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PTPα Activates Lyn and Fyn and Suppresses Hck to Negatively Regulate FcεRI-Dependent Mast Cell Activation and Allergic Responses

Lionel A. Samayawardhana*† and Catherine J. Pallen*†‡

Mast cell activation via FcεRI involves activation of the Src family kinases (SFKs) Lyn, Fyn, and Hck that positively or, in the case of Lyn, negatively regulate cellular responses. Little is known of upstream activators of these SFKs in FcεRI-dependent signaling. We investigated the role of receptor protein tyrosine phosphatase (PTPα), a well-known activator of SFKs in diverse signaling systems, FcεRI-mediated mast cell activation, and IgE-dependent allergic responses in mice. PTPα−/− bone marrow-derived mast cells hyperdegranulate and exhibit increased cytokine and cysteinyl leukotriene secretion, and PTPα−/− mice display enhanced IgE-dependent anaphylaxis. At or proximal to FcεRI, PTPα−/− cells have reduced IgE-dependent activation of Lyn and Fyn, as well as reduced FcεRI and SHIP phosphorylation. In contrast, Hck and Syk activation is enhanced. Syk hyperactivation correlated with its increased phosphorylation at positive regulatory sites and defective phosphorylation at a negative regulatory site. Distal to FcεRI, we observed increased activation of PI3K and MAPK pathways. These findings demonstrate that PTPα activates the FcεRI-coupled kinases Lyn and Fyn and suppresses Hck activity. Furthermore, the findings indicate that hyperactivation of PTPα−/− mast cells and enhanced IgE-dependent allergic responses of PTPα−/− mice are due to the ablated function of PTPα as a critical regulator of Lyn negative signaling. The Journal of Immunology, 2010, 185: 5993–6002.
provided by findings of elevated Lyn activity in Hck−/− mast cells together with some Hck-mediated, Lyn-dependent signaling events and by the elevated Fyn expression or activity detected in Lyn−/− mast cells (9, 11–13). This finding suggests that Lyn suppresses positive regulatory actions of Fyn, whereas Hck may function to suppress Lyn albeit without concomitant effects on Fyn activity. In both cases, the SFK-mediated suppression of another SFK is thought to be effected via activation of the SFK-negative regulatory complex Cbp/PAG-Csk.

Although Cbp/PAG-Csk promotes the phosphorylation of the C-terminal tyrosine residue in SFKs to downregulate mast cell SFK activity, little is known of protein tyrosine phosphatases (PTPs) that counter this phosphorylation and maintain or promote SFK activity downstream of FceRI. Several PTPs with reported activity on SFKs in other signaling systems have been investigated for their roles in FceRI-mediated signaling using PTP-deficient cells. The receptor PTP CD45 regulates SFK activity in immune-receptor signaling (19), and it is required for BMMC degranulation, cytokine release, and IgE-dependent anaphylactic reactions in mice (20, 21). In both unstimulated and Ag-stimulated CD45+/− BMMCs, Lyn is hyperphosphorylated at its inhibitory tyrosine residue (21). Variants of the rat basophilic leukemia cell line RBL-2H3 with greatly reduced CD45 expression exhibit defective recruitment of Lyn to FceRI, reduced FceRI-associated tyrosine kinase activity and FceRI tyrosine phosphorylation, and impaired degranulation (22). Thus, defective CD45-mediated regulation of Lyn is a primary event underlying defective FceRI-mediated responses. BMMCs lacking the nonreceptor PTP SHP-2 exhibit enhanced Ag-induced Lyn activity accompanied by decreased Fyn activation and Fyn-dependent signaling (23). Overall, the lack of SHP-2 did not affect degranulation, but it enhances TNF-α release. The receptor PTPs appears to act via Syk to negatively regulate mast cell activation (24). Ag-stimulated PTPs−/− BMMCs showed enhanced degranulation and cytokine release, but unaffected leukotriene secretion. The most FceRI-proximal alteration detected in these cells was hyperphosphorylation of Syk, whereas Lyn phosphorylation and Fyn-mediated signaling were normal.

PTPx is a receptor PTP that, like CD45 and the closely related PTPγ, has a glycosylated extracellular domain and two tandem catalytic domains, with the majority of activity residing in the membrane proximal domain. In contrast to the mainly hematopoietic expression of CD45 and PTPγ, PTPx is widely expressed. PTPx activates receptor-responsive SFKs in diverse signaling pathways (25); it catalyzes the dephosphorylation of the C-terminal inhibitory tyrosine residue of SFKs (26–29), acting in a manner often coupled to engagement or cross-linking of receptors such as integrins, ligand-gated ion channels, and neural cell adhesion molecules (28, 30–34). In the absence of Ag stimulation in this system, PTPx acted on the active site phosphotyrosine of Lyn to downregulate its basal kinase activity (37). However, the expressed PTPx reportedly exerted these effects even when present at a level approaching that of endogenous Chinese hamster ovary cell PTPx, whereas the endogenous phosphatase did not, raising the possibility of undefined but critical differences between the heterologous and endogenous PTPx. We investigated the role of PTPx in Ag-stimulated FceRI signaling and mast cell activation using BMMCs derived from PTPx−/− mice. We also determined whether the absence of PTPx affected IgE-dependent anaphylactic reactions in mice. We report that PTPx is a negative regulator of these FceRI-dependent responses. The ablation of PTPx inhibited Ag-stimulated Lyn and Fyn activation while enhancing Hck activation. Despite reduced tyrosine phosphorylation of FceRIγ, Syk activity was elevated in PTPx−/− BMMCs, as was the activity of Akt and MAPKs. In conjunction with reduced FceRIJβ tyrosine phosphorylation, SHIP phosphorylation was defective, although that of SHP-1 was increased. Our findings indicate that PTPx plays a major role as an upstream regulator of Lyn-mediated negative signaling, revealing a novel regulatory relationship between Lyn and Hck. Overall, PTPx functions as a key FceRI-proximal phosphatase that initiates and coordinates SFK activities to promote yet limit mast cell activation.

Materials and Methods

Animals

The 129Sv/Ev PTPx−/− mice (29) were backcrossed with C57BL/6 mice for 10 generations. PTPx−/− and wild type (WT; PTPx+/+) C57BL/6 mice were housed under specific pathogen-free conditions. Animal care and use followed the guidelines of the University of British Columbia and the Canadian Council on Animal Care, and were reviewed and approved by the University of British Columbia.

Abs and reagents

Abs to phosphoSer473-Akt, Akt, phospho-Erk1/2, Erk1/2, phospho-p38, ERK1/2, phospho-JNK, JNK, phosphoTyr452-Gab2, phosphoTyr525/526-Syk, phosphoTyr416-Src and phosphoTyr1020-SHIP were purchased from Cell Signaling Technology (Beverly, MA). Ab to phosphoTyr317-Syk was obtained from Invitrogen (Burlington, Ontario, Canada). Abs to Fyn, annexin-V, and 7AAD were obtained from BD Biosciences (San Jose, CA). Abs to Lyn, Fyn, Cbp/PAG, SHP-1, and Syk were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Abs to FceRIγ, FceRIJβ, phosphotyrosine (4G10), Csk, Hck, and Gab2 were obtained from Upstate Biotechnology (Lake Placid, NY). Ab to phosphoTyr529-Src was obtained from BioSource International (Camarillo, CA). HRP-conjugated goat anti-mouse Ab, HRP-conjugated goat anti-rabbit Ab, and Abs for actin and DNP-IgE were obtained from Sigma-Aldrich (St. Louis, MO). Anti-mouse IgE was obtained from MP Biomedicals (Solon, OH). IgE-FITC, c-Kit-PE, rat IgG2a-FITC, and rat IgG2b-PE were obtained from Caltag Laboratories (Burlingame, CA). Recombinant mouse IL-3 was obtained from PeproTech (Rocky Hill, NJ).

IgE-mediated passive cutaneous anaphylaxis

To induce passive cutaneous anaphylaxis, mice (five animals per genotype) were injected intradermally with 10 μg IgE (anti-DNP Ab, clone SPE-7) in saline into the left ear and with saline alone into the right ear. After 24 h, mice were challenged by i.v. injection of 1 mg DNP-human serum albumin (HSA) in saline containing 1% Evans Blue. Control mice were injected with saline alone. Extravasated Evans blue dye in the left ear tissues was extracted with 1 ml of 0.5% Na2SO4 and acetone (3:7) for 24 h, and OD520 nm was measured and normalized to unit weight. The OD from the saline treated right ear was subtracted from the value for the left ear. A second group of mice (three per genotype) was treated essentially as above. Ear tissues were collected 6 h after DNP-HSA challenge, and histologic analysis was performed as described previously (35). Inflamed ear tissue was visualized using a Leica DM4000 B microscope (Leica Microsystems, Deerfield, IL) and Open Laboratory 4.0.2 software (Improvement, Coventry, England).

IgE-mediated passive systemic anaphylaxis

Mice were sensitized by i.v. injection of 2 μg IgE (anti-DNP Ab, clone SPE-7) in 100 μl saline or treated with saline alone. After 24 h, the mice were challenged i.v. with 500 mg DNP-HSA in 100 μl saline for 1.5 min, and blood was immediately collected by cardiac puncture. Serum histamine concentrations were determined by ELISA (Cayman Chemical, Ann Arbor, MI) according to the manufacturer’s specifications.

BMMC culture, stimulation, and lysis

BMMC culture and maintenance were performed as described previously (35). For experiments involving reconstitution of PTPx in PTPx−/− BMMCs, the bone marrow progenitors were infected with murine stem cell virus (MSCV) carrying MSCVpuro (plasmid control) or MSCV-PTPx plasmid and cultured under puromycin selection as described previously (35). Prior to stimulation,
BMMCs were suspended in starvation medium (BMMC medium without IL-3) containing 100 ng/ml anti-DNP IgE and incubated overnight. The cells were washed once in starvation medium and resuspended in prewarmed Tyrodes buffer (10 mM HEPES [pH 7.4], 130 mM NaCl, 5 mM KCl, 1.4 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose, 0.1% BSA) and stimulated with or without 100 ng/ml DNP-HSA (Sigma-Aldrich) at 37°C. Stimulation was stopped with cold PBS containing 0.1 mM Na₃VO₄. Cells were pelleted by centrifugation at 4°C and solubilized in lysis buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 1 mM Na₃VO₄, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 100 μM PMSF) for 30 min at 4°C. Insoluble material was removed by centrifugation at 12,000 rpm for 15 min to generate soluble cell lysates that were used for immunoprecipitation and immunoblotting.

Signals from immunoblotting were quantified by densitometric scanning using Quantity One software (Bio-Rad, Hercules, CA).

**Kinase assays**

Lyn, Fyn, and Hck immunoprecipitates were assayed for kinase activity using the ELISA-based Universal Tyrosine Kinase Assay Kit (GenWay, San Diego, CA) according to the manufacturer’s specifications, and assessed using FACS. Data were statistically analyzed using the Student t test.

**Degranulation**

Degranulation of BMMCs was measured by determining released β-hexosaminidase (38). BMMCs were sensitized with 100 ng/ml anti-DNP IgE and stimulated with varying concentrations of DNP-HSA for 15 min at 37°C. The cells and supernatant of the degranulated material were removed by centrifugation. Cell pellets were solubilized in Tyrodes buffer containing 0.5% Triton X-100 on ice for 15 min followed by centrifugation to remove debris. Supernatants or cell pellet lysates were mixed with 4-nitrophenyl N-acetyl-β-D-glucosaminide (Sigma-Aldrich), a substrate for β-hexosaminidase, in sodium citrate (pH 4.5) for 15 min at 37°C. The enzymatic reaction was stopped by the addition of 0.1 M sodium carbonate (pH 10), and the absorbance at 405 nm was measured. Degranulation was expressed as a percentage of β-hexosaminidase released (absorbance of supernatant divided by absorbance of supernatant plus absorbance of pellet cell lysate). To determine the amount of phosphatidyserine externalization as a measure of degranulation, BMMCs were degranulated essentially as above, followed by labeling with PE annexin V and 7AAD according to the manufacturer’s specifications, and assessed using FACS.

**Mast cell mediator release**

BMMCs were incubated for 5 h with anti-DNP IgE and stimulated with DNP-HSA for 15 h. Culture supernatants were analyzed for IL-2, IL-6, and TNF-α using ELISA kits (R&D Systems, Minneapolis, MN). Alternately, supernatants were collected after 1 h of stimulation and assayed for cysteinyl leukotrienes using an EIA kit (Cayman Chemical).

**Results**

PTPα deficiency leads to FcεRI-dependent hyperdegranulation and augmented cytokine production in BMMCs

To determine whether PTPα regulates FcεRI-dependent release of bioactive mediators from mast cells, we measured mast cell de-
granulation and cytokine production from BMMCs established from WT and PTPα−/− mice. Both types of BMMCs exhibited equivalent expression of the mature mast cell markers c-Kit and FcεRI (Fig. 1A). FcεRI-mediated degranulation of PTPα−/− mast cells was markedly enhanced (up to 2-fold) compared with WT mast cells (Fig. 1B) despite the equivalent β-hexosaminidase content of both types of BMMCs (Fig. 1B, inset). To confirm the observed hyperdegranulation phenotype of PTPα−/− mast cells, annexin V-positive phosphatidylserine on the externalized membrane regions of degranulated mast cells was assessed. Degranulated PTPα−/− mast cells showed significantly higher phosphatidylserine externalization compared with WT cells (Fig. 1C). Activated mast cells not only release cytokines by degranulation, but also synthesize and secrete a variety of cytokines in late phase responses. We therefore determined FcεRI-mediated cytokine release of WT and PTPα−/− mast cells. Significantly elevated levels of IL-2, IL-6, TNF-α, and cysteinyl leukotrienes were released from PTPα-deficient BMMCs (Fig. 1D–G). Collectively, these results demonstrate that PTPα is a negative regulator of FcεRI-dependent BMMC degranulation and cytokine release. To confirm that the observed hyperactivation phenotype of PTPα−/− BMMCs was indeed due to the absence of PTPα, PTPα was re-expressed in the PTPα-null BMMCs by retroviral (i.e., MSCV) infection, to a level that was 86 ± 11% (n = 3) of that in WT BMMCs (Fig. 2A). PTPα re-expression effectively abolished the ability of the BMMCs to hyperdegranulate, because β-hexosaminidase release induced by all concentrations of Ag tested was reduced to levels similar to those of WT BMMCs (Fig. 2B). Likewise, the re-expression of PTPα resulted in the loss of the abilities of these mutant cells to release elevated levels of IL-2 and TNF-α (Fig. 2C). PTPα−/− BMMCs infected with MSCV carrying a (empty) plasmid exhibited degranulation and cytokine release characteristic of the parental PTPα−/− BMMCs (Fig. 2B, 2C).

PTPα-deficient mice show significantly enhanced anaphylactic responses

Anaphylaxis is a profound allergic reaction initiated by allergen-induced cross-linking of specific IgE bound to FcεRI. IgE/FcεRI-stimulated mediator release from mast cells is a major determinant of anaphylaxis (2, 39). Because PTPα-deficient mast cells exhibit FcεRI-dependent hyperdegranulation ex vivo, we questioned whether PTPα plays a role in FcεRI-mediated anaphylaxis in mice. Passive cutaneous anaphylaxis (PCA) was examined upon i.v. challenge with Ag (DNP-HSA in 0.5% Evans blue dye) of sensitized WT and PTPα-deficient mice. As shown in Fig. 2A, mice lacking PTPα demonstrated enhanced PCA as reflected by the significantly elevated Evans blue dye extravasation that specifically occurred in IgE-sensitized ear tissue. We qualitatively assessed IgE-mediated local inflammation responses by measuring inflamed ear tissue thickness and mast cell numbers in inflamed tissue 6 h after Ag challenge. PTPα−/− mice generally had thicker inflamed ears than WT mice (knockout, 839 ± 49 μm; WT, 795 ± 73 μm; n = 15 microscopic fields). Strikingly, we also observed an unusual skin inflammatory response of highly thickened regions of inflamed epidermis that was present only in IgE-sensitized PTPα−/− ears (Fig. 3B, bottom right panel). Passive systemic anaphylaxis (PSA) was assessed in mice sensitized by i.v. injection of DNP-IgE or control saline and challenged 24 h later with an i.v. injection of DNP-HSA. Serum histamine levels, a measure of PSA, were significantly elevated in IgE-sensitized PTPα−/− mice (Fig. 3C). Our data demonstrate that PTPα negatively regulates IgE- and FcεRI-dependent PCA and PSA, consistent with the ex vivo hyperdegranulation of cultured BMMCs. PTPα−/− mice have reduced mast cell numbers in many tissues, including the ear (35); this suggests that the enhanced PCA response, and perhaps other anaphylactic responses in these animals, results from an even higher extent of activation on a per cell basis.

PTPα differentially regulates FcεRI-mediated activation of SFKs

PTPα is well established as an important regulator of SFKs, acting to dephosphorylate their inhibitory C-terminal tyrosine residue and activate them (25). Cross-linking of FcεRI-bound IgE leads to the activation of the SFKs Fyn, Lyn, and Hck—all critical receptor-immediate effectors of mast cell activation (6). We there-

**FIGURE 2.** Restoring PTPα expression in PTPα−/− BMMCs rescues aberrant activation. WT (PTPα+/+), PTPα−/−, and PTPα−/− BMMCs that had been infected with MSCV bearing an empty vector (MSCV) or a PTPα expression vector (MSCV-PTPα) were analyzed for (A) PTPα expression (the halves of each panel were different regions from the same immunoblot), (B) β-hexosaminidase release after sensitization and 15 min stimulation with the indicated concentrations of DNP-HSA, and (C) IL-2 and TNF-α release after 15 h stimulation with 10 ng/ml DNP-HSA. Data represent the mean ± SD from three separate experiments. Asterisks indicate a significant difference (p < 0.05) with results from WT BMMCs.
fore investigated the effects of PTPα ablation on FcεRI-dependent activation of these SFKs. We used phosphospecific Abs to Src phosphoTyr416 to analyze active SFKs, Abs to Src phosphoTyr527 to analyze inactive SFKs, and enzymatic assays to quantify kinase activity. Immunoblotting and kinase assay analysis revealed little to no Lyn activation in stimulated PTPα−/− BMMCs, resulting in significantly reduced Lyn activity for up to 15 min poststimulation in PTPα−/− BMMCs compared with WT BMMCs (Fig. 4A). We also found a lack of Fyn activation in stimulated PTPα−/− BMMCs, as indicated by the absence of Fyn Tyr528 dephosphorylation and reduced kinase activity of Fyn in stimulated PTPα−/− mast cells compared with WT cells (Fig. 4B). In contrast, Hck activation was evident in IgE cross-linked PTPα−/− BMMCs and was higher than that detected in WT cells, as shown by enhanced phosphorylation of Hck at its Tyr416-equivalent residue and by its significantly increased (1.5 and 5 min) and prolonged (15 min) activation in PTPα−/− cells (Fig. 4C). These analyses reveal unexpected differential abilities of PTPα to regulate FcεRI-dependent SFK activities, with PTPα positively regulating Fyn and Lyn activation but negatively regulating Hck activation. PTPα functions as an activator of SFKs in multiple signaling systems, indicating that reduced Lyn and Fyn activities in PTPα−/− BMMCs reflect the loss of PTPα-catalyzed dephosphorylation and activation of these kinases. In contrast, it is

FIGURE 3. Lack of PTPα significantly elevates anaphylaxis in mice. Passive cutaneous anaphylaxis was induced in WT and PTPα−/− mice. A, Thirty minutes after Ag challenge, ears were removed and extravasated Evans blue dye was quantified and normalized for the unit weight (n = 5 animals per genotype). B, Six hours after Ag challenge, ear tissues were assessed for tissue inflammation. One of three representative micrographs per condition is shown (n = 3 animals per genotype). Characteristic thickening of the epidermis of Ag-IgE–treated PTPα−/− mouse ears is marked by the arrow. Scale bar, 100 μm. C, Passive systemic anaphylaxis was induced in mice (n = 5 animals per genotype). Blood was collected 1.5 min after Ag challenge, and serum histamine concentrations were determined by ELISA. The graph represents mean ± SD. Asterisks indicate a significant difference (p < 0.05) between comparative data from animals of each genotype.

FIGURE 4. FcεRI-mediated activation of Fyn, Lyn, and Hck are regulated by PTPα. Immunoprecipitates of Lyn (A), Fyn (B), and Hck (C) were prepared from lysates of WT and PTPα−/− BMMCs that were not stimulated (0) or stimulated with 100 ng/ml DNP-HSA for the indicated times. Immunoprecipitates were immunoblotted with anti-phospho Tyr527-Src Abs or anti-phospho Tyr416-Src Ab (top panels) and with anti-Fyn, anti-Lyn and anti-Hck Ab as appropriate (bottom panels). A representative set from three similar sets of immunoblots is shown. The corresponding graphs below show the densitometric quantification (mean ± SD) of immunoblots from three independent experiments. The graphs to the right show the activity of each immunoprecipitated SFK, as determined by in vitro kinase assays. Kinase activity was calculated relative to the standard kinase activity of c-Src. Data represent the mean ± SD calculated from duplicate assays of each of three independent immunoprecipitates. The asterisks indicate a significant difference (p < 0.05) between comparative data from each genotype. D, Cbp/PAG was immunoprecipitated and probed with phosphotyrosine (4G10, top panel), and stripped and reprobed with Cbp/PAG and Csk Abs (bottom panels). The graph shows the densitometric quantification of the amount of Csk that coimmunoprecipitated per unit Cbp/PAG (mean ± SD) from three independent experiments. n.s., no significant difference (p > 0.05).
likely that the enhanced Hck activity in these cells results from the loss of indirect PTPα-mediated signaling events that normally suppress Hck. One possibility is that reduced Lyn activation results in reduced Cbp/PAG-mediated membrane recruitment of the SFK inhibitor Csk, although we did not detect significantly reduced Cbp/PAG-associated Csk in PTPα−/− BMMCs (Fig. 4D).

**PTPα positively regulates FcεRI receptor phosphorylation and negatively regulates FcεRI-dependent MAPK and PI3K pathways**

Overall protein tyrosine phosphorylation was enhanced in Ag-stimulated PTPα−/− mast cells (Fig. 5A). To further determine how the absence of PTPα affected specific steps in FcεRI-mediated signaling to result in mast cell hyperactivation, we examined the early event of FcεRI phosphorylation and the later activation of downstream molecules in the PI3K and MAPK signaling pathways. Lyn plays a critical role in the phosphorylation of the FcεRI-β and FcεRI-γ subunits to initiate subsequent signaling (3, 7), and Hck can also regulate FcεRI-β and FcεRI-γ phosphorylation (9). Fyn does not phosphorylate FcεRI (11, 18). The FcεRI-γ subunit was immunoprecipitated from WT and PTPα−/− BMMCs and probed for tyrosine phosphorylation (Fig. 5A) and associated phosphotyrosyl β subunit (Fig. 4B, 4C). We observed a large decrease (~70%) in Ag-stimulated phosphorylation of the FcεRI-γ subunit (Fig. 5B) and reduced phosphorylation of the associated β subunit (80% of WT; n = 2; Fig 5C), in PTPα-deficient mast cells compared with WT cells. These results are in accord with the decreased Lyn activity in PTPα-null BMMCs; they also are consistent with the increased Hck activity in PTPα−/− mast cells, because Hck−/− BMMCs exhibit enhanced FcεRI γ and β phosphorylation that may be due to the proposed role of Hck as a negative regulator of Lyn and Lyn-mediated phosphorylation events (9).

Despite reduced FcεRI tyrosine phosphorylation, we detected hyperphosphorylation of Akt (Fig. 5D), ERK1/2 (Fig. 5E), p38 (Fig. 5F), and JNK (Fig. 5G) in PTPα−/− cells, which is indicative of the increased activation of PI3K and MAPK signaling. These findings demonstrate that, despite the action of PTPα to positively regulate FcεRI receptor phosphorylation, it nevertheless acts as a negative regulator of several key downstream signaling molecules.

**Increased activity of the FcεRI-interacting kinase Syk in PTPα−/− mast cells**

Lyn-mediated phosphorylation of the γ and β subunits of FcεRI results in the recruitment of positive and negative regulators of signaling. The tyrosine kinase Syk becomes activated upon interaction with phosphorylated ITAMs of FcεRIγ, subsequently phosphorylating diverse target molecules to elicit responses that include mast cell degranulation and cytokine release (40). Interestingly, we found that Syk phosphorylation at tyrosine 525/526, reflecting Syk activation, was significantly enhanced in PTPα−/− mast cells, because Hck−/− mast cells (Fig. 4D, 5A). In vitro assays of immunoprecipitated Syk

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**FIGURE 5.** Lack of PTPα leads to reduced phosphorylation of FcεRI and hyperphosphorylation of FcεRI downstream molecular targets. WT and PTPα−/− BMMCs were sensitized overnight with anti-DNP-IgE mAb and left unstimulated (0) or stimulated for 1.5, 5, and 15 min with DNP-HSA (100 ng/ml). A, Cell lysates were immunoblotted for phosphotyrosine (pTyr), actin, and PTPα as indicated (n = 3). Numbers on the right indicate m.w. markers. B, FcεRIγ was immunoprecipitated and probed with anti-4G10 to detect tyrosine phosphorylation (pTyr, top panel). The blot was stripped and reprobed for FcεRIγ (bottom panel). Densitometric quantification (mean ± SD) of immunoblots from three experiments is shown in the graph. Asterisks indicate a significant difference (p < 0.05) between comparative data from the two genotypes. C, In other experiments, FcεRIγ immunoprecipitates were probed as in B and reprobed for FcεRIγ (top panels) and FcεRIβ (bottom panels; n = 2). D, Cell lysates were probed for phospho-Ser473 Akt and anti-Akt. (E) phospho-ERK1/2 and anti-ERK. (F) phospho-p38 MAPK and anti-p38 MAPK, and (G) phospho-JNK1/2 and anti-JNK1/2, as indicated (n = 3). Representative immunoblots of three similar independent experiments are shown, and the graphs depict the mean ± SD of quantified data from three experiments. Asterisks indicate a significant difference (p < 0.05) between comparative data from the two genotypes.
demonstrated significantly elevated Syk activity in PTPα−/− BMMCs, compared with WT BMMCs, that was evident for up to 15 min after stimulation (Fig. 6B). These results demonstrate that Syk activation is PTPα dependent, and the enhanced activation of Syk in PTPα−/− mast cells is an early postreceptor signaling event that correlates with the enhanced degranulation and cytokine secretion responses that we observed in PTPα−/− BMMCs.

Syk binding to phospho-FceRIγ is required for Syk activation. Because stimulated PTPα−/− BMMCs have reduced FceRIγ phosphorylation, but have increased Syk activity, we investigated the interaction of Syk with FceRIγ in PTPα−/− and WT cells. Immunoprecipitations of FceRIγ performed using Abs to the γ subunit did not detect any bound Syk in WT mast cells (data not shown). Therefore, we used anti-IgE Abs to immunoprecipitate activated FceRI and then probed for FceRIγ and associated Syk. Although Syk association with FceRI increased upon Ag stimulation of WT BMMCs, no Ag-dependent increase was observed in PTPα−/− BMMCs (Fig. 6C). This finding suggests that Syk is hyperactivated in PTPα-null mast cells via a mechanism other than that involving enhanced Syk binding to FceRI.

We determined Syk phosphorylation at Tyr317, a site in the linker region that inhibits Syk function (41). Ag-stimulated phosphorylation of Tyr317 was not detectable in PTPα−/− mast cells, whereas it increased by ∼30% in WT cells (Fig. 6D). Therefore, the enhanced Syk activity in PTPα-null BMMCs could result from the combined effects of the absence of inhibitory phosphorylation and gain in positive regulatory phosphorylation.

The scaffolding protein Gab2 is a positive regulator of FceRI-mediated mast cell degranulation and anaphylaxis (42). Previous work has shown that Syk can phosphorylate Gab2 (43). Importantly, Fyn and Hck also mediate Gab2 phosphorylation to regulate PI3K activation, microtubule formation, and degranulation (9, 11). In parallel with the enhanced activation of Syk and Hck, we observed hyperphosphorylation of Gab2 in PTPα−/− mast cells at 1.5 min, but not at times of 5 and 15 min after stimulation (Fig. 6E). Therefore, PTPα functions as a negative regulator of FceRI-dependent activation of Syk and Gab2.

**Regulation of FceRI-associated phosphatases by PTPα**

Lyn is associated with and phosphorylates the FceRI-β subunit to mediate negative signaling that includes recruitment and phosphorylation of the inositol phosphatase SHIP and the protein tyrosine phosphatase SHP-1 (7, 13, 44–46). In PTPα−/− BMMCs, Ag-stimulated phosphorylation of SHIP was inhibited while that of SHP-1 was increased (Fig. 7A, 7B).

**Discussion**

We have used PTPα-deficient BMMCs and mice to define roles of PTPα as a negative regulator of FceRI-mediated mast cell activation and IgE-dependent anaphylaxis. Ablation of PTPα has profound effects on the Ag-stimulated activity of three SFKs that critically mediate early events in FceRI signaling. In PTPα-null BMMCs, Ag-stimulated activation of the SFKs Lyn and Fyn is
immunoprecipitates were immunoblotted with anti-phosphotyrosine (pTyr, reduced Lyn activity in PTPα-negative regulator of Hck (Fig. 8).

Fyn and Hck activation, countering PTPα-mediated activation of Lyn and Fyn. Activated Lyn negatively regulates Fyn and Hck activation, countering PTPα-mediated Fyn activation to an unknown extent. In the absence of PTPα, Fyn and Lyn are minimally or not activated, with the latter resulting in elevated Hck activity. The bottom schematic depicts Ag-IgE–dependent (stim) activation of Lyn in the presence or absence of PTPα. PTPα promotes Lyn activation and Lyn negative and positive regulatory signaling (+PTPα). In the absence of PTPα (-PTPα), Lyn activation is significantly reduced, with a concomitant loss of Lyn negative regulatory signaling, including loss of repression of Hck, that results in mast cell hyperactivation.

Enhanced activation of Hck is due to a similar mechanism involving its reduced inhibition, in this case possibly mediated through the loss of PTPα-induced Lyn activation. The elevated Hck activation did not appear to be mediated through a reduction in Lyn-promoted Cbp/PAG-Csk signaling to suppress Hck, and it requires further investigation. It will be of interest to determine whether Hck activity is elevated in Lyn−/− and Lyn−/−/− mast cells that respectively have no Lyn protein or express mutant Lyn with severely reduced activity. A potential for bidirectional signaling between Lyn and Hck is suggested by the observation that Hck−/− mast cells possess elevated Lyn activity, indicating that Hck may also inhibit Lyn (9). Because Lyn can inhibit Fyn (12, 13), PTPα may additionally promote Lyn-mediated inhibition of Fyn, although apparently the direct activation of Fyn by PTPα can effectively overcome this indirect mechanism of inhibition. Obviously, inter-SFK regulatory actions are complex and are determined by the overall SFK expression levels and activities, with the latter controlled both directly and indirectly by PTPα (Fig. 8).

Among Lyn−/−, Fyn−/−, or Hck-deficient BMMCs, only Lyn−/− cells exhibit a hyperactivation phenotype of enhanced degranulation, cytokine release, or both (7, 11–13, 17). The SFK activity profile in Ag-stimulated PTPα−/− BMMCs suggests that the loss of Lyn-mediated negative regulatory signals, which in part is manifested by a consequent gain of Hck-mediated positive regulatory signals, underlies the hyperactivated phenotype of these mast cells (Fig. 8). A role for elevated Hck activity in PTPα−/− mast cell hyperactivation is indicated by findings that the Lyn−/− hyperactivation phenotype is supported by catalytically active Hck, as double-deficient Lyn−/−/− Hck−/− BMMCs show reduced hyperactivation relative to Lyn−/− cells that is restored by the introduction of active Hck into the double-deficient cells (9). Similarly, the amount of Fyn expressed in Lyn−/− BMMCs influences degranulation, because the Lyn−/− hyperdegranulation phenotype is lost in double-deficient Lyn−/− Fyn−/− BMMCs or in Lyn−/− BMMCs with reduced Fyn expression, and the degranulation of Lyn−/− cells is increased by expression of Fyn (8, 12). The abilities of catalytically active Hck and Fyn to promote and enhance the manifestation of the Lyn−/− hyperactivation...
phenotype, together with the overlap of some Hck and Fyn signaling activities (9, 11), suggest that the increased Hck activation in PTPα−/− BMMCs at least partially contributes to PTPα−/− BMMC hyperactivation through positive regulatory actions that are Hck-specific and by compensating for some of the abrogated Fyn-mediated positive signaling effects.

Another aspect of Lyn-negative regulatory signaling involves the activation of molecules such as the inositol phosphatase SHIP (7, 13), which acts to dephosphorylate PIP3 and limit PI3K signaling (44). SHIP activation appears to be defective in the absence of PTPα, because reduced phosphorylation of SHIP and enhanced phosphorylation of the PI3K target Akt occur in PTPα−/− BMMCs. Like SHIP, the PTP SHP-1 undergoes FcεRI-mediated and Lyn-dependent phosphorylation and activation (7, 46, 48). SHP-1 is reported to dephosphorylate various signaling molecules, including FcεRIβ/γ, Syk, LAT, and SLP-76, and to have positive and negative effects on mast cell signaling and activation (49, 50).

We detected only a small increase in SHP-1 tyrosine phosphorylation in WT BMMCs, but a greatly enhanced phosphorylation of SHP-1 was apparent in Ag-stimulated PTPα−/− BMMCs. This finding suggests that SHP-1 catalytic activity and its regulatory actions are unexpectedly enhanced in the absence of PTPα. Nevertheless, this enhancement is insufficient to exert a net inhibition of PTPα−/− BMMC activation. However, SHP-1 targets remain to be clarified, and because a distinct positive role of SHP-1 in FcεRI signaling may involve its function as an adaptor that facilitates SLP-76 and PLCγ activation (50), the significance of enhanced SHP-1 phosphorylation in PTPα-null cells is difficult to interpret.

SHP-1 autodephosphorylation has been reported in RBL-2H3 basophilic leukemia cells (48). An additional mode of action is indicated by our finding that PTPα directly or indirectly limits Ag-stimulated SHP-1 phosphorylation.

A key positive action of Lyn in FcεRI-mediated signaling is the phosphorylation of ITAMs in FcεRI-γ that recruit and activate Syk. In keeping with this role, Lyn−/− mast cells typically exhibit reduced Syk phosphorylation and activation (7–9, 11). In Lyn−/− mast cells, very low Syk activity is sufficient for positive signaling events that are indicative of intact Syk activation, such as the phosphorylation of the Syk substrate LAT (10). In PTPα−/− BMMCs, Ag-stimulated Lyn activity and phosphorylation of FcεRI-γ are reduced, but still detectable, and are sufficient to support Syk activation. In fact, Syk is hyperactivated in these mast cells, despite its reduced association with FcεRI-γ after Ag stimulation. This finding appears in part to be a result of early enhanced phosphorylation of Syk at the Tyr525/6 sites associated with kinase activation and signaling function in addition to the loss of Ag-dependent inhibitory phosphorylation at Tyr317. These findings suggest that the elimination of PTPα-dependent signaling, certainly involving Lyn activation, is manifested through the loss of negative but not positive regulation of Syk.

A recent study of Lyn−/− BMMCs found that this mutant form of Lyn with greatly reduced basal and Ag-stimulated catalytic activity was sufficient for the positive regulatory actions of this SFK in mast cell activation (10). Our study is in agreement with and extends this finding, because it demonstrates that Lyn-positive signaling is functional in PTPα−/− mast cells with WT Lyn expression and basal activity, but with minimal Lyn activation. We also identify PTPα as a critical upstream initiator of the majority of Ag- and FcεRI-induced Lyn activation that drives negative regulatory signaling. Furthermore, the ex vivo hyperactive phenotype of PTPα−/− mast cells is reflected by enhanced PSA and PCA in mice, indicating that PTPα limits FcεRI-initiated anaphylactic allergic reactions. Further investigation of the mechanisms by which Ag engagement of FcεRI promotes PTPα to function in what appears to be one of the earliest enzymatic reactions that triggers subsequent signaling, and of whether PTPα targets a discrete population of Lyn while another is inaccessible to PTPα, will be required to explore the utility of therapeutically manipulating PTPα to control allergic disease.

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Disclosures

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References

1. Brown, J. M., T. M. Wilson, and D. D. Metcalfe. 2008. The mast cell and allergic diseases: role in pathogenesis and implications for therapy. Clin. Exp. Allergy 38: 4–18.
2. Galli, S. J., M. Tsai, and A. M. Piliponsky. 2008. The development of allergic inflammation. Nature 454: 445–454.
3. Rivera, J., and A. M. Gililland. 2008. Molecular regulation of mast cell activation. J. Allergy Clin. Immunol. 117: 1214–1225, quiz 1226.
4. Gillillan, A. M., and C. Tkaczyk. 2006. Integrated signaling pathways for mast-cell activation. Nat. Rev. Immunol. 6: 218–230.
5. Kraft, S., and I. P. Kinet. 2007. New developments in FcεRI signaling, function and inhibition. Nat. Rev. Immunol. 7: 365–378.
6. Gillillan, A. M., and J. Rivera. 2009. The tyrosine kinase network regulating mast cell activation. Immunol. Rev. 228: 149–169.
7. Krnjevic, S., H. Nishimura, J. J. Ryan, H. Kawakami, and T. Kawakami. 2001. Positive and negative regulation of mast cell activation by Lyn via the FcεRI. J. Immunol. 175: 6885–6892.
8. Yasukata, Y., N. Charalambous, Y. Furumoto, S. Odom, T. Yamashita, A. M. Gillilland, S. Constant, M. A. Bower, J. J. Ryan, and J. Rivera. 2007. Cutting edge: genetic variation influences Fc epsilonRI-induced mast cell activation and allergic responses. J. Immunol. 179: 740–743.
9. Kawai, A., T. Kitaura, W. Xiao, V. Horejsi, C. Ra, C. A. Lowell, Y. Kawakami, and T. Kawakami. 2007. The Src family kinase Hck regulates mast cell activation by suppressing an inhibitory Src family kinase. Blood 110: 2511–2519.
10. Podericky, M., Y. Tomomori, T. Ando, W. Xiao, M. Maeda-Yamamoto, K. Sauer, Y. Kawakami, and T. Kawakami. 2010. A minor catalytic activity of Src family kinases is sufficient for maximal activation of mast cells via the high-affinity IgE receptor. J. Immunol. 184: 84–93.
11. Parravicini, V., M. Gadina, M. Kovarova, S. Odom, C. Gonzalez-Espinoza, Y. Furumoto, S. Satoh, L. E. Samelson, J. J. O’Shea, and J. Rivera. 2002. Lyn kinase initiates complementary signals required for IgE-dependent mast cell degranulation. Nat. Immunol. 3: 741–748.
12. Odom, S., G. Gomez, M. Kovarova, Y. Furumoto, J. J. Ryan, H. V. Wright, C. Gonzalez-Espinoza, S. F. Ribas, M. J. Ribbans, W. K. Harder, and J. Rivera. 2004. Negative regulation of immunoglobulin E-dependent allergic responses by Lyn kinase. J. Med. Exp. Med. 199: 1491–1502.
13. Hernandez-Hansen, V., A. J. Smith, Z. Surviladze, A. Chigaev, T. Mazel, J. Kalesnikhoff, C. A. Lowell, G. Krystal, L. A. Sklat, B. S. Wilson, and J. M. Oliver. 2004. Dysregulated FcεRI signaling and altered Lyn and SHP-1 activities in Lyn-deficient mast cells. J. Immunol. 173: 100–112.
14. Nishizumi, H., and T. Yamamoto. 1997. Impaired tyrosine phosphorylation and Ca2+ mobilization, but not degranulation, in lyn-deficient bone marrow-derived mast cells. J. Immunol. 158: 2350–2355.
15. Kalesnikhoff, J., and S. J. Galli. 2008. New developments in mast cell biology. Nat. Immunol. 9: 1215–1223.
16. Roth, K., W. M. Chen, and T. J. Lin. 2008. Positive and negative regulatory mechanisms in high-affinity IgE receptor-mediated mast cell activation. Arch. Immunol. Ther. Exp. (Warsz.) 56: 385–399.
17. Kawakami, Y., J. Kitaura, A. B. Satterthwaite, R. M. Kato, K. Asai, S. E. Hartman, M. Maeda-Yamamoto, C. A. Lowell, D. J. Rawlings, O. N. Witte, and T. Kawakami. 2000. Redundant and opposing functions of two Src kinases, Btk and Lyn, in mast cell activation. J. Immunol. 165: 1210–1219.
18. Gomez, G., C. Gonzalez-Espinoza, S. Odom, G. Baez, M. E. Cud, J. J. Ryan, and J. Rivera. 2005. Impaired FcεRI-dependent gene expression and defective eosinophil and cytokine production as a consequence of Fyn deficiency in mast cells. J. Immunol. 175: 7602–7610.
19. Saunders, A. E., and P. Johnson. 2010. Modulation of immune cell signaling by the leukocyte common tyrosine phosphatase, CD45. Cell. Signal. 22: 339–348.
20. Bierer, S. A., T. W. Mak, and C. J. Paige. 1994. Leukocyte common antigen (CD45) is required for immunoglobulin E-mediated degranulation of mast cells. J. Exp. Med. 180: 471–476.
21. Grochowey, G., M. L. Hermiston, M. Kuhnly, A. Weiss, and M. Huber. 2009. Requirement for CD45 in bone marrow-derived mast cell responses mediated by different ligand-receptor systems. Cell. Signal. 21: 1277–1286.
22. Murakami, K., S. Sato, S. Nagasawa, and T. Yamashita. 2000. Regulation of mast cell signaling through high-affinity IgE receptor by CD45 protein tyrosine phosphatase. Int. Immunol. 12: 169–176.
23. McPherson, V. A., N. Sharma, S. Everingham, J. Smith, H. H. Zhu, G. S. Feng, and A. W. Craig. 2009. SH2 domain-containing phosphatase-2 protein-tyrosine
phosphatase promotes Fc(epsilon)RI-induced activation of Fyn and Erk pathways leading to TNF(alpha) release from bone marrow-derived mast cells. J. Immunol. 183: 4940–4947.

24. Akimoto, M., K. Mishra, K. T. Lim, N. Tani, S. I. Hisanaga, T. Katagiri, A. Elson, K. Mizuno, and H. Yakura. 2009. Protein tyrosine phosphatase epsilon is a negative regulator of FcepsilonRI-mediated mast cell responses. Scand. J. Immunol. 69: 401–411.

25. Pallen, C. J. 2003. Protein tyrosine phosphatase alpha (PTPalpha): a Src family kinase activator and mediator of multiple biological effects. Curr. Top. Med. Chem. 3: 821–835.

26. Zheng, X. M., Y. Wang, and C. J. Pallen. 1992. Cell transformation and activation of protein tyrosine phosphatase. Nature 359: 336–339.

27. den Hertog, J., C. E. Pals, M. P. Peppelenbosch, L. G. Tertoolen, S. W. de Laat, and W. Kruijer. 1993. Receptor protein tyrosine phosphatase alpha activates pp60c-src and is involved in neuronal differentiation. EMBO J. 12: 3789–3798.

28. Su, J., M. Muranjan, and J. Sap. 1999. Receptor protein tyrosine phosphatase alpha regulates Src-family kinases and controls integrin-mediated responses in fibroblasts. Curr. Biol. 9: 505–511.

29. Ponniah, S., D. Z. Wang, K. L. Lim, and C. J. Pallen. 1999. Targeted disruption of the tyrosine phosphatase PTPalpha leads to constitutive downregulation of the kinases Src and Fyn. Curr. Biol. 9: 535–538.

30. Zeng, L., X. Si, W. P. Yu, H. T. Le, K. P. Ng, R. M. Teng, K. Ryan, D. Z. Wang, S. Ponniah, and C. J. Pallen. 2003. PTP alpha regulates integrin-stimulated FAK autophosphorylation and cytoskeletal rearrangement in cell spreading and migration. J. Cell Biol. 160: 137–146.

31. Le, H. T., L. Maksumova, J. Wang, and C. J. Pallen. 2006. Reduced NMDA receptor tyrosine phosphorylation in PTPalpha-deficient mouse synaptosomes is accompanied by inhibition of four src family kinases and Pyk2: an upstream role for PTPalpha in NMDA receptor regulation. J. Neurochem. 98: 1798–1809.

32. Ye, H., Y. L. Tan, S. Ponniah, Y. Takeda, S. Q. Wang, M. Schachner, K. Watanabe, C. J. Pallen, and Z. C. Xiao. 2005. Neural recognition molecules alpha regulates stem cell factor-dependent c-Kit activation and migration of mast cells. J. Biol. Chem. 283: 29175–29185.

33. Le, H. T., L. Maksumova, J. Wang, and C. J. Pallen. 2006. Reduced NMDA receptor tyrosine phosphorylation in PTPalpha-deficient mouse synaptosomes is accompanied by inhibition of four src family kinases and Pyk2: an upstream role for PTPalpha in NMDA receptor regulation. J. Neurochem. 98: 1798–1809.

34. Ye, H., Y. L. Tan, S. Ponniah, Y. Takeda, S. Q. Wang, M. Schachner, K. Watanabe, C. J. Pallen, and Z. C. Xiao. 2005. Neural recognition molecules alpha regulates stem cell factor-dependent c-Kit activation and migration of mast cells. J. Biol. Chem. 283: 29175–29185.

35. Torogoe, C., and H. Metzger. 2001. Spontaneous phosphorylation of the receptor with high affinity for IgE in transfected fibroblasts. Biochemistry 40: 4016–4025.