Hematopoietic Cell Phosphatase, SHP-1, Is Constitutively Associated with the SH2 Domain-containing Leukocyte Protein, SLP-76, in B Cells

By Kazuya Mizuno, Tatsuo Katagiri, Kiminori Hasegawa, Mami Ogimoto, and Hidetaka Yakura

From the Department of Microbiology and Immunology, Tokyo Metropolitan Institute for Neuroscience, Fuchu, Tokyo 183, Japan

Summary

Src homology region 2 (SH2) domain-containing phosphatase 1 (SHP-1; previously named HCP, PTP1C, SH-PTP1, and SHP) is a cytosolic protein tyrosine phosphatase that contains two SH2 domains. Recent data have demonstrated that the gene encoding SHP-1 is mutated in motheaten (*me*) and viable motheaten (*me*^{v}) mice resulting in autoimmune disease. More recently, SHP-1 has been shown to negatively regulate B cell antigen receptor (BCR)–initiated signaling. To elucidate potential mechanisms of SHP-1 action in BCR signal transduction, we studied proteins that interact with SHP-1 in B cells. Both anti-SHP-1 antibody and the two SH2 domains of SHP-1 expressed as glutathione S-transferase fusion proteins precipitated at least three phosphoproteins of ∼75, 110, and 150 kD upon anti-immunoglobulin M stimulation of the WEHI-231 immature B cell line. Binding of SHP-1 to the 75- and 110-kD proteins appeared to be mediated mainly by the NH_2-terminal SH2 domain of SHP-1, whereas both the NH_2- and COOH-terminal SH2 domains are required for maximal binding to the 150-kD protein. Immunoprecipitation and Western blot analysis revealed that the SHP-1–associated 75-kD protein is the hematopoietic cell–specific, SH2-containing protein SLP-76. Further, this protein–protein association was constitutively observed and stable during the early phase of BCR signaling. However, significant tyrosine phosphorylation of SLP-76 as well as of SHP-1 was observed after BCR ligation. Constitutive association of SHP-1 with SLP-76 could also be detected in normal splenic B cells. Collectively, these results suggest possible mechanisms by which SHP-1 may modulate signals delivered by BCR engagement.

Engagement of the B cell antigen receptor (BCR)[1] by Ag or anti-IgM Ab rapidly activates two families of protein tyrosine kinases (PTKs) associated with the BCR through Ig-α (CD79a) and Ig-β (CD79b), including members of the Src-family (Lyn, Blk, Lck, and Fyn) (1–4) and Syk (5, 6). Activation of these PTKs results in phosphorylation of a number of downstream substrates such as phospholipase Cγ (PLCγ) (7), phosphatidylinositol 3-kinase (8), and guanine triphosphatase–activating protein of p21^{ras} (9, 10). Signals are then transmitted to the nucleus through various pathways whose integrations regulate the processes of activation, proliferation, differentiation, or cell death. The degree of substrate tyrosine phosphorylation is tightly controlled by action of PTKs and protein tyrosine phosphatases (PTPs) (11–13). The complexity of these interactions is underscored by the fact that PTK can, in some cases, be a target of PTP action. One such example of this includes CD45, a receptor-type PTP that has been shown to play a critical role in initial activation of PTKs both in T (14, 15) and in B cells (16–19). However, regulatory roles of other PTPs in BCR-mediated signal transduction have not been well characterized.

SHP-1, previously named HCP (20), PTP1C (21), SHP (22), and SH-PTP1 (23), is an intracellular PTP that contains two tandemly linked Src homology region 2 (SH2) domains at the NH_2 terminus followed by a catalytic domain. It has been demonstrated that the immunological abnormalities of the motheaten (*me*) and viable motheaten (*me*^{v}) mice are caused by mutations in the gene encoding

SHP-1 (24, 25). The *me* mutation is a deletion of cytidine residue in the NH₂-terminal SH2 domain resulting in no SHP-1 production, whereas the *me^v* mutation is a thymidine to adenine transversion in the catalytic domain, resulting in an enzymatically deficient SHP-1 variant (24–26). Mice homozygous for the *me* and *me^v* display autoimmune and immunodeficiency disease and die at 3 and 9 wk of age, respectively (27, 28). The abnormality includes expansion of CD5⁺ B-1 B cells (29), reduced numbers of B cell progenitors in the bone marrow and conventional B-2 B cells in the periphery (30), and functional defects in T and NK cells (31, 32). Recent studies (33) have shown that SHP-1 negatively regulates BCR signaling and is involved in determining the threshold for negative selection. SHP-1 has been shown to be involved in negative regulation of BCR signaling by FcγRIIB1 (34). Although the biochemistry of the events remains poorly understood, it has been shown that SHP-1 is constitutively associated with BCR complex and that BCR ligation induces tyrosine phosphorylation of SHP-1 and its dissociation from BCR complex (35). These results strongly suggest a crucial role of SHP-1 in the fine tuning of BCR signaling.

In this study, we investigated the mechanisms whereby SHP-1 regulates BCR-initiated signal transduction by identifying the nature of SHP-1–associated proteins. Our results demonstrate that SHP-1 is associated with at least three species of tyrosine-phosphorylated proteins of ∼75, 110, and 150 kD, both in vitro and in vivo. We identified the protein of 75 kD to be the SH2-containing leukocyte-specific protein of 76 kD, SLP-76. Although BCR ligation is required for the significant induction of tyrosine phosphorylation of SLP-76, the association of SHP-1 with SLP-76 was constitutively observed both in an immature B cell line and in normal splenic B cells. Thus, SLP-76, and two as yet unidentified proteins of 110 and 150 kD, may play roles in the action of SHP-1 in B cells.

Materials and Methods

Mice. C57BL/6 CrSlc (B6) mice were purchased from Japan SLC, Inc. (Hamamatsu, Japan), and were used at 6–8 wk of age.

Cells. WEHI-231 cells were obtained from the American Type Culture Collection (Rockville, MD) and were maintained in RPMI 1640 (Nikken Bio Medical Laboratory, Kyoto, Japan) supplemented with 10% fetal bovine serum (Bioserum, Canterbury, Australia), 50 μM 2-ME, 100 μg/ml streptomycin, and 100 U/ml penicillin (17). Splenic B cells were prepared by treating spleen cells from B6 mice with anti-Thy-1.2 mAb and rabbit C, as described previously (36).

Abs. Goat anti–mouse IgM (anti-μ) Ab was purchased from Cappel, Organon Teknika Corp. (Durham, NC). Anti–mouse HS1 mAb was purchased from Sumitomo Electric Industries (Osaka, Japan). Polyclonal rabbit anti–human SHP-1 Ab, which reacts with the mouse product, and anti-phosphotyrosine (PY) mAb 4G10 were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Polyclonal rabbit anti–mouse Syk Ab was described previously (19). Polyclonal anti-SLP-76 Ab was raised by immunizing a sheep with mouse SLP-76 (amino acids 136–235) expressed as a glutathione S-transferase (GST) fusion protein, and

was provided generously by Dr. Gary Koretzky (University of Iowa). Alkaline phosphatase (AP)–conjugated goat anti–mouse IgG was obtained from Bio-Rad Laboratories (Richmond, CA), and AP-conjugated mouse anti–rabbit IgG and donkey anti–sheep IgG were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

Preparation of GST Fusion Proteins. The SH2 domains of murine SHP-1 were expressed in *Escherichia coli* XL1-Blue as GST fusion proteins. cDNAs corresponding to the NH₂-terminal (amino acids 4–100), COOH-terminal (amino acids 110–213), and both (amino acids 4–213) SH2 domains of SHP-1 were amplified by PCR and cloned into pGEX-4T3 (Pharmacia Biotechnology, Uppsala, Sweden). Fusion proteins were expressed and affinity purified with glutathione–Sepharose 4B (Pharmacia Biotechnology) as described previously (37).

Immunoprecipitation, Protein Precipitation, and Western Blot Analysis. Immunoprecipitation and Western analysis were performed as described previously (18, 19). Cells (3 × 10⁷) were suspended in 5 ml RPMI 1640 supplemented with 20 mM Hepes, incubated for 1 h at 37°C, and then stimulated for 5 min with 25 μg/ml anti-μ Ab. Reactions were stopped with 25 ml ice-cold PBS containing 1 mM Na₃VO₄ and 2 mM EDTA (PBS-VE). The cells were washed twice with PBS-VE, and lysed in 1 ml lysis buffer (1% NP-40, 20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 10% glycerol, 1 mM Na₃VO₄, 2 mM EDTA, 1 mM PMSF, 20 μM leupeptin, and 0.15 U/ml aprotinin). After centrifugation, the supernatants were precipitated with GST-SHP-1-SH2 fusion proteins or immunoprecipitated with protein G–Sepharose coupled with anti-SHP-1 Ab or anti-SLP-76 Ab. Immunoprecipitates were washed five times in lysis buffer, boiled in reducing Laemmli SDS sample buffer, and subjected to 7.5% SDS-PAGE followed by transfer to a nitrocellulose membrane. The membranes were then incubated overnight with anti-PY mAb or anti-HS1, anti-Syk, anti-SHP-1, or anti-SLP-76 Ab. The blots were visualized by incubating with AP-linked goat anti–mouse IgG, mouse anti–rabbit IgG, or donkey anti–sheep IgG, and then developed with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

Results and Discussion

The SH2 Domains of SHP-1 Bind At Least Three Species of Phosphoproteins. To begin to address possible roles for SHP-1 in BCR-mediated signal transduction, we first examined what B cell proteins could associate with the SH2 domains of SHP-1. Total cell lysates were prepared from unstimulated or anti-μ–stimulated WEHI-231 cells. Lysates were precipitated with the SH2 domains of SHP-1 expressed as GST fusion proteins, then subjected to SDS-PAGE and immunoblotting with anti-PY mAb. As shown in Fig. 1 *A*, a GST fusion protein containing NH₂- and COOH-terminal SH2 domains (SH2-N+C) of SHP-1 precipitated two phosphoproteins of ∼75 kD, a 110-kD phosphoprotein, and a 150-kD phosphoprotein from BCR-stimulated cell lysates. To examine the relative contribution of the two SH2 domains to these protein–protein interactions, we constructed GST fusion proteins containing either the NH₂- or the COOH-terminal SH2 domain of SHP-1. The NH₂-terminal SH2 domain, when expressed alone (SH2-N), bound to proteins of ∼75 and 110 kD as efficiently as did the control SH2-N+C, whereas the

binding of SH2-N to the 150-kD protein was significantly reduced (Fig. 1 A). In contrast, the COOH-terminal SH2 domain (SH2-C) alone did not precipitate any of these proteins (Fig. 1 A). Thus, it appears that the SHP-1 association with the 75- and 110-kD proteins is mediated mainly by the SH2-N, but both SH2 domains are required for maximal SHP-1 interaction with the 150-kD protein.

The Binding of SHP-1 to the Associated Proteins Is Observed in Intact Cells. To investigate whether the in vitro binding of SHP-1 to the proteins described above is also observed in intact cells, WEHI-231 cell lysates were immunoprecipitated with anti-SHP-1 Ab, and then subjected to Western blot analysis with anti-PY mAb. The experiment shown in Fig. 1 B demonstrates that SHP-1 immunoprecipitates contain similar species of phosphoproteins of 75, 110, and 150 kD in anti-μ–stimulated cell lysates. Although SHP-1 has been shown to bind to a 44-kD phosphoprotein in BCR-stimulated WEHI-231 cells (38), we did not observe this association. The reason for this discrepancy with previously published work is not known, but may be due to cloned variation in WEHI-231 (39). As previously reported (35, 38, 40), we found that BCR stimulation induced tyrosine phosphorylation of SHP-1. Taken together, these data indicate that SHP-1 is associated both in vitro and in vivo with at least three species of tyrosine-phosphorylated proteins.

Identification of the SHP-1–binding 75-kD Protein as SLP-76. To characterize the nature of the SHP-1–associated protein

of ~75 kD, we used Ab to search for candidate phosphotyrosine-containing signaling molecules in this similar molecular weight range. We first examined whether HS1, a 75-kD protein implicated in B cell apoptosis (41), or Syk, a PTK of 72 kD (5, 6), binds to SHP-1 in WEHI-231 cells. However, neither anti-HS1 nor anti-Syk Ab reacted with anti-SHP-1 immunoprecipitates or the SH2-N+C precipitates, although both Abs could detect a protein of appropriate size in total cell lysates, indicating that phosphoprotein (pp)75 is not HS1 or Syk (data not shown).

We next examined the possibility that pp75 is SLP-76, a recently identified leukocyte-specific phosphoprotein of 76 kD (42). Total cell lysates of WEHI-231 cells, either unstimulated or stimulated with anti-μ Ab for 5 min, were precipitated with anti-SHP-1 Ab or anti-SLP-76 Ab, and then immunoblotted with anti-SLP-76 Ab or anti-PY mAb. Experiments shown in Fig. 2 A revealed that anti-SHP-1 immunoprecipitates contain a protein reactive with anti-SLP-76 Ab, irrespective of anti-μ stimulation. However, significant tyrosine phosphorylation of SLP-76 was observed after BCR ligation (Fig. 2 B). We further tested whether SHP-1 could be detected in anti-SLP-76 immunoprecipitates. Fig. 2 C demonstrates that anti-SLP-76 Ab coimmunoprecipitates SHP-1 even in the absence of anti-μ stimulation, strongly suggesting constitutive association of SHP-1 with SLP-76. The time course experiments revealed that the association of SHP-1 with SLP-76 is detected even at 1 h after BCR ligation (data not shown).
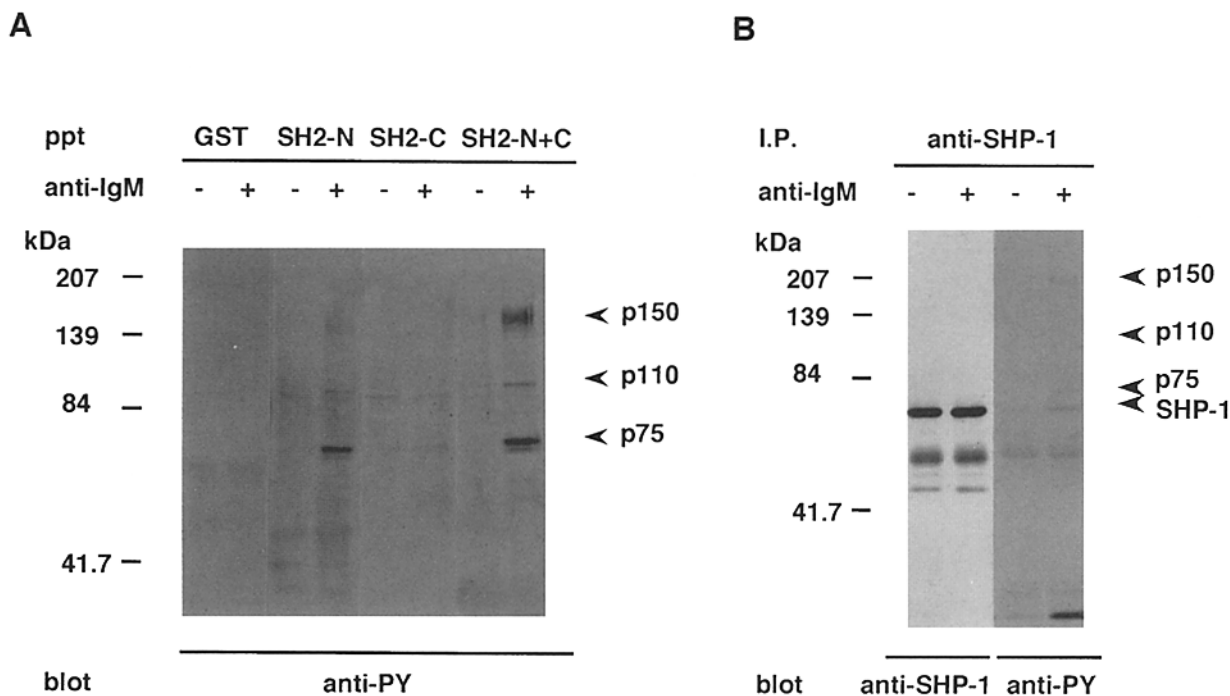
A

B

Figure 1. SHP-1 binds at least three species of proteins both in vitro (*A*) and in vivo (*B*). (*A*) Lysates of WEHI-231 cells, either unstimulated (−) or stimulated with anti-μ Ab (25 μg/ml) for 5 min (+), were incubated with either SHP-1-SH2-N-GST (SH2-N), SHP-1-SH2-C-GST (SH2-C), and SHP-1-SH2-N+C-GST (SH2-N+C) fusion proteins, or GST alone. Bound proteins were resolved by SDS-PAGE and immunoblotted with anti-PY mAb. (*B*) Lysates of WEHI-231 cells, either unstimulated (−) or anti-μ stimulated as above (+), were immunoprecipitated with anti-SHP-1 Ab, subjected to SDS-PAGE, and immunoblotted with anti-SHP-1 Ab or anti-PY mAb. The molecular mass markers used are shown in kilodaltons on the left.
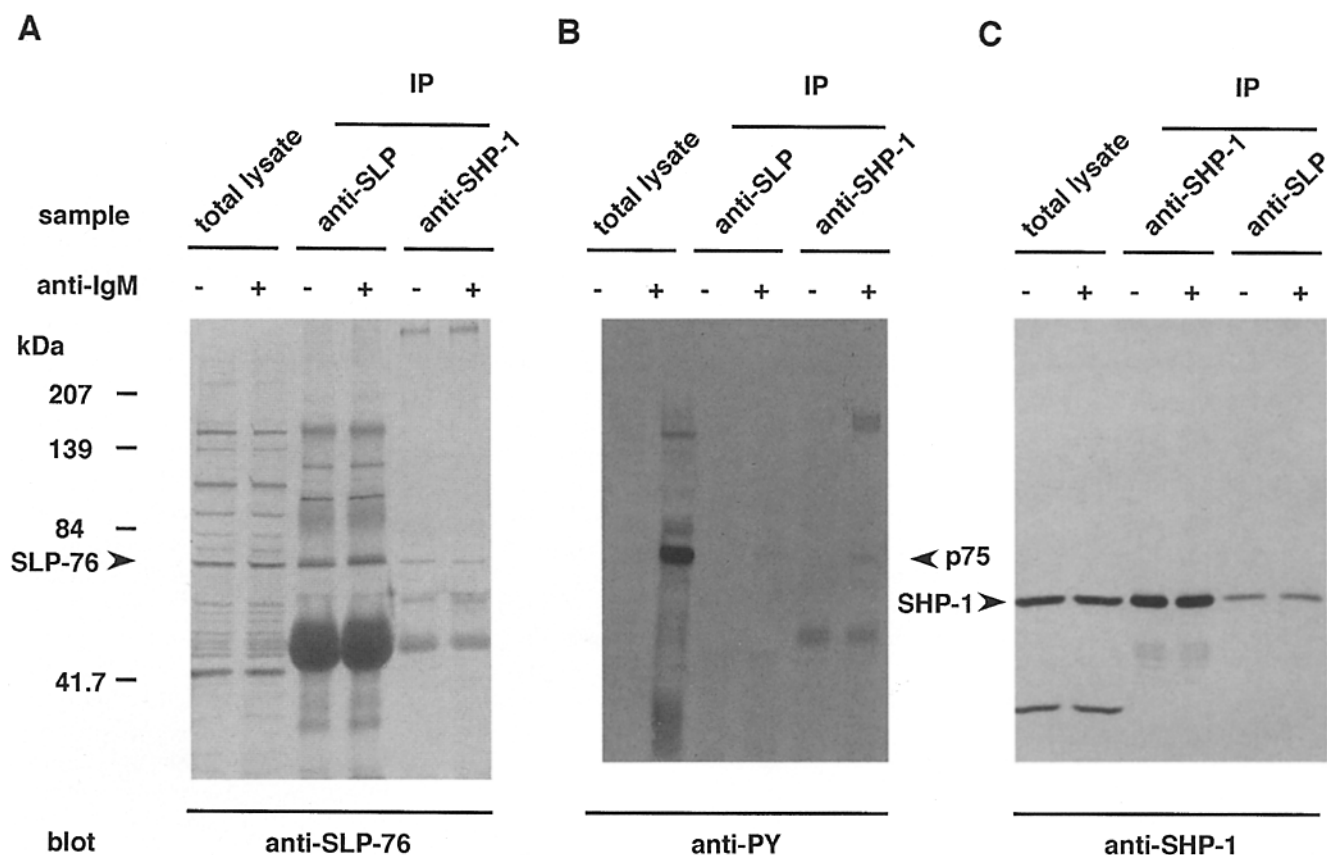
Figure 2. SHP-1 is associated constitutively with SLP-76 in WEHI-231 cells. Lysates of anti-μ–stimulated (+) or unstimulated (−) WEHI-231 cells were precipitated with anti-SLP-76 Ab or anti-SHP-1 Ab, subjected to SDS-PAGE, and immunoblotted with anti-SLP-76 Ab (*A*), anti-PY mAb (*B*), or anti-SHP-1 Ab (*C*). The molecular mass markers used are shown in kilodaltons on the left.

To confirm the association of SHP-1 with SLP-76, depletion experiments were performed. Lysates from anti-μ–stimulated WEHI-231 cells were precipitated with SHP-1-SH2-N+C. The SHP-1–binding proteins were dissociated with heating in SDS sample buffer, diluted in lysis buffer, and then immunoprecipitated with anti-SLP-76 Ab or normal sheep serum. After immunoprecipitation, the supernatants were precipitated with the SH2-N+C, and probed with anti-PY mAb. As shown in Fig. 3, pp75 was almost completely removed by anti-SLP immunoprecipitation, indicating that most of the proteins bound to the SH-N+C are SLP-76. Thus, SHP-1 is constitutively associated with SLP-76 and the association appears to be stable during the early phase of BCR-initiated signal transduction.

Association of SHP-1 with SLP-76 in Normal Splenic B Cells. Finally, we investigated whether the constitutive association of SHP-1 with SLP-76 is detected in normal splenic B cells. Purified B cells were prepared from spleen cells by treatment with anti-Thy-1 mAb and C. As shown in Fig. 4, anti-SHP-1 immunoprecipitates of splenic B cells contained proteins reactive with anti-SLP-76 Ab, suggesting that SHP-1 is constitutively associated with SLP-76 in normal B cells as well.

Accumulating evidence suggests that SHP-1 is involved

in negative regulation or termination of BCR signaling. For instance, using B cells from anti–hen egg lysozyme Ig transgenic mice crossed to *me^v* mice, Cyster and Goodnow (33) have demonstrated that BCR triggers accelerated and greater calcium responses in SHP-1–deficient cells. These investigators also showed that SHP-1 increases a threshold for negative selection of B cells. D'Ambrosio et al. (34) further demonstrated that upon coligation of FcγRIIB1 and BCR, SHP-1 is recruited to a 13–amino acid tyrosine-phosphorylated motif of FcγRIIB1. After binding the receptor, the enzymatic activity of SHP-1 is increased, leading to inhibition of BCR signaling (34). FcγRIIB1-mediated negative signaling is defective in *me* mutant mice (34, 35). Additionally, *me* and *me^v* B cells exhibit an increased response to suboptimal dose of anti-μ Ab (35). It was also shown that SHP-1 is constitutively associated with BCR complex, possibly regulating the phosphorylation state of BCR complex components, and that BCR ligation induces dissociation of SHP-1 from the BCR complex (35). However, to elucidate the exact mechanisms of SHP-1 action and to understand the pathogenesis of *me* mutants, delineation of the molecular interactions involving SHP-1 is essential.

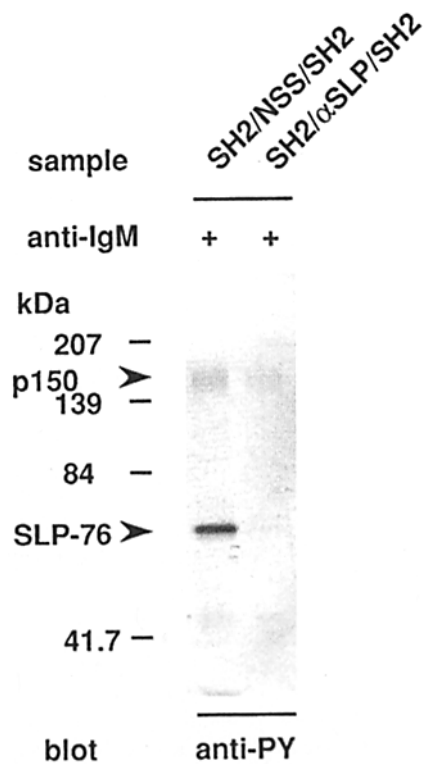The present study was designed to begin such investiga-

Figure 3. Anti-SLP-76 Ab effectively depletes SHP-1–binding pp75. Lysates of anti-μ–stimulated WEHI-231 cells were precipitated with SH2-N+C. The SHP-1–binding fraction was dissociated, and immunoprecipitated with anti-SLP-76 Ab (*right* lane) or normal sheep serum (*NSS; left* lane). The remaining proteins were precipitated again with the SH2-N+C, subjected to SDS-PAGE, and blotted with anti-PY mAb.

Figure 4. Constitutive association between SHP-1 and SLP-76 is observed in splenic B cells. Lysates from T-depleted spleen cells, either before (−) or after (+) anti-μ stimulation, were immunoprecipitated with anti-SHP-1 or anti-SLP-76 Ab, resolved by SDS-PAGE, and immunoblotted with anti-SLP-76 Ab.

tions. The results presented demonstrate that SHP-1 is associated with at least three tyrosine-phosphorylated proteins through its SH2 domains. One of the SHP-1–associated proteins was identified as SLP-76, a hematopoietic cell–specific protein of 533 amino acids that has an NH$_2$-terminal region with potential tyrosine phosphorylation sites, a central proline-rich region, and a COOH-terminal SH2 domain (42). This protein–protein interaction is constitutively observed not only in the WEHI-231 immature B cell line but also in splenic B cells, supporting physiological relevance of SHP-1–SLP-76 association. One possible molecular basis of this interaction is that the binding of the SHP-1 SH2 domain to SLP-76 occurs independently of tyrosine phosphorylation. Alternatively, SH2 domain of SHP-1 can interact even with meagerly tyrosine-phosphorylated SLP-76. Determination of the region(s) of SLP-76 responsible for the SHP-1 binding should resolve this issue.

Functional relevance of SHP-1–SLP-76 interaction in the cascades of BCR signaling also needs to be elucidated. Analysis in T cells has suggested that upon TCR activation, SLP-76 is tyrosine phosphorylated and associated with the adaptor protein Grb2, PLCγ1, and uncharacterized pp36/38 (42). SLP-76 may thus be implicated in linking PTK ac-
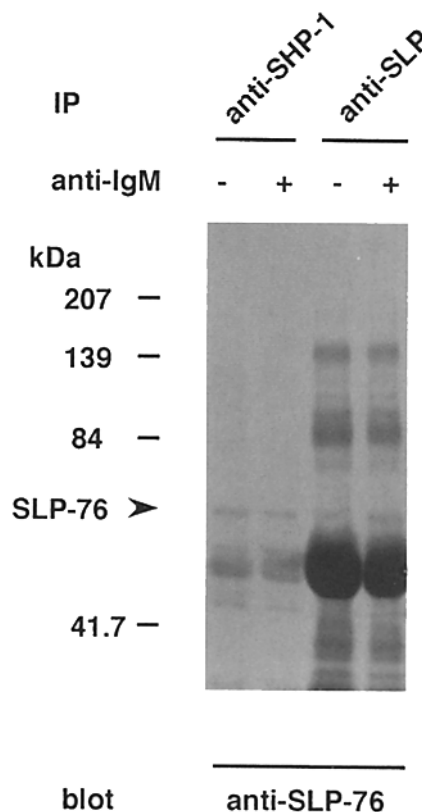
tivation with Ras signaling and/or PLCγ1 activation through its Grb2 association. Whether SLP-76 in B cells behaves in a similar manner or interacts with unique signaling molecules is critical to the understanding of SHP-1 function. Also, information on how the absence of SHP-1 affects the mode of action of SLP-76 may help us to understand better the *me* pathology.

In addition to SLP-76, SHP-1 was shown to bind to 110- and 150-kD phosphoproteins in WEHI-231 cells. It has recently been reported that upon BCR ligation, SHP-1 is recruited to tyrosine–phosphorylated CD22, a BCR complex–associated lectin of 120–135 kD (40, 43), and is activated (40). Our preliminary studies showed that anti-CD22 Ab does not detect a band in anti-SHP-1 or SHP-1 SH2 precipitates. Whether SHP-1 associates with other species of proteins is currently under study.

In summary, our studies demonstrate that SHP-1 is constitutively associated with SLP-76 in WEHI-231 immature B cell line and splenic B cells, mainly through NH$_2$-terminal SH2 domain of SHP-1. Thus, SLP-76 is vital to the understanding of SHP-1–mediated signaling in B cells and of *me* pathogenesis.

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