Src Homology Region 2 (SH2) Domain-Containing Phosphatase-1 Dephosphorylates B Cell Linker Protein/SH2 Domain Leukocyte Protein of 65 kDa and Selectively Regulates c-Jun NH2-Terminal Kinase Activation in B Cells

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Src Homology Region 2 (SH2) Domain-Containing Phosphatase-1 Dephosphorylates B Cell Linker Protein/SH2 Domain Leukocyte Protein of 65 kDa and Selectively Regulates c-Jun NH2-Terminal Kinase Activation in B Cells

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Src homology region 2 (SH2) domain-containing phosphatase-1 (SHP-1) is a cytosolic protein tyrosine phosphatase containing two SH2 domains in its NH2 terminus. That immunological abnormalities of the motheaten and viable motheaten mice are caused by mutations in the gene encoding SHP-1 indicates that SHP-1 plays important roles in lymphocyte differentiation, proliferation, and activation. To elucidate molecular mechanisms by which SHP-1 regulates BCR-mediated signal transduction, we determined SHP-1 substrates in B cells using the substrate-trapping approach. When the phosphatase activity-deficient form of SHP-1, in which the catalytic center cysteine (C453) was replaced with serine (SHP-1-C/S), was introduced in WEHI-231 cells, tyrosine phosphorylation of a protein of about 70 kDa was strongly enhanced. Immunoprecipitation and Western blot analyses revealed that this protein is the B cell linker protein (BLNK), also named SH2 domain leukocyte protein of 65 kDa, and that upon tyrosine phosphorylation BLNK binds to SHP-1-C/S in vitro. In vitro kinase assays demonstrated that hyperphosphorylation of BLNK in SHP-1-C/S-expressing cells was not due to enhanced activity of Lyn or Syk. Furthermore, BCR-induced activation of c-Jun NH2-terminal kinase was shown to be significantly enhanced in SHP-1-C/S transfectants. Taken collectively, our results suggest that BLNK is a physiological substrate of SHP-1 in B cells and that SHP-1 selectively regulates c-Jun NH2-terminal kinase activation.


Tyrosine phosphorylation of cellular proteins is one of the main mechanisms involved in the regulation of cell proliferation, differentiation, and activation (1). In B cells, engagement of the B cell Ag receptor (BCR)4 by Ag or anti-IgM Ab activates Src family (Lyn, Blk, and Fyn), Syk, and Btk protein tyrosine kinases (PTKs). The activated PTKs phosphorylate a number of downstream substrates and then trigger activation of multiple signal transduction pathways, which lead to cell proliferation, differentiation, activation, or cell death (2–5). The degree of tyrosine phosphorylation of intracellular proteins is strictly regulated by two opposing enzymes, PTKs and protein tyrosine phosphatases (PTPs) (6–10).

Src homology region 2 (SH2) domain-containing phosphatase 1 (SHP-1) is a cytoplasmic PTP containing two SH2 domains at the NH2 terminus, followed by a catalytic domain, which is preferentially expressed in hemopoietic cells (11–14). Point mutation in the gene encoding SHP-1 caused a number of immunological abnormalities found in motheaten (me) and motheaten viable (me+) mice (15, 16), including expansion of CD5+ B-1 B cells (17), reduced number of B cell progenitors in the bone marrow and B-2 B cells in periphery (18), and functional defects in T and NK cells (19, 20). SHP-1 has been implicated in negative regulation of the resting BCR complex (21) and in the threshold determination for BCR signaling and negative selection (22). Biochemical studies have demonstrated that SHP-1 is recruited to tyrosine-phosphorylated immunoreceptor tyrosine-based inhibitory motifs present in the cytoplasmic regions of CD22 (23, 24) and CD72 (25, 26), thus negatively regulating BCR-initiating signals. In addition, we have previously demonstrated that SHP-1 is constitutively associated with SLP-76 in the mouse immature B cell line, WEHI-231 (27). SLP-76 is a leukocyte-specific cytoplasmic protein comprised of acidic region at the NH2 terminus containing three tyrosine residues that can be phosphorylated by Syk family PTKs, followed by a proline-rich region and a COOH-terminal SH2 domain (28). This molecule is involved in the regulation of signaling events initiated by TCR (28–33) and FcεRI (34). Recently, it was demonstrated that SLP-76 is a direct substrate of SHP-1 in T cells and NK cells, and that dephosphorylation of SLP-76 by SHP-1 is a crucial mechanism for the negative regulation of lymphocyte activation by inhibitory receptors (35).

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To understand fully the molecular mechanisms of SHP-1 action in B cells, we identified physiological substrates of SHP-1 by using the substrate-trapping approach (36, 37). When cells were transfected with the catalytically inactive form of SHP-1, with the catalytic center cysteine (C453) being replaced with serine (SHP-1-C/S), BCR-induced tyrosine phosphorylation was strongly enhanced in a protein of about 70 kDa. Immunoprecipitation and Western blot analysis revealed that this protein is the B cell linker protein (BLNK) (38), also named SH2 domain leucocyte protein of 65 kDa (SLP-65) (39), preferentially expressed in B cells. Our results also demonstrated that in in vitro conditions, SHP-1-C/S protein binds tyrosine-phosphorylated BLNK and SHP-1 dephosphorylates BLNK. Furthermore, among members of mitogen-activated protein kinase (MAPK) family, only c-Jun NH₂-terminal kinase (JNK) was more strongly induced upon BCR ligation in SHP-1-C/S-expressing cells than in control and wild-type SHP-1 (SHP-1-wt) transfectants. These results suggest that BLNK is a physiological substrate of SHP-1, and JNK is situated downstream of the signaling pathways initiated by SHP-1 and BLNK.

Materials and Methods

Cells

WEHI-231 cells were obtained from the American Type Culture Collection (Manassas, VA) and were maintained in RPMI 1640 (Sigma, St. Louis, MO) supplemented with 10% FBS (JRH Biosciences, Lenexa, KS), 50 mM 2-ME, 100 mg/ml streptomycin, and 100 U/ml penicillin (complete medium).

Antibodies

Goat anti-mouse IgM Ab was purchased from Cappel, Organon Teknika (Durham, NC). Polyclonal rabbit anti-human SHP-1 Ab, reacting with the mouse protein, anti-Lyn Ab, and anti-Syk Ab were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phosphotyrosine (PY) mAb was purchased from Upstate Biotechnology (Lake Placid, NY). Anti-flag epitope M2 mAb was purchased from Sigma. Rabbit anti-BLNK Ab was raised by immunizing a rabbit with mouse BLNK (aa 4–205) expressed in Escherichia coli as a GST fusion protein. Rabbit anti-mouse phospho-specific p38 Ab and phospho-specific JNK Ab were purchased from New England Biolabs (Beverly, MA), and rabbit anti-mouse phospho-specific ERK was purchased from Promega (Madison, WI). Rabbit anti-mouse ERK-2 Ab, anti-JNK Ab, and anti-p38 Ab were purchased from Santa Cruz Biotechnology. Alkaline phosphatase (AP)-conjugated goat anti-mouse IgG and AP-conjugated mouse anti-rabbit IgG were purchased from Bio-Rad Laboratories (Richmond, CA) and Jackson ImmunoResearch (West Grove, PA), respectively. HRP-conjugated anti-rabbit IgG was purchased from Santa Cruz Biotechnology.

Immunoprecipitation, protein preparation, and Western blot analysis

Immunoprecipitation and Western blot analysis were performed as described previously (27). Briefly, cells (2 × 10⁶) were suspended in 5 ml of complete medium and stimulated for the indicated times with 25 mg/ml anti-IgM Ab. The reactions were stopped with 20 ml ice-cold PBS containing 1 mM Na VO₃ and 2 mM EDTA (PBS-VE). After washing twice with PBS-VE, cells were lysed in 2 ml of TNE buffer (1% Nonidet P-40, 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM NaVO₃, 2 mM EDTA) and centrifuged. The supernatants were immunoprecipitated with protein G-Sepharose coupled with anti-flag mAb or Abs against SHP-1 or BLNK.

In vitro kinase assay

In vitro kinase assay was performed as described previously (40). The immunoprecipitates with Abs against Lyn and Syk were washed four times with TNE buffer and then four times with kinase buffer (20 mM HEPES, pH 8, 150 mM NaCl, 10 mM MgCl₂, 20 mM MnCl₂). After washing, each immunoprecipitate was suspended in 20 ml of kinase buffer containing 10 μCi of [γ-³²P]ATP (6000 Ci/mM) and exogenous substrates, enolase for Lyn and myelin basic protein for Syk, incubated for 3 min (Lyn) or 15 min (Syk) at 30°C. The reaction was stopped by adding SDS-PAGE sample buffer. The samples were resolved by 10% SDS-PAGE, and the resulting gels were treated with 1 N KOH at 60°C for 90 min, dried, and subjected to autoradiography. The intensities of bands were measured with a Bio-Rad Imaging Densitometer, and the results were expressed as fold activation with the intensity of unstimulated cells being 1.

Expression plasmids constructs and transfection

To generate transient expression constructs for SHP-1 and BLNK, PCR fragments containing entire open reading frames for SHP-1 and BLNK were cloned downstream of EF promoter in pEF-flag vector (30) (a gift of Dr. Gary Koretzky, University of Pennsylvania, Philadelphia, PA). The resulting expression vectors, pEF-FLAG-SHP-1-wt and pEF-FLAG-BLNK, encode full-length SHP-1 and BLNK, each containing 8-as peptide tag at NH₂ terminus, which is recognized by anti-flag mAb. To introduce a cysteine-to-serine substitution at the position 453 (C453S) in the PTP domain of SHP-1, in vitro mutagenesis was performed on a full-length SHP-1 cDNA cloned in pBluescript using GeneEditor in vitro site-directed mutagenesis system (Promega). Mutation was identified by the presence of an additional restriction site and then verified by sequencing. Mutated SHP-1 cDNA was then cloned into pEF-flag vector (pEF-FLAG-SHP-1-C/S). For transient transfection, WEHI-231 cells (2 × 10⁶ cells/400 ml/cuvette) were electroporated with 40 μg of the indicated plasmid in the cytomix buffer (120 mM KCl, 0.15 mM CaCl₂, 10 mM K HPO₄/KH₂PO₄ (pH 7.6), 25 mM HEPES (pH 7.6), 2 mM EGTA, 5 mM MgCl₂, 2 mM ATP, 5 mM glutathione), using Bio-Rad Gene Pulser II apparatus set at 270 V and 975 mF. After electroporation, cells were placed on ice for 10 min, transferred into 25 ml complete medium, and cultured for 16–18 h.

In vitro binding assay

To generate constructs for GST-SHP-1-wt and GST-SHP-1-C/S fusion proteins, cDNA fragments encoding PTP domain of SHP-1 (aa 274–595) were generated by PCR using pEF-flag-SHP-1-wt and pEF-FLAG-SHP-1-C/S expression plasmids as templates, respectively. The PCR products were ligated into pGEX–4T3 vector (Amersham Pharmacica Biotech, Uppsala, Sweden). The fusion proteins were generated in E. coli and affinity-purified, as described previously (27). For in vitro binding assay, WEHI-231 cells expressing flag-tagged BLNK were either unstimulated or stimulated with pervanadate for 1 min, and lysed with TNE. The cell lysates were incubated overnight at 4°C with glutathione-Sepharose 4B (Amer- sham Pharmacica Biotech) coupled to SHP-1-wtGST, SHP-1-C/S-GST, or GST alone. Samples were washed five times with TNE and subjected to Western blot analysis with anti-flag and anti-PY mAbs.

In vitro dephosphorylation assay

WEHI-231 cells were transfected separately with pEF-FLAG-SHP-1-wt, pEF-FLAG-SHP-1-C/S, or pEF-FLAG-BLNK. Cells expressing BLNK were stimulated with pervanadate for 1 min, lysed with TNE. BLNK was immunoprecipitated with anti-flag mAb and released from protein G-Sepharose by heating in 0.5% SDS. The eluent was diluted into 100 mM citrate buffer, pH 5, containing 1 mM DTT (PTP buffer), and used as substrate for in vitro dephosphorylation assay. SHP-1-wt and SHP-1-C/S were also immunoprecipitated with anti-flag mAb, washed three times with PTP buffer, and mixed with phosphorylated BLNK. The mixture of SHP-1-wtBLNK with or without Na VO₃ (1 mM) and of SHP-1-C/SBLNK was incubated for 60 min on 37°C. After incubation, reactions were terminated by adding SDS-PAGE sample buffer, and the samples were subjected to Western blot analysis with anti-PY and anti-flag mAbs.

Assays for MAPKs

TNE-soluble supernatants from cells, unstimulated and anti-IgM stimulated, were separated on 10% SDS-PAGE gels and transferred to a nitrocellulose membrane. Western blots were incubated with Abs against ERK, JNK, p38, and their phosphorylated forms, followed by incubation with HRP-conjugated anti-rabbit IgG. The blots were visualized by ECL Western blot detection kit (Amerham Pharmacica Biotech). The intensities of bands were measured with a Bio-Rad Imaging Densitometer, and the results were expressed as fold activation with the intensity of unstimulated cells being 1.
Results
A 70-kDa protein is hyperphosphorylated upon BCR ligation in SHP-1-C/S-expressing cells

We first used the substrate-trapping method (36, 37) to identify substrates for SHP-1. This method was developed based on the fact that a mutant PTP with cysteine residue in the catalytic center (C453) substituted by serine (PTP-C/S) loses its PTP activity, but retains the ability to bind to substrate proteins. Expression of PTP-C/S mutant allows cells to compete with endogenous PTP for substrates, resulting in accumulation of PY on the substrate proteins (36, 37). We first examined for proteins in WEHI-231 cells whose tyrosine phosphorylation was altered when they transiently expressed the mutant form of SHP-1, SHP-1-C/S. As shown in Fig. 1, upon anti-IgM stimulation (25 mg/ml), the degree of tyrosine phosphorylation of a protein of about 70 kDa (p70), indicated by arrows, was enhanced in SHP-1-C/S transfectant cells and slightly reduced in SHP-1-wt transfectants compared with control cells. Tyrosine phosphorylation of a protein at a slightly higher m.w., indicated by line, was not significantly different among groups. Hyperphosphorylation of p70 was observed within 1 min after BCR ligation and maintained for up to 1 h. We considered this phosphoprotein of 70 kDa (pp70) as a candidate substrate for SHP-1 and set out to characterize the nature of this protein by searching for PY-containing signaling molecules of about 70 kDa.

Immunoprecipitation and Western blot analyses demonstrated that the phosphorylation state of 75-kDa HS1 (41) and 72-kDa Syk in SHP-1-C/S-transfected cells was not significantly different from that in SHP-1-wt transfectants. These results suggested that pp70 is not HS1 nor Syk (data not shown).

BLNK is a substrate for SHP-1

It has recently been reported that a novel B cell-specific linker protein, BLNK, is tyrosine phosphorylated after BCR stimulation (38, 39). To test whether pp70 is BLNK, pEF-flag-BLNK expression plasmid was introduced in WEHI-231 cells, together with pEF-flag-SHP-1-wt, pEF-flag-SHP-1-C/S, or pEF-flag vector. Each transfectant was either unstimulated or stimulated with 25 mg/ml anti-IgM Ab for 5 min, and exogenously expressed BLNK was immunoprecipitated with anti-flag mAb and immunoblotted with anti-PY or anti-flag mAb. Results shown in Fig. 2A indicate that upon BCR ligation, BLNK immunoprecipitated from SHP-1-C/S transfectants was tyrosine phosphorylated more strongly than that from vector control transfectants, and conversely, transfection of SHP-1-wt significantly reduced tyrosine phosphorylation of BLNK as compared with vector transfection. In this experiment, similar amounts of BLNK were immunoprecipitated in SHP-1-C/S, SHP-1-wt, and control transfectants (Fig. 2A, bottom panel). Furthermore, when total cell lysates from transfectants of SHP-1-C/S or SHP-1-wt and BLNK were immunoblotted with anti-PY and anti-flag mAbs, exogenously expressed BLNK was shown to migrate at a molecular mass similar to pp70 (Fig. 2B). These results suggested that pp70 might be BLNK.

To confirm that BLNK is a substrate for SHP-1, two approaches were taken. First, phosphorylation states of endogenous BLNK in SHP-1-C/S, SHP-1-wt, and vector transfectants were compared. As shown in Fig. 3, BLNK that was immunoprecipitated from SHP-1-C/S transfectants was tyrosine phosphorylated upon BCR ligation more robustly than that from control transfectants, and again the degree of tyrosine phosphorylation of BLNK from SHP-1-wt transfectants was lower than that from vector transfectants. Second, the binding of BLNK and SHP-1 was examined in in vitro conditions. WEHI-231 cells that were transfected with flag-tagged BLNK were either untreated or treated with pervanadate for 1 min, and the total cell lysates were incubated with Sepharose coupled to GST-SHP-1-wt, GST-SHP-1-C/S, or GST alone. The precipitates were then immunoblotted with anti-flag and anti-PY mAbs.

FIGURE 1. Tyrosine phosphorylation of a 70-kDa protein is enhanced in SHP-1-C/S-transfected cells. A, WEHI-231 cells, transfected with vector alone, SHP-1-wt, or SHP-1-C/S, were stimulated with 25 mg/ml anti-IgM Ab for the indicated times. Total cell lysates were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and then immunoblotted with anti-PY mAb. A band at 70 kDa (pp70), indicated by arrows, is hyperphosphorylated in SHP-1-C/S transfectants compared with control cells, and another band, indicated by line, was not significantly different among groups. The molecular mass markers used are shown in kDa on the left. B, Expression of SHP-1-wt and SHP-1-C/S was confirmed by immunoblotting with anti-flag mAb.
results in Fig. 4 demonstrated that GST-SHP-1-C/S, but not GST-SHP-1-wt nor GST, binds tyrosine-phosphorylated BLNK. Taken collectively, it is reasonable to conclude that BLNK is indeed a substrate for SHP-1.

SHP-1 dephosphorylates BLNK in vitro

To test directly whether SHP-1 dephosphorylates tyrosine-phosphorylated BLNK, we performed in vitro dephosphorylation assay. BLNK was immunoprecipitated from pervanadate-stimulated WEHI-231 cells that had been transfected with flag-tagged BLNK. WEHI-231 cells were also transfected with SHP-1, either wt or C/S mutant, and SHP-1 was immunoprecipitated with anti-flag mAb and added to phosphorylated BLNK. As shown in Fig. 5, pervanadate treatment induced tyrosine phosphorylation of BLNK and the addition of SHP-1-wt resulted in almost complete dephosphorylation of BLNK. However, SHP-1-wt with vanadate (a PTP active site competitor) or SHP-1-C/S could not dephosphorylate BLNK, suggesting that SHP-1 directly dephosphorylates BLNK in vitro.

Activity of Lyn and Syk is not affected in SHP-1-C/S-expressing cells

It has been shown that upon Ag receptor stimulation, BLNK and SLP-76 are rapidly tyrosine phosphorylated by Syk in B cells (38) and by ZAP-70 (30, 33) in T cells, respectively. Therefore, Syk or members of Src family PTKs might be activated as a result of competition between SHP-1-C/S and endogenous SHP-1, phosphorylating BLNK. To exclude this possibility, we examined the phosphorylation state and kinase activity of major PTKs in WEHI-231 cells, Lyn and Syk. WEHI-231 cells transfected with SHP-1-wt or SHP-1-C/S were stimulated with anti-IgM Ab for 3 min, and the phosphorylation state and enzymatic activity of Lyn and Syk were examined by immunoblotting with anti-PY mAb and in

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**FIGURE 2.** Exogenously expressed BLNK is tyrosine phosphorylated more strongly in SHP-1-C/S-transfected cells than that in SHP-1-wt transfecants. A, Flag-tagged BLNK was introduced together with flag-tagged SHP-1-wt, SHP-1-C/S, or empty vector in WEHI-231 cells. Lysates prepared from cells, either unstimulated (−) or stimulated (+) with 25 mg/ml anti-IgM Ab for 5 min, were immunoprecipitated with anti-flag mAb and immunoblotted with anti-PY or anti-flag mAb. B, Cells transfected with flag-SHP-1-C/S alone or with flag-SHP-1-C/S and flag-BLNK were stimulated with 25 mg/ml anti-IgM Ab for 5 min, and the total cell lysates were resolved by SDS-PAGE and then subjected to immunoblotting analysis with anti-PY and anti-flag mAbs. Note that BLNK migrates at a molecular mass similar to pp70.

**FIGURE 3.** Tyrosine phosphorylation of endogenous BLNK is enhanced in SHP-1-C/S transfecants after anti-IgM stimulation. WEHI-231 cells transfected with flag-tagged SHP-1-wt, SHP-1-C/S, or empty vector were either unstimulated (−) or stimulated (+) with 25 mg/ml anti-IgM Ab for 5 min. BLNK was then immunoprecipitated with anti-BLNK Ab, resolved by SDS-PAGE, and immunoblotted with anti-PY mAb or anti-BLNK Ab. To assess the level of SHP-1 transfected, total cell lysates were immunoblotted with anti-flag mAb.

**FIGURE 4.** Tyrosine-phosphorylated BLNK binds SHP-1-C/S in vitro. WEHI-231 cells were transfected with pEF-flag-BLNK and treated without (−) or with (+) pervanadate for 1 min. Total cell lysates were incubated with Sepharose coupled to GST, GST-SHP-1-wt, or GST-SHP-1-C/S, and the bound fractions were subjected to Western blot analysis with anti-flag and anti-PY mAbs.
vitro kinase assays, respectively. The results revealed that tyrosine phosphorylation state as well as kinase activity (both autophosphorylation and phosphorylation of exogenous substrates) of Lyn and Syk were not significantly different between SHP-1-C/S- and SHP-1-wt-expressing cells (Fig. 6). This result indicates that enhanced tyrosine phosphorylation of BLNK in SHP-1-C/S-expressing cells was not due to increased activity of Lyn or Syk, but due to direct action of catalytically inactive mutant of SHP-1.

JNK activation is selectively regulated by SHP-1

Finally, to assess the downstream signaling pathways initiated by SHP-1 and BLNK, MAPK activation was examined in SHP-1-wt- and SHP-1-C/S-expressing cells. WEHI-231 cells transfected with SHP-1-wt, SHP-1-C/S, or empty vector, were stimulated with anti-IgM Ab for 5, 15, and 30 min, and activation of MAPK family members, ERK, JNK, and p38, was measured by immunoblotting with anti-phospho-ERK, -JNK, and -p38 Abs. As shown in Fig. 7A, activation of ERK and p38 in SHP-1-C/S transfectants was not significantly different from that in SHP-1-wt transfectants or control cells. However, activation of JNK was strongly increased in SHP-1-C/S transfectants. Densitometric analysis of three independent experiments further confirms that SHP-1 has a selective, negative regulatory effect on JNK activation (Fig. 7B).

Discussion

It is firmly established that SHP-1 is critically involved in the regulation of cells in the immune system (for reviews, see Refs. 8, 42, and 43). Previous studies underscore the importance of SHP-1 in various aspects of BCR-initiated signaling. For example, SHP-1 is physically associated with the resting BCR complex, down-regulating the BCR complex (21). SHP-1 is also implicated in negative regulation of BCR-induced calcium mobilization and in the threshold determination of negative selection (22). Furthermore, SHP-1 is known to be recruited to phosphorylated immunoreceptor tyrosine-based inhibitory motifs found in the cytoplasmic region of CD22 (23, 24) or CD72 (25, 26), negatively regulating BCR signaling. However, precise biochemical mechanisms by which SHP-1 regulates downstream signaling pathways have been largely unknown.

In this study, we try to elucidate the SHP-1 action by identifying its substrate using the substrate-trapping approach (36, 37). The results presented herein demonstrate that expression of SHP-1-C/S in WEHI-231 cells leads to hyperphosphorylation of a 70-kDa protein upon BCR stimulation (Fig. 1), and that this protein turned out to be a recently identified linker protein, BLNK (38, 39). This conclusion is based on the findings that the m.w. of BLNK is identical to a hyperphosphorylated protein of 70 kDa (Fig. 2B).
that both exogenously expressed and endogenous BLNK is hyper-phosphorylated upon BCR ligation in SHP-1-C/S-expressing cells and transfection of SHP-wt decreases the degree of BLNK phosphorylation (Figs. 2A and 3), that upon tyrosine phosphorylation BLNK binds SHP-1-C/S in vitro (Fig. 4), and that catalytically active SHP-1 dephosphorylates tyrosine-phosphorylated BLNK in vitro (Fig. 5). It is thus concluded that BLNK is a physiological substrate for SHP-1 in WEHI-231 cells. Physical association...
between BLNK and SHP-1/C/S in vivo was not detected under our experimental conditions. Given that SHP-1 acts on diverse substrates and BLNK binds many molecules and that in cells, interaction between these two molecules is dynamic and each interaction event may be transient, it may be difficult to constantly observe interaction between PTPs and their substrates in vivo.

BLNK was identified as a phosphorylated adaptor protein that binds multiple signaling molecules such as Grb2, Vav guanine nucleotide exchange factor, PLC-γ1, and Nck adaptor protein (38, 39, 44). BLNK consists of an NH2-terminal basic and acidic domain, a central proline-rich region, and a COOH-terminal SH2 domain (38, 39). The binding of BLNK with Vav, PLC-γ2, and Nck is mediated by tyrosine phosphorylation-dependent mechanisms involving SH2 domains of the latter, whereas BLNK is constitutively associated with SH3 and SH2 domains of Grb2 (38, 39, 44).

The functional significance of tyrosine phosphorylation of BLNK by Syk was clearly illustrated by the findings that overexpression of wild-type BLNK and mutant BLNK, in which tyrosine-72, -84, -96, and -178 are substituted to phenylalanine, respectively, enhances and reduces BCR-induced tyrosine phosphorylation of PLC-γ1 and PLC-γ2 and BCR-mediated calcium mobilization and transcriptional activation of the NF-AT (38). From these results, the following scenario emerges. BCR ligation induces activation of Lyn and subsequently Syk PTKs, which in turn phosphorylate BLNK. Tyrosine-phosphorylated BLNK is then translocated to the membrane and serves as a scaffold protein for SH2-containing signaling molecules, including PLC-γ1. Membrane-located PLC-γ1 is further tyrosine phosphorylated and activated by Syk, initiating the phosphoinositide pathway.

The results presented in this study demonstrate that among MAPK family members, JNK activation is selectively enhanced by transfection of SHP-1/C/S (Fig. 7), suggesting that SHP-1 plays a negative regulatory role in the JNK pathway. Preliminary results showed that calcium responses were not significantly affected in SHP-1/C/S transfectants. Although 20–25% of cells were successfully transfected as revealed by control transfection with green fluorescence protein, the level of phosphorylation of BLNK observed in SHP-1/C-S transfecants may not be high enough to relay fully transfected as revealed by control transfection with green fluorescence protein.

Furthermore, it has been also reported that the activity of Src family PTKs such as Lck and Fyn is enhanced in thymocytes from SHP-1-deficient me/me mice, suggesting that SHP-1 negatively regulates Src family PTKs (47). Thus, it seems that SHP-1 is a common regulator for not only Src family but also Syk PTKs. However, present study shows that PTKs such as Lyn and Syk are not directly regulated by SHP-1 based on the findings that the enzymatic activity of Lyn and Syk in SHP-1/C/S-expressing cells is not significantly different from that in SHP-1/wt-expressing cells (Fig. 6). Definitive reasons for this discrepancy are not clear at present. Given accumulating evidence suggesting that a PTP acts on different substrates depending on the cellular milieu dictated by the cell type, activation stage, or differentiation stage (48, 49), one of the possible reasons for the discrepant phenomena may be due to differences in the cell type assayed: for example, T cell (47) vs B cell (present study, 46), or immature B cells (present study) vs highly differentiated (class-switched) B cells (46).

In summary, we demonstrate that an adaptor protein BLNK constitutes a physiological substrate for SHP-1 in WEHI-231 B cells. In vitro kinase assays reveal that the kinase activity of Lyn and Syk is not altered in SHP-1/C-S transfected cells, suggesting that these PTKs are not substrates for SHP-1. Furthermore, SHP-1 selectively exerts its negative effect on BCR-induced activation of JNK.

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References


