus identifying CD19 as a possible target of SHP-1-mediated dephosphorylation.

In the current study, we have directly investigated the role for SHP-1 in modulating the tyrosine phosphorylation of CD19. The results of these studies confirm that BCR-induced tyrosine phosphorylation of CD19 is enhanced in SHP-1-deficient mice but also suggest that the contribution of SHP-1 to the direct dephosphorylation of CD19 is small. Because of these observations, as well as data revealing CD19 to be associated with the Lyn protein-tyrosine kinase following BCR engagement (25, 26) and identifying a central role for Lyn in modifying CD19 effects on B cell survival (33, 34), we next investigated the possibility that SHP-1 influence on CD19 tyrosine phosphorylation is mediated via the regulation of Lyn activity. The results of this analysis indicate both BCR-induced tyrosine phosphorylation and activation of the Lyn protein-tyrosine kinase to be markedly augmented in me/me and me’me’ compared with wild-type B cells. In addition, Lyn inducibly associates with the SHP-1 N-terminal SH2 domain and is dephosphorylated at its autophosphorylation site (Tyr-397) by incubation with SHP-1. These data identify Lyn as a substrate for SHP-1-mediated dephosphorylation/deactivation and suggest that SHP-1 inhibitory effects on Lyn activity contribute to the down-regulation of CD19 tyrosine phosphorylation and may thereby provide an important mechanism for disrupting CD19 interactions with downstream effectors involved in the relay and amplification of BCR-initiated activation signal.

MATERIALS AND METHODS

Reagents—Antibodies used for these studies included the following: PE-conjugated B220 antibody from Pharmingen (Mississauga, Ontario), rabbit polyclonal anti-Lyn antibody from Santa Cruz Biotechnology Inc. (Santa Cruz, CA), monoclonal anti- phosphotyrosine antibody 4G10 from Upstate Biotechnology Inc. (Lake Placid, NY), and goat F(ab′)2 anti-mouse IgM antibody from Jackson ImmunoResearch (West Grove, PA). Rat anti-mouse CD19 antibody was produced by the ID3 hybridoma (provided by Dr. D. Fearon, University of Cambridge School of Medicine, Cambridge, UK) (35), and a rabbit polyclonal anti-CD19 antibody was derived by immunization with a polylysine-linked peptide corresponding to amino acids 504–523 within the CD19 cytosolic domain (SynPeptide Corp., Dublin, CA). Rabbit polyclonal anti-SHP-1 antibody and monoclonal anti-Thy1.2 antibody from the hybridoma clone J1.10 (ATCC TIB184) were produced in our laboratory as described previously (14, 16). Low-Tox rabbit complement was purchased from Cedarlane (Hornby, ONT), and chemicals for immunoprecipitation/immunoblotting were purchased from Sigma Chemical Co. (St. Louis, MO).

Cells and Cell Lines—Single cell suspensions of splenocytes were obtained from 10- to 14-day-old C3HeB/FeJ-me/me (motheaten), C57BL/6-me/me (viable motheaten), and congenic wild-type (+/+) mice derived at the Samuel Lunenfeld Research Institute breeding institute by mating C3HeB/FeJ-me/+ and +/+ and C57BL/6-J-me/+ and +/+ breeding pairs. Purified populations of splenic B lymphocytes were obtained from me/me, me’me’, and wild-type congenic mice by subjecting splenic cell suspensions to erythrocyte lysis in 0.8% ammonium chloride, followed by treatment with anti-Thy-1.2 antibody for 30 min on ice and a subsequent 45-min incubation with a 1:15 dilution of rabbit complement (S erotec Ltd., Toronto, Ontario). The cells were then washed (3 ×) and resuspended in a Percoll gradient (Amersham Pharmacia Biotech, Baie d’Urfé, Province of Quebec) as described previously (14). The resulting cells were >90% B220 and B220 positive as determined by fluorescence-activated cell sorting (Becton Dickinson, Mountainview, CA) analysis. The CD5+ murine B lymphoma line (36) (provided by Dr. A. Kaushik, University of Guelph, Guelph, Ontario) and the WEHI-231 B lymphoma line (purchased from ATCC, Rockville, MD) were cultured at 37 °C in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 5% fetal bovine serum (Sterile System Inc., Logan, UTI), 50 μg/ml 2-mercaptoethanol, and 100 μg/ml penicillin/streptomycin.

Cell Stimulation and Lysis—WEHI-231, CH12, or purified splenic B cells (2–5 × 107) were resuspended in 5 ml of culture medium and stimulated with 40 μg/ml F(ab′)2 antibody for varying periods of time.

Stimulations were done on ice to retard biochemical reactions when studying the kinetics of CD19 phosphorylation and Lyn kinase activation (37). For biotinylation, 5–6 × 106 cells/ml were suspended at 107 cells/ml in ice-cold PBS and mixed with 0.3 mg/ml sulfo-NHS-Biotin solution (Pierce Chemical Co., Rockford, IL). After 30-min incubation at room temperature, the reaction was quenched by 5-min incubation with 50 mg/ml glycine in PBS. Cells were then washed twice in cold PBS and subjected to stimulation as above. Following stimulation, biotinylated or nonbiotinylated cells were incubated in lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 50 μM NaF, 2 mM phenylmethylsulfonyl fluoride, 2 mM Na3VO4, 50 mM ZnCl2, 50 μM e- phosphostearate, 2 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin) containing either 1% CHAPS (Sigma) or 1% Nonidet P-40. Cell lysates were centrifuged at 14,000 × g for 10 min at 4 °C, and protein concentrations were then determined using the bichinonic acid assay (Pierce).

Antibody Coupling to Protein A-Sepharose—Anti-Lyn or anti-IgG (isotype control) antibody was incubated at 4 °C overnight under rocking conditions with protein A-Sepharose 4B (Amersham Pharmacia Biotech) in PBS (about 10 μg of antibody per 10 μl of beads). Beads were then washed two times in 0.1 M sodium borate (pH 8.6) and two times in 0.2 M triethanolamine (pH 8.2). Beads were then resuspended in 0.2
m tri-ethanolamine solution containing 40 mM dimethyl pimelimidate dihydrochloride (Pierce) and incubated for 1 h at room temperature with continual rocking. The antibody-coupled beads were washed two times in 200 mM ethanolamine (pH 8.2), two times in 0.1 M sodium borate (pH 8.0), two times in PBS, and resuspended in PBS supplemented with 0.1% BSA and 0.01% NaN3.

**Immunoprecipitation and Immunoblotting—**Lysates were precleared before immunoprecipitation by incubating 1 mg of cell lysate with protein A-Sepharose beads (Amersham Pharmacia Biotech) at 4 °C for 1 h and for an additional 1 h with 40 µl of rabbit preimmune serum. Lysates were then incubated for 2 h at 4 °C with the appropriate antibody (anti-Lyn, anti-IgG isotype control) and 25 µl of protein G-Sepharose beads. Immune complexes were then collected by centrifugation, washed four times in lysis buffer, and boiled for 5 min in reducing SDS-gel sample buffer. Samples were then electrophoresed through SDS-polyacrylamide and transferred to nitrocellulose (Bio-Rad Laboratories, Mississauga, Ontario). After 1-h incubation in 3% gelatin, the filters were incubated for 1 h at room temperature with anti-CD19, anti-Lyn, or anti-phosphotyrosine 4G10 antibodies followed by horse-radish peroxidase-labeled secondary antibody (Amersham Pharmacia Biotech, Arlington Heights, ICN) or, for analysis of bintiylated cells, with horse-radish peroxidase-avidin (Pierce). Immune complexes were detected using an enhanced chemiluminescence system (Amersham Pharmacia Biotech). Stripping and reprobing of the blots were performed according to Amersham Pharmacia Biotech’s recommended protocol.

**Assay of Phosphatase Activity—**For analysis of CD19-associated phosphatase activity, anti-CD19 immunoprecipitates were prepared (as described above) from 1 mg of lysates of unstimulated or anti-IgM antibody-stimulated motheaten and wild-type B cells. Phosphatase assays were also performed on anti-CD19 and anti-IgG (control) immunoprecipitates prepared from lysates of wild-type splenic B cells immunodepleted of SHP-1 by overnight incubation with an excess of anti-SHP-1 antibody followed by addition of 100 µl of protein A-Sepharose. For this experiment, the complete immunodepletion of SHP-1 protein was confirmed by Western immunoblotting analysis (data not shown). The amount of SHP-1 antibody utilized to completely immunodeplete SHP-1 protein from lysates was predetermined by titration and Western immunoblotting analysis (data not shown). Immunoprecipitates were washed twice in phosphatase buffer (10 mM Tris-HCl, 1.0 mM EDTA, 1 mg/ml bovine serum albumin, 0.1% 2-β-mercaptoethanol, 0.01% NaN3, pH 7.34) and then incubated for 12 h at 37 °C overnight in phosphatase buffer containing 2 mM p-nitrophenyl phosphatase (Sigma). Under these conditions, SHP-1 activity for the substrate has been shown to increase linearly at least up to 30% of the amount of SHP-1 protein in the reaction (38, 39). Reactions were terminated by addition of 0.2 N NaOH, and absorbance was measured at 410 nm by spectrophotometry.

**In Vitro Kinase Assay—**Lyn kinase activity was evaluated using immunoprecipitates prepared as described above from unstimulated and stimulated splenic B cells. The immunoprecipitates were washed in kinase buffer (20 mM HEPES, pH 7.6, 150 mM NaCl, 5 mM MgCl2, 0.25 mM Na3VO4, 0.1 mM 2-β-mercaptoethanol, 0.01% NaN3, pH 7.34) and then incubated for 12 h at 33 °C in 0.1 ml of kinase buffer containing 1 µCi [γ-32P]ATP (ICN) with or without 10 µg of GST-Igβ fusion protein (provided by Dr. Y. Wu, Toronto, Ontario). Samples were resuspended in SDS-gel sample buffer, boiled, and centrifuged at 14,000 × g for 10 min and resolved over 10% SDS-PAGE gels. The 32P-labeled proteins were electrophoretically transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA) and then visualized by autoradiography. Lyn quantification was performed by anti-Lyn immunoblotting of the membranes using ECL.

**In Vitro Binding Assays—**Glutathione S-transferase (GST)-SHP-1 fusion proteins were generated by subcloning the following murine cDNA or polymerase chain reaction-amplified fragments into pGEX2T: the full-length SHP-1 cDNA (GST-SHP-1), a full-length SHP-1 cDNA containing a Cys-453 → Ser mutation (GST-SHP-1 (C453S)), the SHP-1 N-terminal SH2 domain (amino acids 1-95), the SHP-1 C-terminal SH2 domain (amino acids 110-205), and the SHP-1 N- and C-terminal SH2 domains (amino acids 1-221). These expression plasmids were transfected into Escherichia coli JM101, and the fusion proteins were purified from isopropyl β-D-thiogalactopyranoside-induced bacterial lysates according to the protocol (Amersham Pharmacia Biotech). Equimolar amounts of each GST-SHP-1 fusion protein and GST beads were then incubated at 4 °C for 1 h with 0.9 µg of [γ-32P]labeled purified Lyn protein. Beads were washed seven times, and the complexes were resuspended in SDS-sample buffer, boiled, analyzed by SDS-PAGE, and transferred to nitrocellulose, and the Lyn protein was visualized by autoradiography.

**Cyagen Bronide Cleavage Analysis—**In vitro [γ-32P]ATP-labeled Lyn was immunoprecipitated using anti-Lyn antibody and incubated at 37 °C with equal amounts of either GST-SHP-1 or GST-SHP-1 (C453S) protein in 200 µl of phosphatase buffer (10 mM Tris-HCl, 1.0 mM EDTA, 1 mg/ml bovine serum albumin, 0.1% 2-β-mercaptoethanol, 0.01% NaN3, pH 7.34). The immunoprecipitates were then incubated over SDS-PAGE and transferred to nitrocellulose. The 56-kDa Lyn-containing bands were then excised from the membranes and subjected to CNBr cleavage as described previously (40). The excised Lyn protein was incubated with 60 mg/ml CNBr in 70% formic acid for at least 2 h at room temperature. Samples were then washed and dried, and the CNBr-generated peptide fragments were resuspended in tricine SDS sample buffer, resolved by electrophoresis on 10–20% gradient Tricine SDS-PAGE (Novex, San Diego, CA), transferred to an Immobilon-P membrane, and visualized by autoradiography. Complete digestion of each sample was confirmed by the absence of higher molecular weight 32P-labeled bands, and the CNBr fragments were quantitated by phosphorimaging (Molecular Dynamics).

**RESULTS AND DISCUSSION**

**BCR-evoked CD19 Tyrosine Phosphorylation Is Enhanced, but CD19-associated Phosphatase Activity Is Only Marginally Altered, in SHP-1-deficient B Cells—**Previous data from our laboratory and others have revealed modulation of inhibitory coreceptors to represent a major mechanism whereby SHP-1 mediates its down-regulatory effects on BCR signaling (5, 18–20). However, data garnered from the analysis of mice deficient for both the CD45 and SHP-1 PTPs (31) raised the possibility that SHP-1 also influences the tyrosine phosphorylation and, by extension, signaling functions of the positive regulatory coreceptor, CD19. To begin addressing this issue, SHP-1-deficient B cells from me/me and me/mv me mice were evaluated with regards to the kinetics of CD19 phosphorylation following BCR ligation. As illustrated in Fig. 1, anti-phosphotyrosine immunoblotting analysis of CD19 immunoprecipitates from the SHP-1-deficient cells revealed tyrosine phosphorylation of the 115- to 120-kDa species representing CD19 to be markedly increased both constitutively and inducibly in the me/me and to a lesser extent in the me/me and me/me mice compared with wild-type cells. The increased phosphorylation of CD19 detected in the motheaten cells cannot be attributed to expansion of the CD5− B-1 cell population in these mice, because CD5− CH12 cells exhibited normal levels of CD19 phosphorylation both before and after BCR cross-linking (data not shown). These data support the contention that SHP-1 modulates CD19 tyrosine phosphorylation and are consistent with previous data revealing BCR-evoked CD19 phosphorylation to be augmented in a B cell line derived from me/me mice (41).

To more directly investigate the possibility that CD19 represents a SHP-1 substrate, the SHP-1-deficient B cells were next compared with wild-type B cells in terms of the levels of tyrosine phosphatase activity coimmunoprecipitated with CD19 from these respective cell populations. The results of this analysis demonstrated phosphorylated phosphatase activity to be markedly increased following BCR ligation in both wild-type and me/me B cells (Fig. 2). Although levels of phosphatase activity coimmunoprecipitated with CD19 were lower in me/me compared with wild-type B cells and were also relatively reduced in B cells immunodepleted for SHP-1, the reduction in coprecipitated phosphatase activity observed in the SHP-1-deficient cells was only modest. These findings indicate that SHP-1 is not the major CD19-associated tyrosine phosphatase and suggest that SHP-1 contribution to the direct dephosphorylation of CD19 may be small. This interpretation of the data is consistent with the data shown in Fig. 1A, indicating that tyrosine dephosphorylation of CD19 proceeds normally in motheaten B cells and is not diminished in the SHP-1-deficient compared with wild-type B cells at the 10-min time point following BCR stimulation. This conclusion is also supported by
SHP-1 Modulates CD19 Tyrosine Phosphorylation and Lyn Activation

Fig. 1. CD19 tyrosine phosphorylation is increased in SHP-1-deficient motheaten B cells. Purified splenic B cells isolated from C57BL/6J-wild-type (+/+ ) or viable motheaten (Me−/−) mice (A) or from C3HeFfJ-wild-type (+/+ ) or motheaten (Me−/−) mice (B) were stimulated with 40 μg/ml goat F(ab′)2 anti-mouse IgM antibody for the indicated times. Lysate proteins (1 mg) were then immunoprecipitated with anti-CD19 antibody (ID3), resolved on 10% SDS-PAGE, and transferred to nitrocellulose membranes, and the membranes were then immunoblotted with anti-phosphotyrosine antibody. Loading of equivalent amounts of CD19 protein was confirmed by reblotting with either horseradish peroxidase-avidin (A) or anti-CD19 (B) antibodies (lower left and right panels, respectively). Arrows indicate the position of CD19. Mobilities of molecular mass standards are shown on the left.

Fig. 2. BCR-induced increases in CD19-associated PTP activity are only modestly influenced by SHP-1 deficiency. Splenic B cells purified from C3HeFfJ-wild-type (C3H+/+) or motheaten (Me−/−) mice were stimulated with goat F(ab′)2 anti-mouse IgM antibody (40 μg/ml) for 5 min. Cells were lysed in 1% CHAPS lysis buffer, and lysate proteins (1 mg) were then immunoprecipitated with anti-CD19 or isotype IgG control antibodies. CD19 was also immunoprecipitated from normal anti-IgM antibody-stimulated splenic B cell lysates pretreated with an excess of anti-SHP-1 antibody (SHP-1-depleted). The precipitated proteins were incubated for 12 h at 37 °C in phosphatase buffer containing 1 mM p-nitrophenyl phosphate. After addition of 0.2 M NaOH, absorbance was measured at 410 nm using an enzyme-linked immunosorbent assay plate reader. Phosphatase activity was quantified in millimoles based on a standardized concentration curve of the product p-nitrophenol (PNP). Bars indicate the standard deviations obtained from three independent experiments.

our observation of minimal amounts of SHP-1 in anti-CD19 immunoprecipitates from unstimulated and stimulated B cells (data not shown), a finding also consistent with the lack of ITIMs in the CD19 cytosolic region. Along similar lines, SHP-1 deficiency has been shown to have negligible impact on the enhanced dephosphorylation of CD19, which occurs in conjunction with FcγRIII-B-mediated inhibition of BCR signaling (42, 43). Taken together, these data suggest a minimal role for SHP-1 in the direct dephosphorylation of CD19.

BCR-induced Activation of the Lyn PTK Is Enhanced in SHP-1-Deficient Cells—Although the precise profile of effectors that mediate induction of CD19 tyrosine phosphorylation following BCR ligation is unclear, an important role for the Lyn PTK has been suggested by the detection of Lyn kinase activity in anti-CD19 immunoprecipitates and by data implicating the CD19-Lyn complex in regulation of B cell survival (25, 26, 34). In view of these observations, the kinase activity contained in immunoprecipitates from me−/− and me+/− B cells was next investigated using an in vitro assay of kinase activity. The results of this analysis revealed Lyn-mediated autophosphorylation and phosphorylation of exogenous substrate (GST-Igα/β) to be both constitutively and inducibly higher in the me−/− and me+/− B cells than in wild-type B cells (Fig. 3, A and B). Similarly, analysis of the Lyn kinase activity contained in CD19 immunoprecipitates from resting and BCR-stimulated cells demonstrated CD19-associated Lyn activity to be both constitutively and inducibly enhanced in SHP-1-deficient compared with wild-type cells (Fig. 3C). Lyn association with CD19 was also increased in the context of SHP-1 deficiency, an observation consistent with previous data linking increases in Lyn activity to enhanced phosphorylation of the CD19 tyrosine residues mediating Lyn binding to CD19 (44). As shown in Fig. 3D, tyrosine phosphorylation status of Lyn was also evaluated by anti-phosphotyrosine immunoblotting analysis of Lyn immunoprecipitates from the cells under study. The results of this analysis revealed tyrosine phosphorylation of Lyn both before and after BCR ligation to be increased in the motheaten cells. These results, therefore, indicate SHP-1 deficiency to be associated with heightened Lyn activity and strongly suggest that Lyn is a SHP-1 substrate. Interestingly, increases in Lyn activity have also been detected in pre-B cell lines derived from motheaten bone marrow (45). However, in this latter system, the augmentation in Lyn activity was ascribed to the expression of increased Lyn protein in these mutant cells. By contrast, in the current analysis, levels of Lyn protein were found to be comparable in splenic B cells isolated from either wild-type or motheaten mice. Thus, these data suggest that SHP-1 induces the dephosphorylation and deactivation of Lyn and that the SHP-1 effect on CD19 phosphorylation may be mediated via the down-regulation of Lyn activity.

Lyn Associates with the SHP-1 N-terminal SH2 Domain—In view of the apparent role for SHP-1 in modulating Lyn kinase activity, the possibility that these enzymes associate with one another in resting and stimulated B cells was next investigated. As shown in Fig. 4A (upper panel), anti-SHP-1 immunoblotting analysis of Lyn immunoprecipitates prepared from resting and BCR-ligated WEHI-231 B cells revealed SHP-1 to be present in Lyn immunoprecipitates from stimulated but not unstimulated cells. Association of SHP-1 with Lyn has also been previously detected in U937 myeloid leukemia cells, although, in the latter study the association of these enzymes appeared to occur constitutively (46). By contrast, in the current study, SHP-1 binding to Lyn was up-regulated by cell stimulation, a result that suggests the interaction may be phosphotyrosine-dependent and involve binding of the SHP-1 SH2 domains with phosphorylated tyrosine residues on Lyn. To investigate this possibility, GST fusion proteins containing full-
length SHP-1, a catalytically inert form of SHP-1 (SHP-1 C453S), and one or both of the SHP-1 SH2 domains were evaluated for their capacities to interact with in vitro phospho-rylated Lyn. The results of this analysis revealed the interaction of phosphorylated Lyn with the N-terminal, but not the C-terminal, SHP-1 SH2 domain (Fig. 4B). As shown in Fig. 4B, the fusion protein containing the C- and the N-terminal SHP-1 SH2 domain precipitated more Lyn protein than did the GST-N-terminal SHP-1 fusion protein. This observation is consistent with previous data indicating that a single SH2 domain may be sufficient for SHP-1 interaction with target substrate, but may be less efficient than the combined SH2 domains in promoting such interactions (47). In addition, these data do not preclude the possibility that SHP-1 association with Lyn involves other sites within these respective proteins. The Lyn SH3 domain has, for example, been implicated in the Lyn-SHP-1 interaction detected in myeloid cell lines (46), and the association of SHP-1 with the related Src PTK appears to involve interactions between not only the SHP-1 SH2 domains and phosphorylated Src, but also the Src SH2 domain and phosphorylated SHP-1 (48, 49). In addition, both SHP-1 and Lyn associate with other signaling effectors, such as PI3K (50, 51), that are recruited to the BCR following its stimulation and that may create a structural framework at the membrane that enhances SHP-1 association with Lyn. Although the relative importance of these various interactions to SHP-1-Lyn binding remains to be determined, the current data are consistent with the conclusion that SHP-1 physically associates with Lyn in B cells and is therefore appropriately positioned to induce dephosphorylation and deactivation of the kinase.

The data shown in Fig. 4B also reveal the signal intensity of the Lyn species precipitated with the SHP-1 C453S fusion protein to be greater than that of the species precipitated with the wild-type SHP-1 fusion protein. This observation most likely reflects the substrate trapping properties of SHP-1 C453S and, by extension, indirectly supports the contention
that Lyn represents a SHP-1 substrate. Conversely, the association between SHP-1 and Lyn may also provide a framework for Lyn to phosphorylate SHP-1. SHP-1 has in fact been shown to represent a substrate for Lyn in an exogenous expression system (46), and several Src-family kinases (Src, Lck, and Lyn) can phosphorylate SHP-1 in vitro (46, 49, 52). Moreover, in the BCR-stimulated splenic B cells studied here, the tyrosine phosphorylation of Lyn-associated SHP-1 was found to be increased compared with that detected in the total pool of SHP-1 (data not shown). These findings suggest that SHP-1 is phosphorylated by Lyn in vivo and raise the possibility that SHP-1 and Lyn engage in a reciprocal functional relationship wherein Lyn phosphorylates and potentially activates SHP-1 and SHP-1 then dephosphorylates and deactivates Lyn. This functional paradigm has been suggested with respect to SHP-1 modulation of Lyn-dependent apoptotic responses to DNA damage (46) and also in relation to SHP-1 modulation of ZAP-70-dependent proliferative response to T cell antigen receptor engagement (53). However, at present the relationship between SHP-1 tyrosine phosphorylation status and its catalytic activity is unclear (52, 54), and accordingly, the relevance of Lyn to SHP-1 activation remains to be determined. By contrast, the available data provide compelling evidence that SHP-1 negatively regulates Lyn activity and thus suggest that SHP-1 modulation of Lyn plays a role in SHP-1-mediated inhibition of BCR signaling.

**SHP-1 Catalyzes the Dephosphorylation of Tyr-397 within the Lyn Kinase Domain—**As for other Src-family PTKs, Lyn contains two major sites of tyrosine phosphorylation, these being the autophosphorylation site (Tyr-397) within the kinase domain and the negative regulating tyrosine phosphorylation site (Tyr-508) within the C-terminal tail (55). Thus phosphorylation and activation of Lyn appears to be regulated in a fashion highly similar to that of the related Lck PTK, the latter of which is also tyrosine-dephosphorylated by SHP-1 (52). However, although phosphorylation of the Lyn regions represented by both the 8.0- and the 4.0-kDa Lyn CNBr cleavage fragments was diminished following treatment with wild-type SHP-1, the reduction in phosphorylation of the 8-kDa cleavage fragment was much more dramatic than that of the 4-kDa fragment, particularly in the context of Lyn pretreatment with 1.0 μg of SHP-1. These data suggest that both Tyr-397 contained within the 8-kDa fragment and Tyr-508 contained within the 4-kDa fragment can be dephosphorylated by SHP-1, but also indicate that SHP-1 preferentially dephosphorylates Tyr-397. Thus, these findings reveal the capacity of SHP-1 to directly dephosphorylate Lyn at the autophosphorylation site as is consistent with a role for SHP-1 in negatively regulating Lyn activity. The current data also suggest that the C-terminal inhibitory phosphotyrosine on Lyn is not regulated by SHP-1, a conclusion supported by previous data revealing the phosphorylated C-terminal tyrosine on Lyn to be targeted by the CD45 PTP (55). Thus phosphorylation and activation of Lyn appears to be regulated in a fashion highly similar to that of the related Lck PTK, the latter of which is also tyrosine-dephosphorylated at its autophosphorylation site by SHP-1 (52) and at its inhibitory C-terminal site by CD45 (56). A role for CD45 in activating Lyn is also suggested indirectly by the diminution of BCR-induced CD19 tyrosine phosphorylation detected in CD45-deficient mice (32). Thus CD45 and SHP-1 appear to exert opposing effects on Lyn activity and thereby engender a biochemical counterbalance critical to the modulation of CD19 and...
SHP-1 realizes its inhibitory effects on BCR signaling. The data reported in this study establish the capacity of SHP-1 to modulate CD19 tyrosine phosphorylation but link this effect to SHP-1-mediated dephosphorylation of Lyn rather than to dephosphorylation of CD19 per se. Importantly, although Lyn interactions with CD19 appear to exert a positive effect on BCR signal delivery, recent data derived largely from the analysis of Lyn-deficient mice, have revealed a pivotal role for this PTK in the negative regulation of BCR signaling (57, 58). Interestingly, SHP-1 association with CD22 also appears involved in BCR signal delivery. Potentially to the regulation of other signaling effectors in-
The SH2 Domain Containing Tyrosine Phosphatase-1 Down-regulates Activation of Lyn and Lyn-induced Tyrosine Phosphorylation of the CD19 Receptor in B Cells
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J. Biol. Chem. 2001, 276:1938-1944.
doi: 10.1074/jbc.M006820200 originally published online October 20, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M006820200

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