

Positive and Negative Regulation of High Affinity IgE Receptor Signaling by Src Homology Region 2 Domain-Containing Phosphatase 1¹

Kazuko Nakata,* Tetsuro Yoshimaru,[†] Yoshihiro Suzuki,[†] Toshio Inoue,[†] Chisei Ra,[†] Hidetaka Yakura,^{2*} and Kazuya Mizuno^{3*}

Src homology region 2 domain-containing phosphatase 1 (SHP-1), a cytoplasmic protein tyrosine phosphatase, plays an important role for the regulation of signaling from various hematopoietic cell receptors. Although SHP-1 is shown to be a negative signal modulator in mast cells, its precise molecular mechanisms are not well defined. To elucidate how SHP-1 regulates mast cell signaling, we established bone marrow-derived mast cells from SHP-1-deficient *motheaten* and wild-type mice and analyzed downstream signals induced by cross-linking of high affinity IgE receptor, FcεRI. Upon FcεRI ligation, *motheaten*-derived bone marrow-derived mast cells showed enhanced tyrosine phosphorylation of Src homology region 2 domain-containing leukocyte protein of 76 kDa (SLP-76) and linker for activation of T cells, activation of mitogen-activated protein kinases and gene transcription and production of cytokine. Because the activity of Syk, responsible for the phosphorylation of SLP-76 and linker for activation of T cells, is comparable irrespective of SHP-1, both molecules might be substrates of SHP-1 in mast cells. Interestingly, the absence of SHP-1 expression disrupted the association between SLP-76 and phospholipase Cγ, which resulted in the decreased phospholipase Cγ phosphorylation, calcium mobilization, and degranulation. Collectively, these results suggest that SHP-1 regulates FcεRI-induced downstream signaling events both negatively and positively by functioning as a protein tyrosine phosphatase and as an adaptor protein contributing to the formation of signaling complex, respectively. *The Journal of Immunology*, 2008, 181: 5414–5424.

Mast cells play a central role in the induction of allergic and inflammatory reactions. Mast cells express the high affinity receptor for IgE (FcεRI),⁴ and cross-linking of FcεRI with IgE and multivalent Ag leads to mast cell activation that results in degranulation, release of granules containing preformed chemical mediators, and the synthesis and secretion of cytokines and chemokines (1, 2). FcεRI belongs to the multichain

immune receptor superfamily, consisting of one IgE-binding α-chain, one β-chain, and a homodimer of disulfide-linked γ-chains (3). β- and γ-chains that contain ITAMs in their cytoplasmic regions are essential for mediating downstream signaling events (4). Upon aggregation of FcεRI, the Src-family protein tyrosine kinase (PTK), Lyn, is activated and phosphorylates tyrosine residues in the ITAMs of the β- and γ-chains. Syk PTK is then recruited to the phosphorylated ITAMs of γ-chain via its Src homology region 2 (SH2) domains, becomes tyrosine phosphorylated, and is activated by Lyn (5–7). The activated PTKs phosphorylate a number of signaling molecules, including phospholipase Cγ (PLCγ) (8, 9), Vav (10), SH2 domain-containing leukocyte protein of 76 kDa (SLP-76) (11), linker for activation of T cells (LAT) (12), and linker for activation of B cells/non-T cell activation linker (13). Phosphorylation of these proteins induces calcium (Ca²⁺) mobilization and activation of MAPKs and is ultimately converged into degranulation and production of cytokines. Among these cellular substrates, LAT and SLP-76 play critical roles in the integration of signaling. Following receptor ligation, tyrosine phosphorylated LAT recruits a number of SH2-containing molecules such as Grb2-Sos complex responsible for the activation of Ras-ERK pathway (14, 15), Gads-SLP-76 complex (16), and PLCγ (15, 17). SLP-76 brought to the plasma membrane by interacting with phosphorylated LAT is then tyrosine-phosphorylated by Syk, which provides docking sites for other SH2-containing molecules such as Vav (18, 19), Nck (20), and Itk (21, 22). The central proline-rich region of SLP-76 mediates a constitutive interaction with PLCγ (23). Thus, PLCγ translocated to the plasma membrane is phosphorylated by Syk/Itk PTKs and activated. Accordingly, mast cells deficient for LAT (12) or SLP-76 (24) showed impaired tyrosine phosphorylation of PLCγ and Ca²⁺ mobilization following FcεRI ligation.

*Department of Immunology and Signal Transduction, Tokyo Metropolitan Institute for Neuroscience, Tokyo Metropolitan Organization for Medical Science, Fuchu, Tokyo, Japan and [†]Division of Molecular Cell Immunology and Allergy, Advanced Medical Research Center, Nihon University Graduate School of Medical Sciences, Itabashi-Ku, Tokyo, Japan

Received for publication April 17, 2008. Accepted for publication August 18, 2008.

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¹ This work was supported in part by grants-in-aid from the Japanese Ministry of Education, Culture, Sports, Science and Technology and by a grant-in-aid High-Tech Research Center Project (2002–2006) for Private Universities matching fund subsidy from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

² Current address: Université Paris 1 Panthéon-Sorbonne, 17 rue de la Sorbonne, Paris, France.

³ Address correspondence and reprint requests to Dr. Kazuya Mizuno, Department of Immunology and Signal Transduction, Tokyo Metropolitan Institute for Neuroscience, Tokyo Metropolitan Organization for Medical Science, 2-6 Musashidai, Fuchu, Tokyo, Japan. E-mail address: kzmizuno@tmin.ac.jp

⁴ Abbreviations used in this paper: FcεRI, high affinity IgE receptor; PTK, protein tyrosine kinase; SH2, Src homology region 2; PLCγ, phospholipase Cγ; SLP-76, SH2 domain-containing leukocyte protein of 76 kDa; LAT, linker for activation of T cell; PTP, protein tyrosine phosphatase; SHP-1, SH2 domain-containing phosphatase 1; *me*, *motheaten*; BMMC, bone marrow-derived mast cells; WT, wild type; PY, phosphorylation; HSA, human serum albumin; TCL, total cell lysates; TG, thapsigargin; CRAC, Ca²⁺ release-activated Ca²⁺; STIM1, stromal interacting molecule 1; ER, endoplasmic reticulum.

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Given that the extent of tyrosine phosphorylation of cellular proteins is strictly balanced by PTKs and protein tyrosine phosphatases (PTPs), much attention has recently been focused on a role of PTPs in immune receptor-mediated signaling (25). The cytosolic SH2-domain containing phosphatase-1 (SHP-1) has two SH2 domains in N terminus, one catalytic domain and the C-terminal regulatory domain and is highly expressed in hematopoietic cells. Mutation of the *Shp-1* gene is responsible for the *motheaten* (*me*) and *viable motheaten* phenotypes that develop a severe autoimmune and immunodeficiency syndrome (26, 27). SHP-1 is generally considered as a negative regulator of receptor-mediated signaling in lymphocytes, because B and T cells derived from SHP-1-deficient *me* mice are hyperresponsive to Ag receptor stimulation (28, 29). In mast cells, following FcεRI engagement, SHP-1 is recruited to the phosphorylated tyrosine residues in ITIMs found in the cytoplasmic regions of inhibitory receptors (30–34). As a result, SHP-1 is activated and is predicted to dephosphorylate various signaling molecules including the β/γ subunits of FcεRI and Syk (35), mediating negative regulation of FcεRI-initiated downstream signal transduction. However, knowledge of the molecular mechanisms underlying negative regulation by SHP-1 is still limited.

Recently, by introducing catalytically inactive form of SHP-1 (SHP-1-C/S) into rat basophilic leukemia cell line, RBL-2H3, we have shown that SHP-1 dephosphorylates Tyr-536 of SHP-1, leading to the decrease of its PTP activity (36). Because the overexpression of dominant negative mutants might have nonspecific effects and the presence of endogenous molecules might diminish the effects of mutants, it is necessary to use cells lacking the expression of specific molecules for understanding their physiological roles in cell signaling. In the present study, to elucidate the role of SHP-1 in FcεRI-signaling, we established bone marrow-derived mast cells (BMMC) from *me* and wild-type (WT) mice and analyzed tyrosine phosphorylation states of cellular proteins, activation of MAPKs, Ca^{2+} mobilization, and final outputs such as degranulation and cytokine production, all of which are induced by FcεRI cross-linking. Our results indicate that SHP-1 plays a negative role in FcεRI-induced activation of MAPKs and cytokine gene transcription and production possibly by dephosphorylating SLP-76 and LAT, whereas the phosphorylation of PLC γ , Ca^{2+} mobilization, and degranulation were positively regulated by SHP-1. For the positive regulation, SHP-1 appears to function as an adaptor molecule mediating the association of SLP-76 with PLC γ .

Materials and Methods

Abs and reagents

Anti-DNP IgE mAb (SPE-7), and DNP-human serum albumin (HSA) were purchased from Sigma-Aldrich. HRP-conjugated anti-phosphotyrosine (PY) mAb (PY20) and Abs against SHP-1, Lyn, Syk, ERK2, JNK2, p38, PLC γ -1, and PLC γ -2 were from Santa Cruz Biotechnology. Anti-LAT Ab and Abs against phosphotyrosine 191-human LAT (equivalent to pY195 murine LAT) and phosphotyrosine 226-human LAT (equivalent to pY235 murine LAT) were from Upstate Biotechnology. Anti-phospho-specific JNK, anti-phospho-specific p38, and anti-phosphotyrosine 171-human LAT (equivalent to pY175 murine LAT) Abs were from Cell Signaling Technology. Anti-phospho-specific ERK Ab was from Promega. Anti-SLP-76 Ab was raised in rabbits by immunizing with SH2 domain of mouse SLP-76 (aa 421–533) fused with glutathione S-transferase. All HRP-conjugated secondary Abs were described (37). ECL Western blotting detection kit and protein G Sepharose were obtained from GE Healthcare Bioscience.

Mice

Mice for the present experiments were obtained by mating C3HeB/FeJ *me/+* breeding pairs originally provided by The Jackson Laboratory. To detect *Shp-1* gene mutation, PCR-based genotyping was performed as pre-

viously described (38). All animal experiments were conducted according to the guideline of Tokyo Metropolitan Institute for Neuroscience.

Cell culture and stimulation

Bone marrow cells from femurs of *+/+* and *me/me* mice were cultured in IL-3-containing medium for 4–6 wk to generate WT- and *me*-BMMC, respectively, with >95% purity (c-Kit⁺, FcεRI⁺ as evaluated by flow cytometry). For cell stimulation, cells were incubated with 100 ng/ml SPE-7 overnight. After washing cells twice with PBS to remove excess SPE-7, the cells were precultured in the medium in the absence of IL-3 for 4 h and, then, stimulated with DNP-HSA (50 ng/ml otherwise indicated) for various periods of times.

Immunoprecipitation and Western blot analysis

After simulation, the reaction was stopped with ice-cold PBS containing 1 mM Na₃VO₄ and 2 mM EDTA (PBS-VE). The cells were washed twice with PBS-VE and lysed in TNE buffer (1% Nonidet P-40, 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM Na₃VO₄, and 2 mM EDTA). Subcellular fractionation to obtain cytoplasmic and membrane fractions was performed as described (39). The total cell lysates (TCL), cytoplasmic, or membrane fraction thus prepared were subjected to immunoprecipitation and Western blot analysis as described (37). The intensities of phosphorylated protein bands were measured with a Bio-Rad Imaging Densitometer (Bio-Rad Laboratories), and the results were standardized with the intensities of unphosphorylated protein bands and expressed as fold increase with the intensity of unstimulated cells being 1.0.

In vitro kinase assay

In vitro kinase assay for Syk was performed as described previously (37). In brief, immunoprecipitated Syk was washed three times with TNE buffer and then twice with kinase buffer (20 mM HEPES (pH 8.0), 150 mM NaCl, 10 mM MgCl₂, 20 mM MnCl₂). After washing, the immunoprecipitates were suspended in 10 μ l of kinase buffer containing 10 μ Ci of [γ -³²P] ATP (6000 Ci/ml) and myelin basic protein as an exogenous substrate, and incubated for 15 min at 37°C. The samples were resolved by SDS-PAGE and the resulting gels were dried and subjected to autoradiography.

Measurement of cytokines

SPE-7-sensitized cells were stimulated with 50 ng/ml DNP-HSA for 0.5 h (real-time PCR analysis) or 3 h (enzyme-linked immunosorbent assay). To quantify the transcription of TNF- α and IL-6 genes, real-time PCR was performed as described (36). Levels of TNF- α and IL-6 were first normalized with GAPDH levels and determined relative to the unstimulated WT-BMMC, which was arbitrarily designated a value of 1.0. The amounts of TNF- α and IL-6 in the cell culture supernatants were measured with a Cytoscreen ELISA kit (BioSource International). The results were expressed as amounts/ml per 1×10^6 cells.

Measurement of intracellular Ca^{2+} concentration

BMMC were labeled with the fluorescent Ca^{2+} indicator, fluo-3/AM as previously described (40), after which cells were washed and resuspended in HBSS containing 1 mM CaCl₂. For measuring Ca^{2+} store release, cells were suspended in HBSS containing 1 mM EGTA. Fluo-3 fluorescence was monitored at 5-s intervals up to 3 min with a microplate fluorometer (Fluoroskan Ascent CF; Labsystems; excitation and emission at 485 and 527 nm, respectively). The cytosolic free calcium concentration ($[\text{Ca}^{2+}]_i$) was calculated using the equation: $[\text{Ca}^{2+}]_i = \text{KDa} [(F - F_{\text{min}})/(F_{\text{max}} - F)]$, where KDa is the dissociation constant of the Ca^{2+} -Fluo-3 complex (450 nM), F_{max} represents the maximum fluorescence (obtained by treating cells with 5 μ M A23187), F_{min} represents the minimum fluorescence (obtained for A23187-treated cells in the presence of 1 mM EGTA), and F is the actual sample fluorescence.

Degranulation assay

Degranulation was determined by measuring β -hexosaminidase release. Cells were sensitized with SPE-7 overnight, washed, and precultured in Tyrode's buffer (10 mM HEPES, pH 7.4, 130 mM NaCl, 5 mM KCl, 1.4 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose and 0.1% (wt/vol) BSA) for 4 h. After stimulation of cells with 5 or 50 ng/ml DNP-HSA at 37°C for 30 min, 40 μ l of supernatant or cell lysates and 100 μ l of 2 mM p-nitrophenyl-N-acetyl- β -D-glucosaminide (in 0.4 M citrate and 0.2 M phosphate buffer (pH 4.5)) were added to each well of a 96-well plate, and color was developed for 30 min at 37°C. Enzyme reaction was terminated by adding 200 μ l of 0.2 M glycine-NaOH (pH 10.7). The absorbance at 405 nm was measured in a microplate reader (Bio-Rad

550; Nippon Bio-Rad Laboratories). Cells were lysed with 0.1% Triton X-100, and the β -hexosaminidase activity of the extracts was measured (total). The β -hexosaminidase activity in nonstimulated cells (the spontaneous release, <5% of the total) was subtracted from the enzyme activity in stimulated cells (test). The percentage of β -hexosaminidase released into the supernatant was calculated using the following formula: release (%) = (test - spontaneous)/(total - spontaneous) $\times$ 100.

Results

Tyrosine phosphorylation of 75- and 39-kDa proteins is increased in *me*-derived BMMC

We have previously identified SHP-1 as a substrate of SHP-1 itself based on the findings that tyrosine phosphorylation of SHP-1 is increased in RBL-2H3 cells transfected with SHP-1-C/S and that SHP-1 dephosphorylates Tyr-536 of SHP-1 (36). To identify further substrates of SHP-1 in mast cells, we established BMMC from SHP-1-deficient *me* and control WT mice, yielding *me*- and WT-BMMC, respectively. We cultured bone marrow cells in the presence of IL-3 for 4 wk to generate highly pure BMMC as assessed by the expression of Fc ϵ RI and *c-Kit* (data not shown). In addition, the expression level of Fc ϵ RI and *c-Kit* in *me*-BMMC was comparable to that in WT-BMMC (data not shown), suggesting that IL-3-dependent mast cell differentiation *in vitro* is not impaired in the absence of SHP-1. At first, WT-BMMC thus established were incubated with DNP-specific IgE mAb, SPE-7 (100 ng/ml), overnight, precultured in the medium without IL-3 for 4 h, and stimulated with increasing amounts of DNP-HSA (up to 100 ng/ml) for 5 min to determine the optimal condition of stimulation. Cells cultured overnight in the absence of SPE-7 were used as a negative control. The TCL from these cells were subjected to SDS-PAGE and Western blotting with anti-phosphotyrosine (PY) mAb. Tyrosine phosphorylation was comparable between cells after overnight incubation with or without SPE-7 (Fig. 1A, lanes 1 and 2) and was increased as an Ag dose-dependent manner to 50 ng/ml DNP-HSA (Fig. 1A). Based on this result, we concluded that SPE-7 itself failed to induce considerable tyrosine phosphorylation of cellular proteins and that cells would be stimulated with 50 ng/ml DNP-HSA for additional experiments otherwise indicated. We next compared tyrosine phosphorylation of WT- and *me*-BMMC after stimulation for the indicated periods. As shown in Fig. 1B, increased tyrosine phosphorylation was observed in proteins of 75 kDa (pp75), 70–72 kDa, 55 kDa, and 39 kDa (pp39) in *me*-BMMC before and after Fc ϵ RI cross-linking, whereas tyrosine phosphorylation of 130-kDa protein was enhanced in WT-BMMC (pp130). Almost equal protein loading was verified by anti-ERK2 immunoblotting (Fig. 1, A and B, bottom panels). Among the proteins whose tyrosine phosphorylation was increased in *me*-BMMC, we focused on pp75 and pp39 because, based on the m.w., they are predicted to be SLP-76 and LAT, respectively, both of which play critical roles to transduce signals from Fc ϵ RI to downstream. Although tyrosine phosphorylation of 70–72-kDa and 55-kDa proteins was strongly increased in *me*-BMMC before and after Fc ϵ RI ligation, the nature of these proteins are currently unidentified.

SLP-76 and LAT are hyperphosphorylated in *me*-BMMC

To assess whether pp75 and pp39 are SLP-76 and LAT, respectively, we performed combined immunoprecipitation and Western blot analysis. After sensitizing with SPE-7 overnight, BMMC were either left unstimulated or stimulated with 50 ng/ml DNP-HSA for 2 and 5 min, and SLP-76 and LAT were immunoprecipitated and immunoblotted with anti-PY mAb. The results shown in Fig. 2A demonstrated that basal as well as stimulation-induced tyrosine phosphorylation of SLP-76 and LAT was higher in *me*-BMMC than in WT-BMMC. To

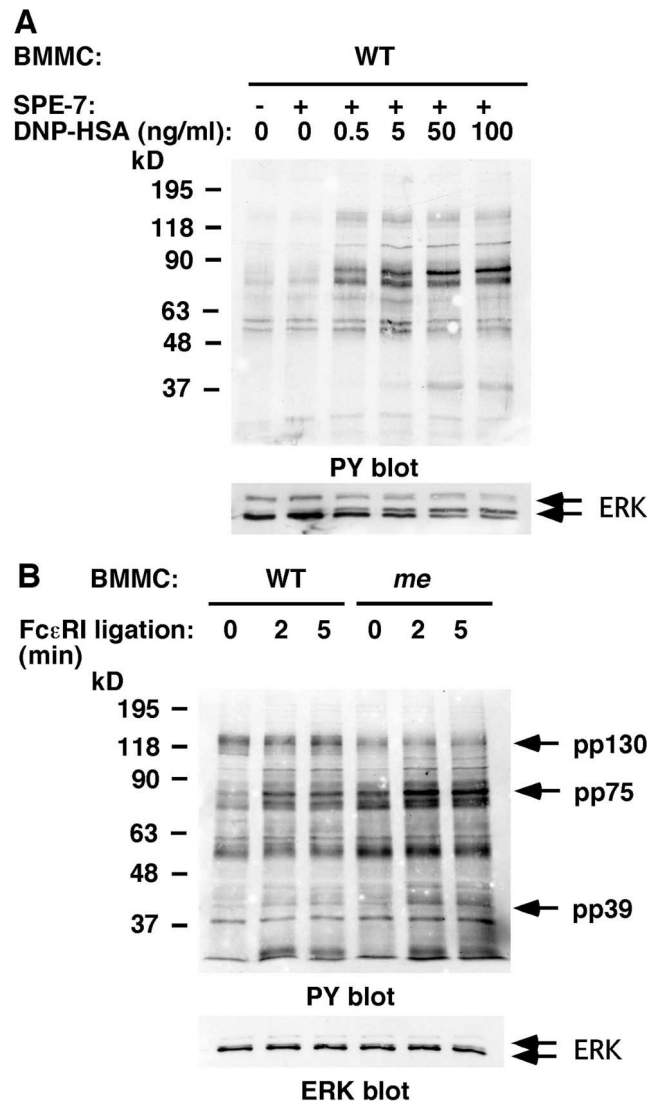


FIGURE 1. Tyrosine phosphorylation of 75- and 39-kDa proteins is increased in *me*-BMMC. **A**, BMMC established from control WT mice were cultured in the presence or absence of 100 ng/ml SPE-7 overnight and stimulated with increasing amounts of DNP-HSA (0–100 ng/ml) for 5 min. TCL were size-fractionated by SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted with anti-PY mAb or anti-ERK Ab. **B**, WT- and *me*-BMMC were sensitized with 100 ng/ml SPE-7 overnight and stimulated with 50 ng/ml DNP-HSA for the indicated periods of time. TCL were subjected to anti-PY and anti-ERK immunoblots. The bands migrating at 75 kDa (pp75) and 39 kDa (pp39), indicated by arrows, showed enhanced phosphorylation in *me*-BMMC whereas phosphorylation of 130-kDa protein (pp130) was increased in WT-BMMC. The m.w. markers used are shown in kDa on the left. The results were confirmed to be reproducible by five independent experiments.

evaluate which Tyr residues in LAT showed increased phosphorylation, we made use of Abs against individual phosphotyrosine residues on LAT and found that the phosphorylation of Tyr-175, -195, and -235 was increased in *me*-BMMC (Fig. 2B). Although these results might suggest that both SLP-76 and LAT are substrates of SHP-1 in mast cells, it is also possible that the lack of SHP-1 expression results in increased activation of PTKs. To examine this possibility, we determined the activity of Syk, a PTK responsible for the phosphorylation of both SLP-76 (41) and LAT (42) in mast cells. Cells were either unstimulated or stimulated for 15 min, and the immunoprecipitated Syk was subjected to

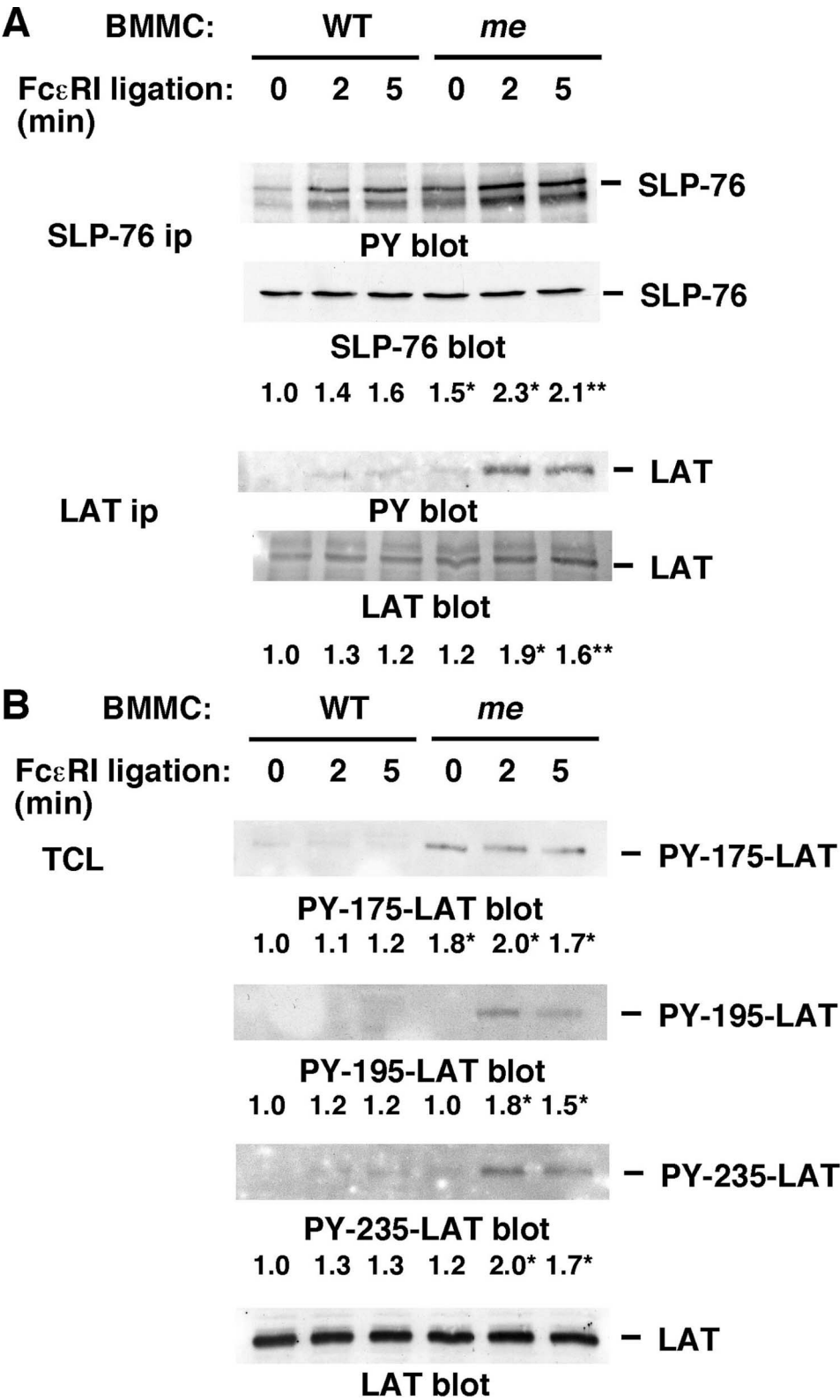


FIGURE 2. Tyrosine phosphorylation of SLP-76 and LAT is increased in *me*-BMMC. **A**, BMMC were sensitized with SPE-7 and stimulated with DNP-HSA as in Fig. 1. Following stimulation of 2 and 5 min with DNP-HSA, SLP-76 and LAT were immunoprecipitated and the samples were divided into two parts, one for anti-PY immunoblot and another for anti-SLP-76 or anti-LAT immunoblot. Numbers at the bottom of each panel indicate average fold increase in SLP-76 or LAT phosphorylation of three independent experiments. *, $p < 0.01$; **, $p < 0.05$, vs WT-BMMC values. **B**, TCL prepared as **A** were subjected to immunoblots with Abs against phospho-Tyr 171-human LAT (equivalent to phospho-Tyr175 murine LAT), phospho-Tyr 191-human LAT (equivalent to phospho-Tyr 195 murine LAT), phospho-Tyr 226-human LAT (equivalent to phospho-Tyr 235 murine LAT), and LAT. Numbers at the bottom of each panel indicate average fold increase in phosphorylation of each Tyr residue of three independent experiments. *, $p < 0.01$ vs WT-BMMC values.

anti-PY immunoblotting and in vitro kinase assay using myelin basic protein as an exogenous substrate. The results clearly demonstrated that the phosphorylation state and kinase activity of Syk were almost identical between in *me*- and in WT-BMMC (Fig. 3). Taken together with the previous findings that SHP-1 can directly dephosphorylate SLP-76 (39, 43) and LAT (44) in other cell types, these results suggest that both proteins are physiological substrates of SHP-1 in mast cells.

FcεRI-induced MAPK activation and cytokine production are increased in me-BMMC

Given that phosphorylated Tyr residues in LAT (14–16) and SLP-76 (18–20) would provide docking sites for several molecules that mediate MAPK activation, hyperphosphorylation of both molecules found in *me*-BMMC is expected to lead to an enhanced activation of MAPKs. To address this possibility, we sensitized BMMC with

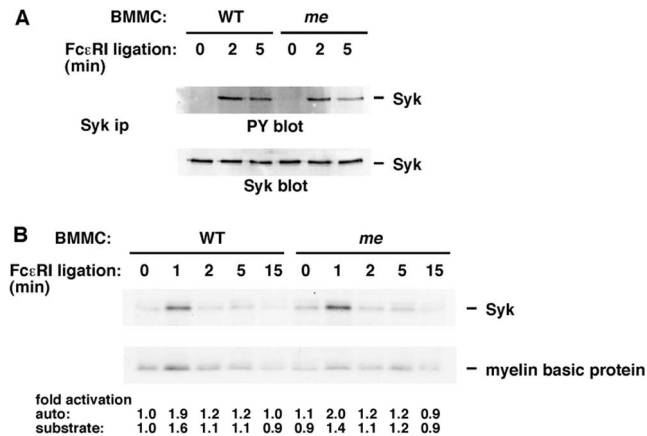


FIGURE 3. Activity of Syk is comparable between in *me*- and WT-BMMC. BMMC were sensitized with SPE-7 and stimulated with 50 ng/ml DNP-HSA for the times indicated. *A*, Syk immunoprecipitated from each BMMC was subjected to immunoblotting with anti-PY mAb. The amount of Syk immunoprecipitated was estimated by immunoblotting using anti-Syk Ab. *B*, The activity of Syk was determined by in vitro kinase assay of Syk and using myelin basic protein as an exogenous substrate. Kinase activity was expressed as average fold activation, the density of the sample from unstimulated WT-BMMC being arbitrarily assigned to 1.0. The results are representative of three independent experiments.

SPE-7 and stimulated them with 50 ng/ml DNP-HSA for 5 and 15 min, and examined the activation status of MAPKs by immunoblot with phospho-specific Abs against ERK, JNK, and p38. Consistent with the phosphorylation states of LAT and SLP-76, lack of SHP-1 expression resulted in an increased phosphorylation of ERK, JNK, and p38 (Fig. 4). It should be noted that increased phosphorylation of ERK was evident even in the absence of FcεRI cross-linking. Because FcεRI-mediated MAPK activation has been demonstrated to lead to gene transcription of several cytokines (35), we next compared the

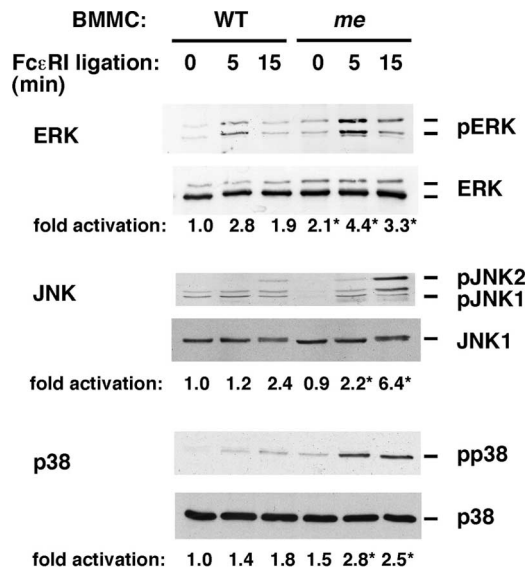


FIGURE 4. FcεRI-induced activation of MAPKs is increased in *me*-derived BMMC. Cells were sensitized with SPE-7 and either unstimulated or stimulated with 50 ng/ml DNP-HSA for 5 and 15 min. TCL were immunoblotted with Abs against ERK, JNK, and p38, and their phosphorylated (active) forms (pERK, pJNK, and pp38). Data are representative of three independent experiments. Numbers at the bottom of each panel indicate average fold increase in MAPK phosphorylation of three experiments. *, $p < 0.01$ vs WT-BMMC values.

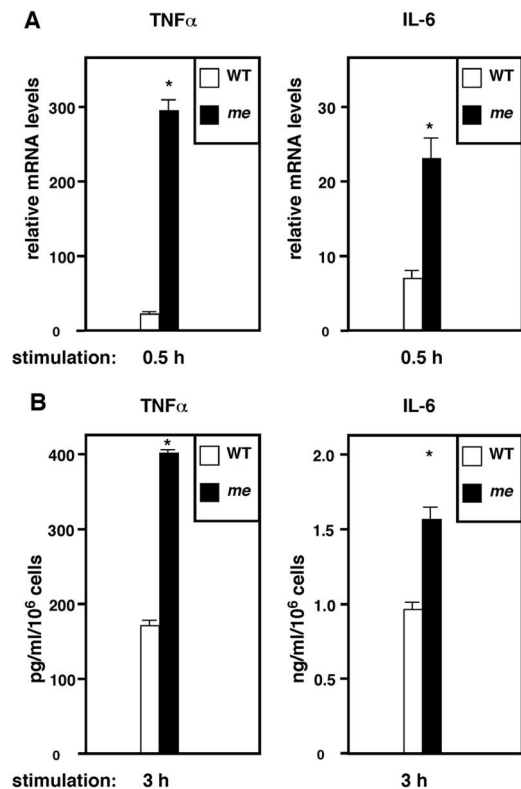


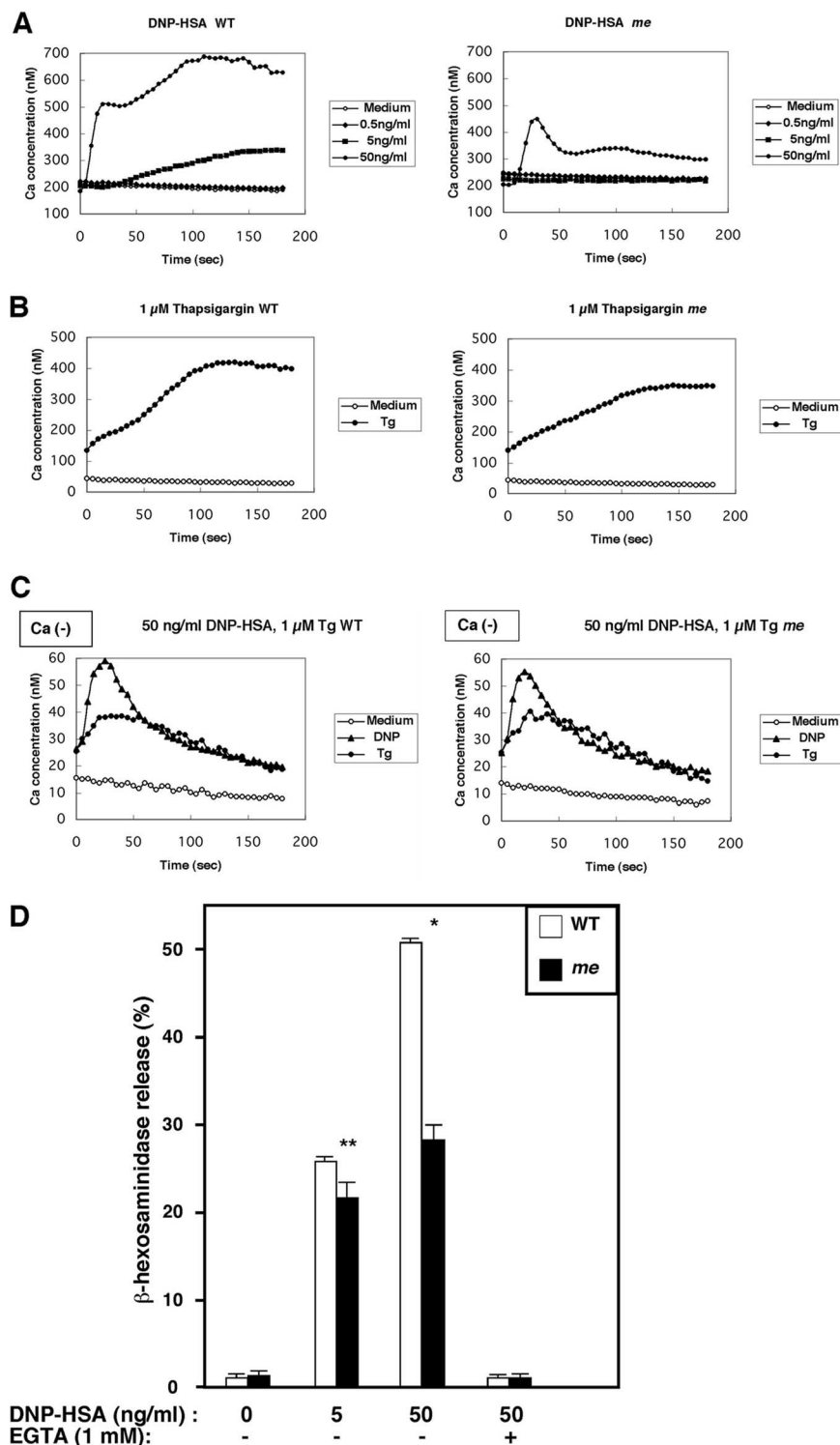
FIGURE 5. Cytokine gene transcription and production are enhanced in *me*-BMMC. *A*, Real-time PCR analysis to quantify TNF- α and IL-6 mRNA production. BMMC were sensitized with SPE-7 and either unstimulated or stimulated with 50 ng/ml DNP-HSA for 0.5 h. Total RNA was prepared and first strand cDNA was synthesized with random hexamer primers. Values obtained were normalized with an internal GAPDH control and fold increases were determined relative to the unstimulated WT-BMMC, which was arbitrarily designated a value of 1.0. Data are shown as the mean $\pm$ SEM (error bars) from five independent RNA samples. *, $p < 0.01$. *B*, Enzyme-linked immunosorbent assay to measure TNF- α and IL-6 mRNA production. BMMC were sensitized with SPE-7 and stimulated with DNP-HSA for 3 h. Data (mean $\pm$ SEM) are representative of three independent experiments. *, $p < 0.01$, vs WT-BMMC values.

transcription of mRNA and production for TNF- α and IL-6 that are the major cytokines induced by FcεRI cross-linking in mast cells (35). Real-time PCR analysis revealed that mRNA production for TNF- α and IL-6 was strongly increased in the *me*-BMMC that were stimulated with SPE-7 and DNP-HSA for 0.5 h (Fig. 5*A*). Accordingly, the production of both cytokines was much more increased in *me*-BMMC, as measured by enzyme-linked immunosorbent assay (Fig. 5*B*). These results indicate that SHP-1 might negatively regulate MAPK-cytokine production pathway by possibly dephosphorylating LAT and SLP-76, critical linker/adaptor proteins for MAPK activation.

FcεRI-induced Ca^{2+} mobilization is impaired in *me*-BMMC

After binding to phosphorylated LAT and SLP-76 via SH2 domain, PLC γ -1 and -2 were tyrosine phosphorylated by Syk and ITK, and activated. This leads to the generation of inositol 1,4,5-triphosphate (IP₃) and the release of Ca^{2+} from endoplasmic reticulum (ER), followed by the influx of extracellular Ca^{2+} (45). We next analyzed another downstream signaling event induced by FcεRI ligation, Ca^{2+} mobilization. As in the case of MAPK activation, it is also likely that hyperphosphorylation of LAT and SLP-76 in *me*-BMMC might enhance Ca^{2+} signaling because increased amount of PLC γ would be recruited to strongly phosphorylated Tyr residues of LAT and SLP-76. Cells were sensitized with

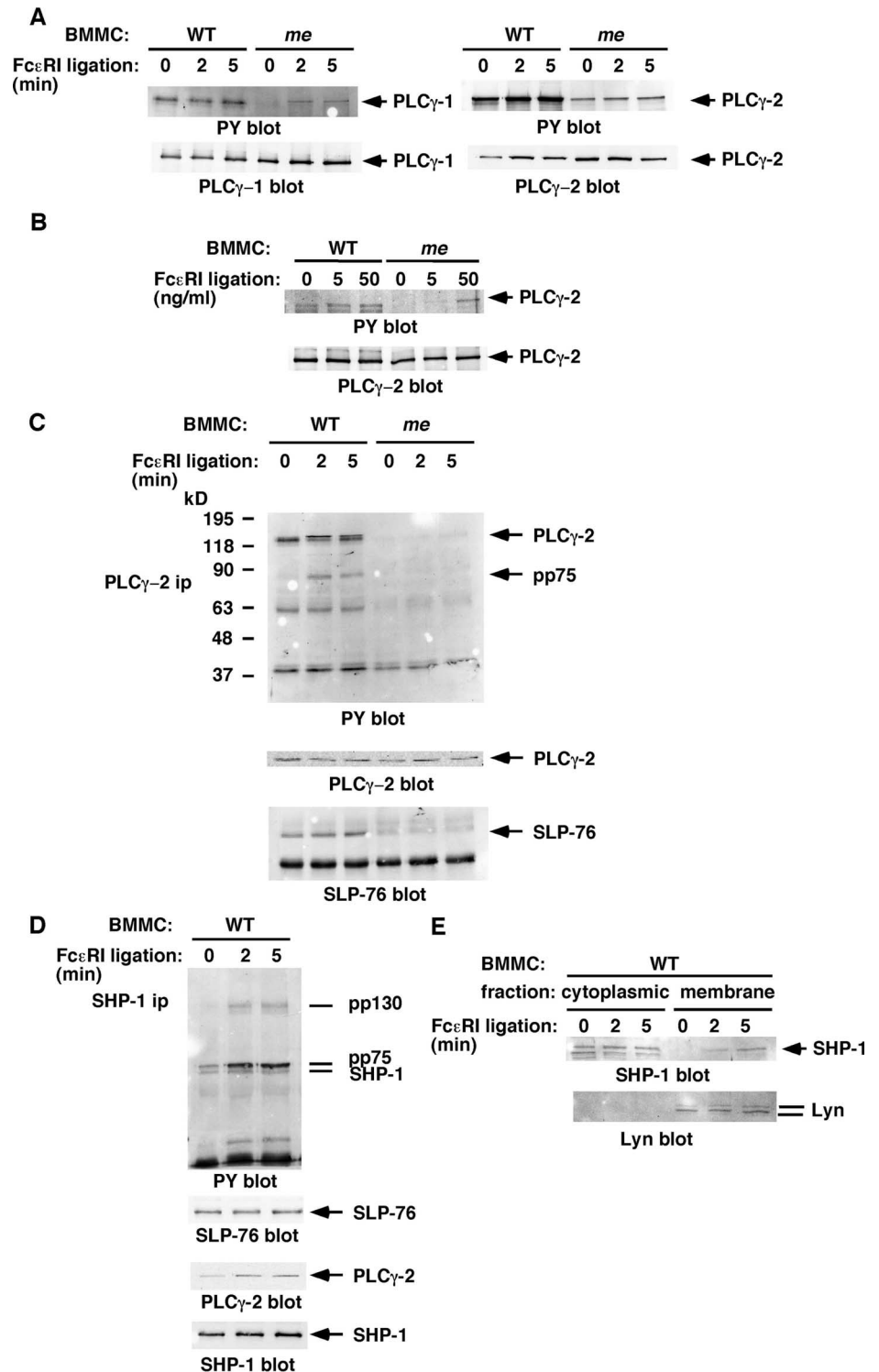
FIGURE 6. Ca^{2+} mobilization and degranulation are suppressed in *me*-BMMC. **A**, Cells were sensitized with SPE-7 overnight and labeled with Fluo-3/AM. After washing, cells were either unstimulated or stimulated with 0.5–50 mg/ml DNP-HSA and then Ca^{2+} mobilization profile was measured by using a microplate fluorometer. **B**, Fluo-3/AM-labeled BMMC were either unstimulated or stimulated with thapsigargin (TG, 1 μM) and Ca^{2+} mobilization profile was measured as in **A**. **C**, Fluo-3/AM-labeled BMMC were suspended in Ca^{2+} free medium (HBSS containing 1 mM EGTA), and either unstimulated or stimulated with 50 ng/ml Ag or TG (1 μM). Ca^{2+} mobilization profile was measured as in **A**. The data are representative of three independent experiments. **D**, Degranulation of WT- and *me*-BMMC, assessed as the release of β -hexosaminidase. Cells were sensitized with SPE-7 overnight and, after washing, were precultured in Tyrode's buffer for 4 h. Then, cells were either unstimulated or stimulated with 5–50 ng of DNP-HSA for 30 min. Degranulation from cells stimulated with 50 ng/ml DNP-HSA in Ca^{2+} -free conditions was measured in the presence of 1 mM EGTA. Data (mean $\pm$ SEM) are representative of seven independent experiments. *, $p < 0.01$; **, $p < 0.05$ vs WT-BMMC values.



SPE-7 overnight, labeled with Fluo-3/AM, and stimulated with 0.5–50 ng/ml DNP-HSA. Surprisingly, the results shown in Fig. 6A clearly demonstrated that Ca^{2+} mobilization was almost completely abrogated with lower than 5 ng/ml DNP-HSA in *me*-BMMC. When cells were stimulated with 50 ng/ml Ag, an initial Ca^{2+} spike was comparable between WT- and *me*-BMMC (Fig. 6A), but a sustained Ca^{2+} plateau was decreased in *me*-BMMC, suggesting that extracellular Ca^{2+} influx induced by ER Ca^{2+} depletion was impaired in *me*-BMMC. To test this possibility, in addition to Ag, we used thapsigargin (TG), a strong and selective inhibitor of the ER Ca^{2+} -ATPase (46). In the medium containing

Ca^{2+} , TG is capable of depleting ER Ca^{2+} stores, thus activating store operated Ca^{2+} channels in the plasma membrane. Although TG-induced Ca^{2+} mobilization was slightly but reproducibly reduced in *me*-BMMC (Fig. 6B), the difference between *me*- and WT-BMMC was marginal compared with that observed when cells were stimulated with Ag (Fig. 6A). Next, we stimulated BMMC with TG or Ag (50 ng/ml DNP-HSA) in the presence of the Ca^{2+} chelator EGTA. This condition allows Ca^{2+} release from intracellular stores but not extracellular Ca^{2+} entry. As shown in Fig. 6C, *me*-BMMC showed similar Ca^{2+} responses induced by Fc ϵ R1 ligation or TG to WT-BMMC. These results suggest that

FIGURE 7. FcεRI-induced tyrosine phosphorylation of PLCγ is severely suppressed in *me*-BMMC by disruption of SLP-76/PLCγ association. **A**, BMMC were sensitized with SPE-7 and either unstimulated or stimulated with 5 ng/ml DNP-HSA for 2 and 5 min. Immunoprecipitated PLCγ-1 (left panel) and -2 (right panel) were divided into two parts, one for immunoblotted with anti-PY mAb and another for with Ab against PLCγ-1 or -2. **B**, Sensitized BMMC were either unstimulated or stimulated with increasing amounts of DNP-HSA for 5 min. PLCγ-2 was immunoprecipitated, divided into two parts, and was subjected to immunoblot one with anti-PY mAb and another with anti-PLCγ-2 Ab. **C**, PLCγ-2 immunoprecipitated from BMMC sensitized and stimulated as in **A** was immunoblotted with anti-PY mAb (top), anti-PLCγ-2 Ab (middle), or anti-SLP-76 (bottom). Tyrosine phosphorylated protein of 75 kDa coimmunoprecipitated with PLCγ-2 is indicated as arrow (top panel, pp75). **D**, WT-BMMC were sensitized and stimulated as in **A**. Immunoprecipitated SHP-1 was subjected to immunoblot with anti-PY mAb (top), anti-SLP-76 Ab (second), anti-PLCγ-2 Ab (third), or anti-SHP-1 Ab (bottom). Tyrosine phosphorylated protein of 130 kDa and 75 kDa coimmunoprecipitated with SHP-1 is indicated by an arrow (top panel, pp130 and pp75). **E**, SHP-1 located in membrane fraction is increased after stimulation. SPE-7-sensitized WT-BMMC were stimulated with 50 ng/ml DNP-HSA for 2 and 5 min. After solubilizing cells with hypotonic buffer, cytosolic, and membrane fractions were separated by ultracentrifugation as previously described (39). The samples thus obtained were subjected to immunoblots with anti-SHP-1 Ab (top) or anti-Lyn Ab (bottom). All the results were representative of three independent experiments.



impaired Ca^{2+} response elicited with the high concentration of Ag (50 ng/ml) in *me*-BMMC was caused by defective extracellular Ca^{2+} influx, although ER Ca^{2+} depletion-dependent Ca^{2+} influx machinery per se seem to be intact in *me*-BMMC.

Although it is widely accepted that Ca^{2+} mobilization is required for the degranulation (47), additional evidence implies Ca^{2+} -independent pathways in degranulation (48–51). To elucidate the potential role of Ca^{2+} in degranulation, we next measured the release of β -hexosaminidase from WT- and *me*-BMMC following FcεRI ligation. As shown in Fig. 6D, lack of SHP-1 resulted in a marked decrease of degranulation when cells were stimulated with 50 ng/ml DNP-HSA,

consistent with the impaired Ca^{2+} influx in *me*-BMMC. The stimulation with 5 ng/ml DNP-HSA, by which *me*-BMMC showed marginal Ca^{2+} response (Fig. 6A), still induced β -hexosaminidase release, although slightly but significantly less than WT-BMMC. In addition, the presence of EGTA almost completely abolished FcεRI-mediated degranulation even after stimulation with high dose of Ag. These results collectively suggest that SHP-1 is required both for the entry of Ca^{2+} from the extracellular fluid when cells were stimulated with higher concentration of Ag and for the initial Ca^{2+} release from ER after weak FcεRI ligation, and that Ca^{2+} mobilization, especially extracellular Ca^{2+} influx, is required for the degranulation process.

*Tyrosine phosphorylation of PLC γ and the association of SLP-76 with PLC γ are impaired in *me*-BMMC*

Finally, we investigated the molecular mechanisms underlying the positive regulation of Ca²⁺ signaling by SHP-1. At first, we examined tyrosine phosphorylation of PLC γ -1 and -2 following the stimulation with 5 ng/ml Ag for 2 and 5 min. Consistent with Ca²⁺ mobilization, robust tyrosine phosphorylation of immunoprecipitated PLC γ -1 and -2 was not detected in *me*-BMMC (Fig. 7, A and B). In WT-BMMC, there was no difference in the degree and the kinetics of phosphorylation between PLC γ -1 and -2. This increased phosphorylation of PLC γ -1 and -2 might, at least in part, explain the hyperphosphorylation of p130 as observed in TCLs from WT-BMMC (pp130 in Fig. 1). When cells were stimulated with increased concentrations of Ag for 5 min, phosphorylation of PLC γ -2 was detectable at 5 ng/ml in WT-BMMC whereas tyrosine phosphorylation was detectable at 50 ng/ml Ag but not at 5 ng/ml in *me*-BMMC (Fig. 7B). In the course of these analyses, we found another tyrosine-phosphorylated protein of 75 kDa coimmunoprecipitated with PLC γ -2 (Fig. 7C) and PLC γ -1 (data not shown) in WT- but not in *me*-BMMC. Sequential immunoprecipitation and immunoblot revealed that this protein was SLP-76 (Fig. 7C). The association between SLP-76 and PLC γ was detectable in the absence of stimulation (Fig. 7C), possibly through interaction between proline-rich domain of SLP-76 and SH3 domain of PLC γ as reported previously in T cells (23). To confirm that the observed molecular complex was indeed present in mast cells, we examined whether SHP-1 immunoprecipitates contain SLP-76 and PLC γ in WT-BMMC. A constitutive association between SHP-1 and SLP-76 was observed in WT-BMMC as in B cells (52) and PLC γ -1 (data not shown) and PLC γ -2 were coimmunoprecipitated with SHP-1 before and after Fc ϵ RI stimulation (Fig. 7D). Although the association among three proteins appeared to be constitutive, SLP-76 and PLC γ were inducibly tyrosine-phosphorylated following Fc ϵ RI ligation (Fig. 7, C and D). This might be explained, at least in part, by the translocalization of SHP-1 from cytosol to membrane after Fc ϵ RI stimulation (Fig. 7E), where trimolecular complex is likely to be localized in the vicinity to the activated PTKs. Taken together, these results might indicate that SHP-1 is required for the association of PLC γ with SLP-76, the event being essential for the phosphorylation and activation of PLC γ , subsequent Ca²⁺ mobilization and degranulation.

Discussion

SHP-1 is generally considered as a negative signal transducer, essentially by dephosphorylating critical signaling molecules. Accordingly, our present study using *me*- and WT-BMMC has shown that SHP-1 functions as a negative signal modulator for MAPK-cytokine production pathway possibly by dephosphorylating LAT and SLP-76 in mast cells. After Fc ϵ RI ligation, Lyn and Syk PTKs are rapidly activated (5–7), and activated Syk phosphorylates LAT and SLP-76, which then provide docking sites for SH2-containing Grb2/Sos complex (14, 15) and Vav (18, 19)/Nck (20), respectively. We conclude that SLP-76 and LAT are physiological substrates of SHP-1 based on the following findings. First, tyrosine phosphorylation of both molecules induced by Fc ϵ RI aggregation was increased in *me*-BMMC. Secondly, Syk activity was comparable between WT- and *me*-BMMC. Direct dephosphorylation of SLP-76 (39, 43) and LAT (44) by SHP-1 demonstrated in other cell types may support our conclusion. Fc ϵ RI-induced Lyn activation leads to tyrosine phosphorylation and activation of SHP-1, which results in the dephosphorylation and inactivation of SHP-1 itself (36). Thus, it is likely that the tyrosine phosphorylation state of LAT and SLP-76 is strictly balanced by the actions of three

enzymes, Lyn, Syk, and SHP-1. Regarding the localization of SHP-1 in mast cells, many reports demonstrate that, after stimulation, SHP-1 is recruited to phosphorylated tyrosine residues in ITIMs of inhibitory receptors (30–34). Alternatively, a previous report by Xiao et al. (53) demonstrates another possible localization of SHP-1. They showed that SHP-1 is constitutively associated with Fc ϵ RI- β -chain (53), thus exerting an inhibitory control on the Fc ϵ RI- β ITAM (54) and Fc ϵ RI- β -associated Lyn (53). For the strict and effective regulation on the function of LAT and SLP-76 by Lyn, Syk, and SHP-1, all three enzymes should be localized properly during the course of Fc ϵ RI signaling. Although the fraction of membrane-localized SHP-1 appeared to increase after stimulation (Fig. 7E), the experiments to determine the precise subcellular localization of SHP-1 before and after Fc ϵ RI ligation are currently in progress.

In T cells, a previous study demonstrated that Grb2 associates with LAT following TCR stimulation by binding its SH2 domain to three distal phospho-Tyr residues, Tyr-171, -191, and -226 (equivalent to Tyr-175, -195, and -235 in murine LAT) and that phospho-Tyr-171 and 191 in LAT are necessary for Gads binding (55). We found that SHP-1 deficiency resulted in an increase in phosphorylation of all three Tyr residues of LAT (Fig. 2B), which might lead to enhancement of LAT-Grb2-Sos and LAT-Gads-SLP-76 complex formation and of MAPK activation (Fig. 4). Consistent with the published evidence that MAPK activation leads to gene transcription of several cytokines in mast cells (35), mRNA transcription and production of TNF- α and IL-6 were increased in *me*-BMMC (Fig. 5). Based on these results, we concluded that SHP-1 regulates MAPK-cytokine pathway negatively, possibly by dephosphorylating LAT and SLP-76. However, Xie et al. (35) demonstrated that the Fc ϵ RI-induced JNK activation and mRNA transcription of TNF- α were increased in RBL-2H3 cells expressing WT-SHP-1 whereas the expression of phosphatase-deficient SHP-1 had the opposite effect, indicating a positive regulation of these signaling events by SHP-1. The possible explanations for the discrepancy in the role of SHP-1 for the regulation of MAPK-cytokine pathway might be the difference in cell types used and/or experimental system. In contrast to *me*-BMMC that lack SHP-1 expression, RBL-2H3 cells expressing WT- or dominant negative SHP-1 also contain endogenous SHP-1. Thus, it is possible that the introduction of PTP activity-deficient SHP-1 enhances the activity of endogenous SHP-1, leading to strong suppression of certain signaling pathway as suggested our previous report (36).

This study also showed that SHP-1 has a positive role in the pathway leading to the activation of PLC γ and subsequent Ca²⁺ mobilization in mast cells. Regarding PLC γ activation, it has been demonstrated that, following stimulation, PLC γ is recruited to multiple binding sites in the cytoplasmic region of LAT, such as phospho-Tyr-132, -171, -191, and -226 of human LAT, where PLC γ is phosphorylated and activated (57). Given that at least Tyr-175, -195, and -235 of LAT were hyperphosphorylated in *me*-BMMC (Fig. 2B), it is expected that Fc ϵ RI stimulation should induce the increased PLC γ recruitment to LAT and activation of PLC γ in *me*-BMMC. However, the phosphorylation of PLC γ -1 and -2 was considerably reduced in *me*-BMMC (Fig. 7, A–C), implying the presence of LAT-independent pathway for the phosphorylation/activation of PLC γ . Despite ~55% sequence identity between SHP-1 and another SH2-containing phosphatase, SHP-2, it is generally believed that they have opposite roles in cell signaling. SHP-2 is considered as a positive signal transducer mainly based on the findings that cells expressing catalytically inactive SHP-2 or SHP-2-deficient cells exhibit reduced signal activation induced by growth factors and cytokine (56, 57). However, a recent study revealed that SHP-1 as well as SHP-2 plays a positive

role in epidermal growth factor signaling (58). Knock down of either SHP-1 or SHP-2 causes significant reduction of ERK activation. This effect is mediated by the linker function of SHP-1. SHP-1 and SHP-2 interact with each other and SHP-2 binds directly to tyrosine-phosphorylated Gab1, thus forming trimolecular signaling complex. In our system, positive regulation of FcεRI-signaling is likely to be mediated by protein-protein interactions. In the presence of SHP-1, PLCγ-1/2 and SLP-76 can form stable complex (Fig. 7, C and D) that might be required for the tyrosine phosphorylation of PLCγ. In fact, it has been shown that SHP-1 constitutively associated with SLP-76 in B cells (52) and that PLCγ binds directly to the proline-rich region of SLP-76 via its SH3 domain (23). Then, how does this SHP-1-mediated protein-protein interactions function in the signaling pathway? One possibility is that SHP-1 determines the localization of SLP-76/PLCγ complex where PLCγ becomes readily phosphorylated upon stimulation. Again, the association of SHP-1 with FcεRIβ-chain would localize SLP-76/PLCγ complex in the vicinity of FcεRIγ-chain where Syk is recruited and activated. Indeed, although trimolecular complex was constitutively observed, SLP-76 and PLCγ were inducibly phosphorylated in WT-BMMC (Fig. 7, C and D). Furthermore, SHP-1 translocated to the membrane fraction was increased following FcεRI ligation. Alternatively, it is also possible that the formation of trimolecular complex causes the structural change of PLCγ, which would facilitate the exposure of the phosphorylation sites of PLCγ.

When BMMC are stimulated with high concentration of Ag, the initial spike is evoked but a sustained Ca^{2+} plateau is impaired in *me*-BMMC (Fig. 6A), suggesting that there is additional defect of Ca^{2+} mobilization in *me*-BMMC, extracellular Ca^{2+} influx. The initial and later phases of Ca^{2+} responses are reflected by the rapid release of ER Ca^{2+} store and extracellular Ca^{2+} entry, respectively. It is believed that the second stage of Ca^{2+} response is initiated as a result of depletion of endoplasmic Ca^{2+} stores, which activates store-operated Ca^{2+} channels such as Ca^{2+} release-activated Ca^{2+} (CRAC) channels (59, 60). Although little is known about how CRAC channels are activated, recent identification of two important components, Orai1 (61) and stromal interaction molecule 1 (STIM1) (62, 63), has shed light on the molecular mechanisms of Ca^{2+} entry through CRAC channels. Although Orai1 is an essential component of the CRAC channel complex, STIM1 is diffusely dispersed throughout the ER membrane and possesses Ca^{2+} -binding motif that may sense the stores. Once the ER Ca^{2+} stores are depleted, STIM1 becomes redistributed into discrete spots under the plasma membrane and activates CRAC channels. The importance of STIM1 in mast cell function has been demonstrated that STIM1-deficient fetal liver-derived mast cells have impaired FcεRI-induced Ca^{2+} influx, degranulation, and cytokine production (64). In addition, Orai1 colocalizes with STIM1 after store depletion and both proteins move in coordinated fashion to form closely apposed clusters in the ER and plasma membrane, thereby creating the elementary unit of store-operated Ca^{2+} entry (65). There might be at least two possible explanations as to why Ca^{2+} influx is impaired in *me*-BMMC. One possibility is that lack of SHP-1 causes dysfunction of STIM1/Orai1. In our study, almost comparable increase in intracellular Ca^{2+} concentration was observed in WT- and *me*-BMMC when cells were treated with TG that is able to activate CRAC channel by depleting ER Ca^{2+} stores (Fig. 6B). This result is in strong contrast to the previous study (64) in which lack of STIM1 completely abrogates Ca^{2+} influx following the treatment with TG or ionomycin, implying the relatively intact function of STIM1/Orai1 in *me*-derived BMMC. Because TG-induced Ca^{2+} response is slightly decreased in *me*-BMMC (Fig. 6B) and the internal Ca^{2+} elevation induced by the stimula-

tion with Ag or TG is comparable between *me*- and WT-BMMC (Fig. 6C), we cannot rule out the possibility that SHP-1 exerts some effects on the STIM1/Orai1-dependent activation of Ca^{2+} channels such as the function of STIM1, the process of STIM1 redistribution, or puncta formation of STIM1 underneath the plasma membrane. The second possibility is that lack of SHP-1 expression might lead to insufficient Ca^{2+} store depletion following Ag stimulation. This possibility is supported by our finding that both *me*- and WT-BMMC showed similar Ca^{2+} mobilization when cells were stimulated with TG to completely deplete Ca^{2+} store in the presence of extracellular Ca^{2+} (Fig. 6B). In addition, TG- or Ag-induced increase in intracellular Ca^{2+} concentration in the absence of extracellular Ca^{2+} is identical between WT- and *me*-BMMC (Fig. 6C). Although these results appear to indicate that there is little difference in the Ca^{2+} release from the ER between two groups, it should be considered that the increase in Ca^{2+} concentration is also mediated by the release from other intracellular Ca^{2+} stores such as mitochondria. Apart from the major role of mitochondria as cellular energy sources and regulator for cell death, recent studies have placed mitochondria as a Ca^{2+} regulator (60). To discriminate the intracellular Ca^{2+} store contributing to the increase of Ca^{2+} concentration following the stimulation with Ag or TG, it may be required to monitor the Ca^{2+} concentration in ER as well as in mitochondria simultaneously. Because mitochondria have been shown to reduce Ca^{2+} -dependent inactivation of CRAC channels by quickly removing the inflowing Ca^{2+} (66, 67), determination of mitochondrial Ca^{2+} concentration might provide further evidences on the regulation Ca^{2+} influx thorough CRAC channel.

Although the importance of Ca^{2+} flux in mast cell degranulation has been widely accepted (47, 68), enhanced or normal degranulation was detected in Lyn-deficient mast cells despite defective Ca^{2+} mobilization (48–51). However, the Ca^{2+} response in Lyn-deficient mast cells was impaired at early transient stage of the response and was almost intact at the later prolonged phase (48, 50, 51) with one exception in which Lyn deficiency caused a marked decrease in overall Ca^{2+} response (49). It should be noted that Stim1-deficient mast cells showed severely impaired sustained Ca^{2+} increase and degranulation (64). Consistent with these published evidences, *me*-BMMC had a decrease in later, sustained Ca^{2+} response and degranulation when cells were stimulated with high dose of Ag (50 ng/ml) (Fig. 6, A and D). Furthermore, treatment of the cells with EGTA almost completely abolished extracellular Ca^{2+} influx and degranulation in WT- and *me*-BMMC. All these observations suggest a prolonged Ca^{2+} increase is required for degranulation. However, stimulation of *me*-BMMC with 5 ng/ml DNP-HSA elicited almost no Ca^{2+} increase but still evident degranulation (Fig. 6, A and D). Taken together with exceptional Lyn-deficient mast cells that had severely reduced Ca^{2+} response but normal degranulation (49), it is possible that slight Ca^{2+} increase in specialized region, such as fusing sites between plasma membrane and granule, might be sufficient to induce degranulation.

In summary, to elucidate the regulatory mechanisms of SHP-1 in mast cell signaling, we identified substrates for SHP-1 by using BMMC established from *me* and WT mice and analyzed downstream signaling events induced by FcεRI ligation. Tyrosine phosphorylation of SLP-76 and LAT was increased in *me*-BMMC. Combined with the almost identical activity of Syk in both BMMC and the previous observations that SHP-1 directly dephosphorylates SLP-76 and LAT in other cell types, we concluded that SLP-76 and LAT serve as substrates for SHP-1 in mast cells. In

accordance with hyperphosphorylation of both molecules, the activation of MAPKs and transcription of cytokine genes are enhanced in *me*-BMMC. In contrast, FcεRI-induced Ca²⁺ mobilization was severely impaired in *me*-BMMC, especially at the later plateau phase when cells were stimulated with high dose of Ag. Upon stimulation with lower concentration of Ag, Ca²⁺ response was abrogated in *me*-BMMC. In the absence of SHP-1, PLCγ failed to associate with SLP-76, which might result in the impairment of Ca²⁺ responses and degranulation. These results suggest a novel molecular mechanism leading to Ca²⁺ mobilization independent of LAT, and provide evidence that SHP-1 can positively regulate signaling events by acting as a linker/adaptor molecule in mast cells.

Disclosures

The authors have no financial conflict of interest.

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