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Positive Regulation of c-Jun N-Terminal Kinase and TNF-α Production But Not Histamine Release by SHP-1 in RBL-2H3 Mast Cells

Zhi-Hui Xie, Juan Zhang, and Reuben P. Siraganian

The SH2-containing protein tyrosine phosphatase (SHP-1) is important for signaling from immune receptors. To investigate the role of SHP-1 in mast cells, we overexpressed the wild-type and the phosphatase-inactive forms of SHP-1 in rat basophilic leukemia 2H3 (RBL-2H3) mast cell line. The phosphatase-inactive SHP-1 (C453S or D419A) retains its ability to bind tyrosine phosphorylated substrates and thereby competes with the endogenous wild-type enzyme. Overexpression of wild-type SHP-1 decreased the FcεRI aggregation-induced tyrosine phosphorylation of the β and γ subunits of the receptor whereas the dominant negative SHP-1 had the opposite effect. The substrate-trapping mutant SHP1/D419A identified pp25 and pp30 as two major potential substrates of SHP-1 in RBL-2H3 cells. Therefore, SHP-1 may play a role in allergy and inflammation by regulating mast cell cytokine production.

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Protein tyrosine phosphorylation is an early and critical event in FcεRI-mediated signal transduction in basophils and mast cells (1). FcεRI, like other immune receptors such as the B cell receptor (BCR) and TCR, lack intrinsic enzymatic activity but contain cytoplasmic immunoreceptor tyrosine-based activation motifs (ITAM) that are critical for cell activation (2–4). After receptor aggregation, there is phosphorylation of the tyrosines in these motifs, which then recruit the Syk/ZAP-70 family protein tyrosine kinase (PTK) and propagate downstream signals (5–7). The phosphorylation of these downstream molecules then results in cell activation. The balance between the action of protein tyrosine phosphatases (PTPs) and kinases regulates the extent of these tyrosine phosphorylations and therefore the signal transduction that results in degranulation and the release of inflammatory mediators.

Identifying possible substrates of PTPs is important for understanding their function in cell signaling. The catalytic tyrosine dephosphorylation reaction requires both the cysteine and the aspartic acid residues of the catalytic domain of PTPs (8), with the cysteine reacting with the phosphate in the substrate to form a thio-phosphate intermediate while the aspartic acid then facilitates the cleavage of the P-O bond in the substrate (9, 10). PTPs that have a mutation of this aspartic acid to alanine (D/A mutant) retain binding affinity for the substrate, but, because of reduced catalytic activity, they form a stable enzyme-substrate complex (11). Such D/A mutants have been used to identify possible substrates of PTPs (12, 13).

The Src homology 2 (SH2)-containing cytoplasmic PTPs (SHP) have two SH2 domains in the N-terminal half and one catalytic domain in the C-terminal half. The SH2 domains function not only to recruit the enzyme to tyrosine-phosphorylated molecules but also to regulate the enzymatic activity. SHP-1 and SHP-2 are the two members of this family of nonreceptor PTP. SHP-1 (also known as PTP1C, hemopoietic cell phosphatase, and SHPTP-1) is primarily expressed in hemopoietic tissues but is also present in epithelial cells (14). In contrast, SHP-2 (also called Syk, PTP1D, PTP2C, or SHPTP-2) is more ubiquitously expressed. SHP-1 is predominantly a negative regulator of signal transduction; for example, B cells and T cells from SHP-1-deficient mice are hyperresponsive to immune receptor stimulation (15). Such hyperresponsiveness is a result of the loss of the activity of SHP-1 that normally dephosphorylates signal transduction molecules (16–18). Similarly, the recruitment of SHP-1 to the agonist-stimulated erythropoietin receptor results in dephosphorylation and inactivation of Janus kinase 2 (JAK2) and the termination of receptor-stimulated cell proliferation (19).

There are several PTPs in basophils and mast cells (20, 21). CD45 is a receptor-type PTP present on some basophils or mast cells that may play a role in some aspects of signaling in these cells (22–24). There is also PTP activity that is present in basophils or mast cells that may be important for reversing or regulating the phosphorylation of the receptor subunits (1, 25, 26). Previously, we observed that both SHP-1 and SHP-2 were present in the rat basophilic leukemia 2H3 (RBL-2H3) mast cell line, and, like SH2 domain-containing inositol phosphatase (SHIP), both SHP-1 and SHP-2 were tyrosine phosphorylated in Syk-deficient cells (21). In the present experiments we transfected RBL-2H3 cells with the

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2 Abbreviations used in this paper: BCR, B cell receptor; ITAM, immunoreceptor tyrosine-based activation motif; JNK, c-Jun N-terminal kinase; PTK, protein tyrosine kinase; PTP, protein tyrosine phosphatase; pTyr, phospho-tyrosine; RBL-2H3, rat basophilic leukemia 2H3 cell line; SH2, Src homology 2; SHP, SH2-containing protein tyrosine phosphatase; SHP1/DA, SHP-1 D419A mutant; SHP1/CS, SHP-1 C453 mutant; ERK, extracellular signal-related kinase.
wild-type and negative-dominant forms of SHP-1 to examine the role of this PTP in FcεRI-mediated cellular signaling and mast cell functions. The results demonstrate that SHP-1 decreases the tyrosine phosphorylation of early signaling molecules such as the receptor subunits or Syk but enhances the phosphorylation of c-Jun N-terminal kinase (JNK) and the production of TNF-α. However, SHP-1 did not affect the extent of the FcεRI-induced histamine release.

Materials and Methods

Materials

Aprotinin, Triton X-100, iodoacetic acid, DTT, and protein A-conjugated agarose were obtained from Sigma (St. Louis, MO). Polyclinylidene difluoride transfer membrane was purchased from Millipore (Bedford, MA), and the enhanced chemiluminescence reagent was from NEN Life Science (Boston, MA). The materials for electrophoresis were purchased from Novex (San Diego, CA). The plasmid-containing human full-length SHP-1 was a GST fusion protein in the pGEX-2T vector (Pharmacia) and was kindly provided by Dr. Benjamin G. Neel, (Beth Israel Hospital, Boston, MA). The source of other materials was as described previously (27).

Antibodies

Mouse monoclonal anti-SHP-1 was obtained from Transduction Laboratories (Lexington, KY). The rabbit polyclonal anti-phospho-JNK (pThr-183 and pTyr-185) and anti-SHP-1 Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-JNK Ab and HRP-conjugated anti-phosphotyrosine (pTyr) mAb, 4G10, were obtained from Upstate Biotechnology (Lake Placid, NY). Rabbit polyclonal anti-pERK (pThr-202 and pTyr-204) and anti-extracellular signal-regulated kinase (ERK) were purchased from New England BioLabs (Beverly, MA). All other Abs have been described previously (27).

Construction of plasmids and stable transfection

Full-length SHP-1 cDNA was excised by EcoRI from pGEX-2T and cloned into pBlueScript and then subcloned between the XhoI and XbaI sites of the pSV expression vector (Pharmacia). Cys453 or Asp419 of SHP-1 were mutated to Ser (C/S) mutant and Ala (D/A) mutant respectively using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and confirmed by DNA sequencing. For stable transfection, 20 μg of linearized expression constructs together with 2 μg pSV2-neo vector were cotransfected into 5 × 10^6 RBL-2H3 cells by electroporation (960 mV) as described previously (7). The following constructs of SHP-1 were used: wild-type, C/S, and D/A respectively using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and confirmed by DNA sequencing. For stable transfection, 20 μg of linearized expression constructs together with 2 μg pSV2-neo vector were cotransfected into 5 × 10^6 RBL-2H3 cells by electroporation (960 mV, 310 V) as described previously (7). The following constructs of SHP-1 were used: wild-type, C/S, and D/A mutant. Clones were selected with 350 μg/ml of active G418 (Life Technologies, Grand Island, NY). Cell lines were screened for the level of SHP-1 expression by immunoblotting of total cell lysates with anti-SHP-1 Ab using Western blotting with anti-FcεRI Ab as an internal control. Cell lines that expressed high levels of these proteins were selected for additional experiments.

Expression of GST fusion proteins

The SHP1/D419A cDNA from the pSVL vector was subcloned into the EcoRI sites of the pGEX-2T vector. The GST fusion proteins of wild-type and D419A mutant of SHP-1 were expressed in Escherichia coli and affinity purified using glutathione-Sepharose 4B beads as recommended by the manufacturer (Pharmacia).

Cell culture and activation

RBL-2H3 cells and SHP-1 transfectants were cultured as monolayers in Eagle’s MEM supplemented with 15% heat-inactivated FBS, penicillin, streptomycin, amphotericin, and glutamine (28). For activation, cells were cultured overnight with or without the Ag-specific anti-trinitrophenyl IgE. For kinase release assays, the cell monolayers were washed twice with MEM containing 0.1% BSA and 10 mM pH 7.4 Tris. The cells incubated with IgE were stimulated with the Ag dinitrophenyl coupled to human IgE were stimulated with the Ag dinitrophenyl coupled to human IgE and cultured overnight with or without the Ag-specific anti-trinitrophenyl IgE.

Immunoprecipitation and immunoblotting

After stimulation, the cell monolayers were rinsed once with ice-cold PBS containing Na3VO4 (1 mM) and protease inhibitors (2 mM aminoethylbenzenesulfonyl-fluoride hydrochloride, 10 μg/ml leupeptin, 5 μg/ml pepstatin A, and 0.2 U/ml aprotinin). Cells were then solubilized in Triton lysis buffer (1% Triton X-100, 50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM Na3VO4, and protease inhibitors). The postnuclear supernatants were first preclarified by mixing with protein A-conjugated agarose beads, and then proteins were immunoprecipitated with Abs prebound to protein A-agarose beads. Rabbit anti-mouse IgG Ab was used to couple mouse mAb with protein A-agarose beads. After gentle rotation for 1 h at 4°C, the beads were washed four times with ice-cold Triton wash buffer (lysis buffer with Triton concentration decreased to 0.5%), and the precipitated proteins were eluted by boiling for 15 min with SDS-PAGE sample buffer as described previously (29). For the preparation of total cell lysates, monolayers were rinsed twice with PBS as described above and directly lysed by the addition of 2× SDS-PAGE sample buffer.

Immunoprecipitated proteins or whole cell lysates were separated by SDS-PAGE under reducing conditions and electrotransferred to polyvinylidene difluoride membranes. The membrane was incubated with 4% BSA blocking buffer (10 mM Tris (pH 7.4), 150 mM NaCl, and 0.1% Tween 20) for 1 h at room temperature, and the tyrosine-phosphorylated proteins were detected by HRP-conjugated anti-pTyr Ab 4G10. The membranes were then stripped and reprobed with primary Abs. In all these blots, the proteins were visualized by the enhanced chemiluminescence reagent (NEN life Science, Boston, MA).

Results

Overexpression of wild-type and dominant negative SHP-1,

SHP1/D419A, or SHP1/D419A, in RBL-2H3 cells

To investigate the role of the protein tyrosine phosphatase SHP-1, we transfected different forms of the molecule into the RBL-2H3 mast cell line. To reduce the catalytic activity of SHP-1, we transfected different forms of the molecule into the RBL-2H3 mast cell line. To reduce the catalytic activity of SHP-1, two residues in the catalytic domain, Cys453 and Asp419, were mutated to Ser (C453S) and Ala (D419A), respectively. These mutations in the catalytic domain decrease enzymatic activity but still allow enzyme-substrate binding and, when overexpressed, have a negative-dominant effect in cells (30). The binding to substrate will also allow the identification of major substrates. The clones selected with G418 were screened by immunoblotting with SHP-1, and cell lines were picked in which there was at least 10-fold overexpression of this protein compared with the parental RBL-2H3 cells.
FIGURE 1. Generation of stable cell lines overexpressing the wild-type or dominant negative forms (C453S and D419A) of SHP-1. RBL-2H3 cells were transfected with the various SHP-1 cDNAs and stable clones were selected with 350 μg/ml G418. Two positive cloned lines expressing each of the different forms of SHP-1 were selected for detailed analysis. Total cell lysates (2 × 10^5 cell equivalents/lane) from the RBL-2H3, and cells transfected with the wild-type (SHP1/WT), C453S (SHP1/CS), or D419A (SHP1/DA) SHP-1 or empty vector (Vector) were immunoblotted with anti-SHP-1 and anti-FceRIβ Abs, respectively.

(Fig. 1). Six independent lines isolated with each different form of SHP-1 were analyzed for histamine release. Two clones from each transfection were then used to further define signaling in these cells. There was no change in SHP-1 expression in the cells transfected with only the pSV2-neo plasmid.

The earliest event after FcεRI stimulation is the phosphorylation of proteins on tyrosine residues, which is critical for downstream signal propagation (1, 31, 32). As compared with that in RBL-2H3

FIGURE 2. The effect of overexpression of wild-type SHP-1 or dominant negative SHP-1 on FcεRI-induced tyrosine phosphorylation of cellular protein. RBL-2H3 and cell lines expressing wild-type (SHP1/WT) or D419A (SHP1/DA) SHP-1 were primed with IgE and stimulated with the 0.1 μg/ml of the Ag DNP-HSA for the indicated times. Total cell lysates (1.5 × 10^5 cell equivalents/lane) were separated by 10% SDS-PAGE and analyzed by immunoblotting with anti-pTyr Ab. Similar results were obtained when the other cell lines transfected with wild-type or the D419A mutant form of SHP-1 were examined.

The effects of SHP-1 on cellular tyrosine phosphorylation

The indicated cell lines were either nonstimulated or stimulated with Ag, and lysates from 5 × 10^6 were immunoprecipitated with anti-FceRIβ Ab and analyzed by immunoblotting with anti-pTyr Ab (A and B). By immunoblotting, there were equal amounts of β and γ in the immunoprecipitates (data not shown). In the time course experiments (A), Ag concentration was 0.01 μg/ml and in the dose response experiments (B) the reaction was for 30 min. The intensities of anti-pTyr signal of FceRI β subunit (C) and γ subunits (D) of SHP-1 transfectants and RBL-2H3 cells were determined by densitometric analysis. The intensity of the signal at each time point is expressed as the percentage of the maximum signal for RBL-2H3 cells. Data represents the mean ± SEM from four separate experiments.

FIGURE 3. SHP-1 negatively regulates the tyrosine phosphorylation of FceRI. The indicated cell lines were either nonstimulated or stimulated with Ag, and lysates from 5 × 10^6 were immunoprecipitated with anti-FceRIβ Ab and analyzed by immunoblotting with anti-pTyr Ab (A and B). By immunoblotting, there were equal amounts of β and γ in the immunoprecipitates (data not shown). In the time course experiments (A), Ag concentration was 0.01 μg/ml and in the dose response experiments (B) the reaction was for 30 min. The intensities of anti-pTyr signal of FceRI β subunit (C) and γ subunits (D) of SHP-1 transfectants and RBL-2H3 cells were determined by densitometric analysis. The intensity of the signal at each time point is expressed as the percentage of the maximum signal for RBL-2H3 cells. Data represents the mean ± SEM from four separate experiments.
cells, the intensity of the total cellular tyrosine phosphorylation induced by FcεRI aggregation was consistently decreased in cells overexpressing wild-type SHP-1, whereas these were increased in the SHP-1 D419A mutant (SHP1/DA) transfected cells (Fig. 2). However, the decrease in these phosphorylations in cells expressing wild-type SHP-1 was less dramatic than the increase in the cells transfected with SHP1/DA. These results suggest that SHP-1 could play a role in regulating signaling in mast cells.

Negative regulation of FcεRI tyrosine phosphorylation by SHP-1

FcεRI, the high affinity receptor for IgE, is an αβγ₂ tetramer with the α subunit responsible for extracellular binding of IgE and the βγ subunits for transducing intracellular signals. Both the β and γ subunits are tyrosine phosphorylated after FcεRI aggregation (33). The phosphorylation of the tyrosine in the ITAMs of these receptor chains then recruits signaling molecules such as Syk to transduce downstream activation signals (4, 34). Therefore, the extent of the tyrosine phosphorylation of the β- and γ-chains could be important for regulating intracellular signals initiated by receptor aggregation. Since we had previously observed that in vitro SHP-1 dephosphorylated the β- and γ-chains of FcεRI, we examined whether FcεRI tyrosine phosphorylation is modulated in vivo by SHP-1. The overexpression of wild-type SHP-1 decreased the Ag-stimulated tyrosine phosphorylation of both the β- and γ-chains, whereas the overexpression of the dominant negative SHP-1 had the opposite effect and caused an increase in these receptor subunit phosphorylations (Fig. 3). In time course and in dose-response experiments, these differences were consistently observed. However, as seen from the densitometric analysis, there was usually less than 2-fold difference in the extent of the tyrosine phosphorylation of the receptor subunits in the transfected cell lines compared with that in the parental RBL-2H3 cells (Figs. 3, C and D). Therefore, the in vivo tyrosine phosphorylation of both the β and γ subunits of FcεRI are negatively regulated by SHP-1.

Negative regulation of Syk tyrosine phosphorylation by SHP-1

Syk tyrosine phosphorylation and activation are critical for FcεRI-mediated mast cell signaling. After FcεRI aggregation, Syk is recruited to the γ subunits and binds to the phosphorylated tyrosines in the ITAM, which results in enzymatic activation and propagation of downstream signals (29, 34, 35). Since the tyrosine phosphorylation of both the β and γ subunits of FcεRI were regulated by SHP-1, we examined whether Syk tyrosine phosphorylation is also affected by SHP-1. Overexpression of wild-type SHP-1 decreased the tyrosine phosphorylation of Syk, whereas the dominant negative form of SHP-1 increased the tyrosine phosphorylation of Syk (Fig. 4). This negative regulation of the tyrosine phosphorylation of Syk (Fig. 4). This negative regulation of the tyrosine phosphorylation of Syk. RBL-2H3 and cells expressing the wild-type (SHP1/WT) or the D419A (SHP1/DA) forms of SHP-1 were either nonstimulated or stimulated with Ag for the indicated times. Lysates were immunoprecipitated with anti-Syk Ab and analyzed by immunoblotting with anti-pTyr and anti-Syk Abs.

Positive regulation by SHP-1 of JNK pathway

Aggregation of FcεRI leads not only to degranulation but also to activation of the mitogen-activated protein kinase ERK, JNK, and p38 pathways that lead to gene transcription and generation of cytokines (36–39). We therefore examined whether transfection with SHP-1 had effects on these pathways. There was a similar amount of phospho-ERK after FcεRI aggregation in the different transfected cell lines (Fig. 5A). However, there were differences in the phosphorylation of the JNK pathway (Fig. 5B). Overexpression of wild-type SHP-1 increased the phosphorylation of JNK whereas overexpression of the dominant negative SHP1/DA decreased this phosphorylation, indicating a positive modulation of JNK phosphorylation by SHP-1. The anti-phospho-JNK Abs recognize pThr-183 and pTyr-185 within the Thr-Pro-Tyr motif, and phosphorylation of these residues leads to the activation of JNK (40). Therefore, SHP-1 positively regulates the FcεRI-induced activation of the JNK but not the ERK pathways.

The activation of the JNK pathway links to cytokine gene transcription. Using a multiprobe RNA protection assay, we examined the FcεRI-mediated induction of cytokine mRNA in the different cell lines (Fig. 6). Among the eleven different cytokines that were tested, the message for IL-4, IL-6, and TNF-α were the main ones induced by FcεRI aggregation in RBL-2H3 cells. The overexpression of wild-type SHP-1 had no detectable effects on IL-4 or IL-6 mRNA production. However, there were significant changes in the mRNA for TNF-α. FcεRI-induced TNF-α mRNA production was slightly increased in the cells that overexpressed the wild-type SHP-1 whereas it was decreased in the cells expressing the dominant negative molecule. In
the cells that overexpressed negative-dominant SHP1/DA, the TNF-α mRNA production was inhibited about 50% compared with the parental RBL-2H3 cells (Fig. 6B). When tested at different Ag concentrations (1 ng/ml and 10 ng/ml) the results for the different cytokines were still similar (data not shown). Therefore, SHP-1 positively regulates the activation of JNK and selectively increases TNF-α mRNA level.

Overexpression of wild-type or dominant negative SHP-1 has no effect on Ag-stimulated histamine release

SHP-1 has been shown to play a negative regulatory role in immune receptor systems such as in B cells, T cells and NK cells (17, 18, 41). The killer inhibitory receptor (KIR)-related inhibitory molecule gp49B1 binds to SHP-1 (42) and co-ligation of gp49B1 with FcεRI inhibits FcεRI-mediated exocytosis (43). SHP-1 in vitro dephosphorylates the β and γ subunits of FcεRI (21). Therefore, we examined the effect of overexpression of the different forms of SHP-1 on mast cell degranulation. Six to eight cloned lines transfected with each of the different forms of SHP-1 (wild-type, SHP1/CS or SHP1/DA) were tested at least three times. The overexpression of wild-type or dominant negative SHP-1 (SHP1/CS or SHP1/DA) had no effect on FcεRI or ionophore mediated histamine release. Both the dose-response curve and the maximum histamine release were similar in all the different transfected cell lines and in cells transfected with vector alone or the parental RBL-2H3 cells. Results with two clones of wild-type or SHP1/DA transfectants are showed in Fig. 7. Therefore these results suggest that histamine release is not regulated by SHP-1.

Identification of pp25 and pp30 as potential substrates of SHP-1 in RBL-2H3 cells

Substrate trapping experiments were used to identify possible substrates of SHP-1. From lysates of FcεRI-stimulated cells two tyrosine phosphorylated proteins of 25 kDa (pp25) and 30 kDa (pp30) were co-immunoprecipitated with SHP-1 (Fig. 8A). As expected, the signal for these two proteins by anti-phosphotyrosine immunoblotting was decreased in the cells that overexpressed wild-type SHP-1 and was increased in the cells overexpressing the dominant negative SHP1/DA. The enzymatic inactive D419A mutant of SHP-1 by still binding to phosphorylated substrate would protect the substrate from being dephosphorylated by endogenous SHP-1. Therefore, the increased tyrosine phosphorylation of pp25

FIGURE 6. Positive regulation of TNF-α mRNA production by SHP-1. A, RBL-2H3 and cell lines expressing wild-type (SHP1/WT) or D419A (SHP1/DA) SHP-1 were either nonstimulated or stimulated for the indicated times with Ag (10 ng/ml). Total RNA was prepared, and 20 μg was used for hybridization with 32P-labeled multiple cytokine probe. After purification, protected mRNA was resolved on denaturing polyacrylamide gels and visualized by autoradiography. B, The intensity of autoradiography signals was determined by densitometric analysis. The data for TNF-α are presented and are expressed as the percentage at each time point compared with the maximum for RBL-2H3 cells. Data represent the average of three separate experiments and are expressed as the mean ± SEM.

FIGURE 7. FcεRI-induced histamine release is not affected by overexpression of wild-type or dominant negative SHP-1. RBL-2H3 and two cell lines expressing wild-type (SHP1/WT) or D419A (SHP1/DA) forms of SHP-1 were cultured overnight with Ag-specific IgE and then stimulated for 45 min with the indicated concentrations of the Ag DNP-HSA. Histamine release was measured in the supernatants and calculated as the percentage of total histamine. Release in nonstimulated cells and in cells activated with the calcium ionophore A23187 was similar in all the cell lines. Data represent the mean ± SEM from three independent experiments.
Indeed, in 10 mM vanadate, the association of pp25 and pp30 with SHP1/DA was tested by immunoprecipitation of SHP-1 in the presence of increasing vanadate concentrations (Fig. 8B). The association of pp25 and pp30 with SHP1/DA transfectants was either nonstimulated or stimulated with Ag for 30 min. Cell lysates were immunoprecipitated with anti-SHP-1 Ab in the presence of 1 mM vanadate and analyzed by immunoblotting with anti-pTyr and anti-SHP-1 Abs. A, The parental RBL-2H3 or the cells transfected with SHP1/DA were treated with pervanadate to inhibit endogenous PTPs. The GST-SHP-1 precipitated pp25 and pp30 from the lysates of both RBL-2H3 cells and SHP-1 dominant negative transfectants. The amount of proteins trapped in RBL-2H3 cells was much less than that in SHP-1 dominant negative cells, probably due to dephosphorylation by the endogenous SHP-1 that may not have been fully inactivated. The GST-SHP1/DA precipitated more proteins than the wild-type SHP-1 fusion protein. The lesser amount of pp25 and pp30 precipitated by the wild-type GST-SHP-1 was probably due to phosphatase activity of SHP-1. A fusion protein containing the two SH2 domains of SHP-1 did not precipitate these two molecules, indicating that these proteins are not interacting with the SH2 domains of SHP-1 (data not shown). Although similar in size to FcεRIβ, these proteins were not blotted with anti-β Ab (data not shown). These data therefore strongly suggest that pp25 and pp30 are two major potential substrates of SHP-1 in RBL-2H3 mast cells and that they are tyrosine phosphorylated after receptor aggregation.

**FIGURE 8.** Identifying of pp25 and pp30 as potential substrates of SHP-1 in RBL-2H3 cells. A, The indicated cell lines were either nonstimulated or stimulated with Ag for 30 min. Cell lysates were immunoprecipitated with anti-SHP-1 Ab in the presence of 1 mM vanadate and analyzed by immunoblotting with anti-pTyr and anti-SHP-1 Abs. B, The D419A SHP-1 (SHP1/DA) transfectants were either nonstimulated or stimulated with Ag for 30 min, and then lysates were prepared and used for immunoprecipitation with anti-SHP-1 Ab in the presence of the indicated concentrations of vanadate. C, RBL-2H3 or the cells expressing D419A SHP-1 were treated with pervanadate for 15 min, and the lysates then used for precipitation with the indicated Sepharose 4B-coupled GST-fusion proteins. The bound proteins were analyzed by immunoblotting with anti-pTyr Abs.

and pp30 in the SHP1/DA transfectant suggests that these molecules could be substrates for SHP-1. Vanadate is a phosphate analogue that competitively inhibits the binding of PTP with their substrates (44). With increasing concentrations, vanadate will replace substrate that is associated with PTPs (12, 13). The association of pp25 and pp30 with SHP1/DA was tested by immunoprecipitation of SHP-1 in the presence of increasing vanadate concentrations (Fig. 8B). Indeed, in 10 mM vanadate, the association of pp25 and pp30 with SHP-1 was disrupted in both stimulated and nonstimulated SHP-1 dominant negative transfectants. The trapping of pp25 and pp30 by endogenous SHP1/DA and the disruption of this association by vanadate strongly suggest that these molecules are in vivo substrates of SHP-1.

The pp25 and pp30 were also trapped in vitro by GST-SHP-1 fusion proteins (Fig. 8C). The parental RBL-2H3 or the cells transfected with SHP1/DA were treated with pervanadate to inhibit endogenous PTPs. The GST-SHP-1 precipitated pp25 and pp30 from the lysates of both RBL-2H3 cells and SHP-1 dominant negative transfectants. The amount of proteins trapped in RBL-2H3 cells was much less than that in SHP-1 dominant negative cells, probably due to dephosphorylation by the endogenous SHP-1 that may not have been fully inactivated. The GST-SHP1/DA precipitated more proteins than the wild-type SHP-1 fusion protein. The lesser amount of pp25 and pp30 precipitated by the wild-type GST-SHP-1 was probably due to phosphatase activity of SHP-1. A fusion protein containing the two SH2 domains of SHP-1 did not precipitate these two molecules, indicating that these proteins are not interacting with the SH2 domains of SHP-1 (data not shown). Although similar in size to FcεRIβ, these proteins were not blotted with anti-β Ab (data not shown). These data therefore strongly suggest that pp25 and pp30 are two major potential substrates of SHP-1 in RBL-2H3 mast cells and that they are tyrosine phosphorylated after receptor aggregation.

**Discussion**

We examined the effect of overexpression of wild-type and negative-dominant SHP-1 on activation pathways in mast cells. The overexpression of wild-type SHP-1 should have the opposite effect to the expression of the catalytically inactive forms (C453S and D419A) that can compete with endogenous wild-type SHP-1 for association with intracellular substrates. Multiple mechanisms could account for the changes in tyrosine phosphorylation of specific cellular proteins in cells expressing the different forms of SHP-1. The increased phosphorylation of proteins in the cells overexpressing the catalytically inactive form could indicate that these molecules are substrates of SHP-1. The binding of the catalytically inactive SHP-1 to the tyrosines of these substrates would prevent the binding and subsequent dephosphorylation by the endogenous functional phosphatase. The FcεRI-induced tyrosine phosphorylation of the FcεRIβ, FcεRIγ, and of Syk was regulated by the expression of wild-type and negative-dominant forms of SHP-1. However, we did not detect the binding of these molecules by the catalytically inactive SHP-1. This suggests that these molecules are not direct substrates of SHP-1. Interestingly, SHP-1 associates and/or dephosphorylates Syk and the related kinase ZAP-70 in B and T cells (45, 46). A second mechanism could be that SHP-1 regulates the enzymatic activity of PTKs that phosphorylate these proteins. For examples, SHP-1 enhances the kinase activity of Src by dephosphorylating the inhibitory C-terminal phosphorylated tyrosine (Tyr-530) (47). Changes in the activity of Lyn could affect the tyrosine phosphorylation of Syk and the receptor subunits. A third mechanism could be by the recruitment of SHP-1 to the membrane; the SH2 domains of SHP-1 bind to immunoreceptor tyrosine-based inhibitory motif sequences present in a number of membrane proteins (48). In mast cells, immunoreceptor tyrosine-based inhibitory motif sequences are present in gp49B1 and the mast cell function-associated Ag (MAFA) (43, 49). The tyrosine in this motif is phosphorylated during receptor aggregation and can then recruit SHP-1 to the membrane. At the membrane, the SHP-1 is phosphorylated and activated by Src-family kinases and can dephosphorylate molecules that are in its...
vicinity (18, 19, 21, 50). Another mechanism could be the association of SHP-1 with immune receptors and signaling molecules such as Vav, Grb2, mSOS, and SLP76 (51–53).

The FcεRI aggregation results in tyrosine phosphorylation of proteins that then transduce the intracellular signals for degranulation. Since FcεRI lacks intrinsic enzymatic activity, it depends on associated and recruited molecules to propagate the intracellular signals. One of the main mechanisms for this signaling is the tyrosine phosphorylation of the ITAM of the β and γ subunits of the receptor. The tyrosine phosphorylated β and γ ITAM then recruits SH2-containing molecules such as Syk (34). The interaction of Syk with the ITAM results in activation, which is pivotal for the pathway that leads to calcium mobilization and histamine release (7, 35). By overexpression, we observed that active SHP-1 decreases the receptor-induced tyrosine phosphorylation of the β and γ subunits of FcεRI, whereas the catalytically inactive form of SHP-1 increased this phosphorylation. Because phosphorylation of the subunits of FcεRI is essential for signaling, one may have predicted that the negative regulation of these phosphorylations by SHP-1 would also result in a decrease in histamine release. Although SHP-1 negatively regulated the tyrosine phosphorylation of the receptor subunits and of Syk, it did not affect the FcεRI-mediated histamine release. Therefore, this level of the tyrosine phosphorylation of the receptor and of Syk in the transfected cell lines was enough for signal transduction to result in histamine release. These results dissociate the extent of tyrosine phosphorylation of the receptor from the eventual histamine release. It is also possible that SHP-1 selectively dephosphorylates molecules that negatively regulate signal transduction.

SHP-1 appears to exert dual effects on FcεRI-mediated mast cell signaling: it negatively regulated tyrosine phosphorylation of Syk and FcεRI, but positively modulated the phosphorylation of JNK and TNF-α production. The phosphorylation and activation of JNK required the phosphatase activity of SHP-1, suggesting that SHP-1 does not function simply as an adapter molecule. The FcεRI aggregation-induced phosphorylation of JNK was enhanced by the overexpression of SHP-1. Activated JNK phosphorylates transcription factors such as c-Jun, leading to gene transcription (40). The increase in the mRNA for TNF-α would then be expected to result from this increase in JNK phosphorylation. In mast cells, the FcεRI aggregation-induced activation of the JNK pathway leads to the expression and production of several cytokines such as TNF-α, IL-2, and IL-6 (38, 39). In HeLa cells, overexpression of wild-type SHP-1 increased epidermal growth factor (EGF)-induced activation of the ERK kinase pathway whereas catalytically inactive SHP-1 had the opposite effect (54). However, in the present experiments with mast cells, SHP-1 did not regulate the activation of the ERK kinase pathway. Although the activation of both ERK and JNK are downstream of Syk, there are differences in the pathways that lead to their activation. The ERK pathway is thought to involve Shc–Grb2, Sos, and Ras, whereas the activation of JNK probably requires Vav and Rac1 (37, 55, 56). The positive effects of SHP-1 may be due to changes in some inhibitory or activating molecules upstream of JNK. Nevertheless, it is possible that SHP-1 regulates FcεRI-mediated TNF-α production via the modulation of the JNK pathway in these cells.

Protein tyrosine phosphorylation plays an important role in signal transduction in cells. PTKs and phosphatases cooperatively regulate the level of the tyrosine phosphorylation of molecules and therefore intracellular signals. Identification of specific substrates of PTPs could help our understanding of the role of these enzymes in signaling. We identified pp25 and pp30 as potential substrates of SHP-1 in RBL-2H3 cells. In vivo, both pp25 and pp30 were hyperphosphorylated in the cells expressing the dominant negative form of SHP-1, and they both coprecipitated with SHP-1. In vitro the association of SHP-1 with pp25/pp30 was disrupted by vandare, and both proteins were dephosphorylated by SHP-1. FcεRI aggregation increased the tyrosine phosphorylation of these proteins, suggesting that they are involved in receptor-mediated signaling.

In summary, by overexpression of wild-type or dominant negative SHP-1, we observed that tyrosine phosphorylation of Syk and the β and γ subunits of FcεRI were negatively regulated by SHP-1. However, the FcεRI-mediated histamine release was unchanged, suggesting that the level of the tyrosine phosphorylation of the receptor subunits and Syk are not the sole determinant of the extent of the signal transduction in the cell. In contrast, SHP-1 positively regulated FcεRI-mediated JNK pathway and TNF-α. SHP-1, by controlling the extent of the production of TNF-α, could play an important role in allergic inflammation.

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References


