

PTP α Activates Lyn and Fyn and Suppresses Hck to Negatively Regulate Fc ϵ RI-Dependent Mast Cell Activation and Allergic Responses

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Mast cell activation via Fc ϵ RI 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 Fc ϵ RI-dependent signaling. We investigated the role of receptor protein tyrosine phosphatase (PTP) α , a well-known activator of SFKs in diverse signaling systems, Fc ϵ RI-mediated mast cell activation, and IgE-dependent allergic responses in mice. PTP α ^{-/-} bone marrow-derived mast cells hyperdegranulate and exhibit increased cytokine and cysteinyl leukotriene secretion, and PTP α ^{-/-} mice display enhanced IgE-dependent anaphylaxis. At or proximal to Fc ϵ RI, PTP α ^{-/-} cells have reduced IgE-dependent activation of Lyn and Fyn, as well as reduced Fc ϵ RI 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 Fc ϵ RI, we observed increased activation of PI3K and MAPK pathways. These findings demonstrate that PTP α activates the Fc ϵ RI-coupled kinases Lyn and Fyn and suppresses Hck activity. Furthermore, the findings indicate that hyperactivation of PTP α ^{-/-} mast cells and enhanced IgE-dependent allergic responses of PTP α ^{-/-} mice are due to the ablated function of PTP α as a critical regulator of Lyn negative signaling. *The Journal of Immunology*, 2010, 185: 000–000.

Mast cells are important effectors of IgE-dependent immune responses that mediate acute and chronic inflammation and allergies (1, 2). Mast cell activation is initiated by Ag binding and cross-linking of IgE that is complexed with the high-affinity IgE receptor Fc ϵ RI at the mast cell surface. Fc ϵ RI engagement and cross-linking triggers intracellular signaling events that culminate in the release, both by degranulation and secretion, of a variety of stored and newly synthesized bioactive compounds that have profound effects on multiple other cells and tissues (3–5).

The Src family kinases (SFKs) Lyn, Fyn, and Hck are critical Fc ϵ RI-proximal regulators of signaling (6). Their individual actions and roles, as well as their functional interrelationships, in Fc ϵ RI signaling and mast cell activation have been investigated using bone marrow-derived mast cells (BMMCs) from mice deficient in one or more of these kinases. Lyn exerts both positive and negative regulatory effects that appear to be determined by genetic background and the intensity of mast cell stimulation (7, 8). Indeed, Lyn-null

BMMCs have been found to have reduced (7–10), enhanced (7, 8, 11–13), or unaltered (14) Ag-induced degranulation. Lyn associates with the β - and γ -chains of Fc ϵ RI and is activated upon receptor clustering, phosphorylating ITAMs in the β - and γ -chains of Fc ϵ RI that recruit and promote the activation of other signaling molecules (6, 15, 16). Among these is the tyrosine kinase Syk that recognizes phospho-ITAMs in Fc ϵ RI γ . Lyn and Syk phosphorylate several other proteins, such as the transmembrane scaffolding–adaptor proteins linker for activation of T cells (LAT) and non-T cell activation linker, to initiate downstream signaling pathways that stimulate Ca²⁺ mobilization and degranulation as well as gene transcription. The positive actions of Lyn in mast cell signaling are manifested in BMMCs that receive low-intensity stimulation or that express a mutant form of Lyn with impaired kinase activity (7, 10). A key role of Lyn in negatively regulating mast cell activation was revealed by the hyperactive phenotype of Lyn-null BMMCs and the finding that Lyn-deficient mice are prone to allergic disease (7, 11–13). Lyn-mediated signaling events that are implicated in negatively regulating Fc ϵ RI-dependent mast cell responses include the phosphorylation of Fc ϵ RI β subunits, activation of the inhibitory inositol phosphatase SHIP, and phosphorylation of the transmembrane protein Cbp/PAG (C-terminal Src kinase [Csk]-binding protein/phosphoprotein associated with glycosphingolipid-enriched microdomains) to promote recruitment of the SFK inhibitory kinase Csk (7, 12, 13). Overall, hyperresponsive Lyn-null BMMCs exhibit enhanced or prolonged activation of Fyn, PI3K, phospholipase C (PLC) γ 1, and MAPK (12, 13, 17).

The SFKs Fyn and Hck positively regulate mast cell activation, which is defective in Fyn^{-/-} or Hck^{-/-} BMMCs (9, 11, 18). Ag stimulation induces the association of Fyn with Fc ϵ RI β , but Fyn does not phosphorylate the receptor (9, 11). Both Fyn and Hck phosphorylate the adaptor protein Gab2 to activate PI3K signaling and promote microtubule formation that is important for granule translocation to the plasma membrane (9, 11). Hck also positively regulates Syk activation and that of other downstream kinases, such as Btk, Akt, and JNK (9). Evidence of inter-SFK regulation is

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Abbreviations used in this paper: BMMC, bone marrow-derived mast cell; Cbp/PAG, Csk-binding protein/phosphoprotein associated with glycosphingolipid-enriched microdomains; Csk, C-terminal Src kinase; c-Src, cellular Src; HSA, human serum albumin; LAT, linker for activation of T cells; MSCV, murine stem cell virus; PCA, passive cutaneous anaphylaxis; PLC, phospholipase C; PSA, passive systemic anaphylaxis; PTP, protein tyrosine phosphatase; pTyr, phosphotyrosine; SFK, Src family kinase; WT, wild type.

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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 Fc ϵ RI. Several PTPs with reported activity on SFKs in other signaling systems have been investigated for their roles in Fc ϵ RI-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 Fc ϵ RI, reduced Fc ϵ RI-associated tyrosine kinase activity and Fc ϵ RI tyrosine phosphorylation, and impaired degranulation (22). Thus, defective CD45-mediated regulation of Lyn is a primary event underlying defective Fc ϵ RI-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 PTP ϵ appears to act via Syk to negatively regulate mast cell activation (24). Ag-stimulated PTP ϵ ^{-/-} BMMCs showed enhanced degranulation and cytokine release, but unaffected leukotriene secretion. The most Fc ϵ RI-proximal alteration detected in these cells was hyperphosphorylation of Syk, whereas Lyn phosphorylation and Fyn-mediated signaling were normal.

PTP α is a receptor PTP that, like CD45 and the closely related PTP ϵ , 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 PTP ϵ , PTP α is widely expressed. PTP α 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 mast cells, PTP α is required for Fyn activation in stem cell factor-dependent c-Kit signaling and mast cell migration (35). Overexpression of Lyn in epithelial Chinese hamster ovary cells stably expressing Fc ϵ RI resulted in constitutive phosphorylation of Fc ϵ RI because of the dysregulated high basal activity of Lyn, and this was suppressed by co-overexpression of PTP α (36, 37). In the absence of Ag stimulation in this system, PTP α acted on the active site phosphotyrosine of Lyn to downregulate its basal kinase activity (37). However, the expressed PTP α reportedly exerted these effects even when present at a level approaching that of endogenous Chinese hamster ovary cell PTP α , whereas the endogenous phosphatase did not, raising the possibility of undefined but critical differences between the heterologous and endogenous PTP α . We investigated the role of PTP α in Ag-stimulated Fc ϵ RI signaling and mast cell activation using BMMCs derived from PTP α ^{-/-} mice. We also determined whether the absence of PTP α affected IgE-dependent anaphylactic reactions in mice. We report that PTP α is

a negative regulator of these Fc ϵ RI-dependent responses. The ablation of PTP α inhibited Ag-stimulated Lyn and Fyn activation while enhancing Hck activation. Despite reduced tyrosine phosphorylation of Fc ϵ RI γ , Syk activity was elevated in PTP α ^{-/-} BMMCs, as was the activity of Akt and MAPKs. In conjunction with reduced Fc ϵ RI β tyrosine phosphorylation, SHIP phosphorylation was defective, although that of SHP-1 was increased. Our findings indicate that PTP α plays a major role as an upstream regulator of Lyn-mediated negative signaling, revealing a novel regulatory relationship between Lyn and Hck. Overall, PTP α functions as a key Fc ϵ RI-proximal phosphatase that initiates and coordinates SFK activities to promote yet limit mast cell activation.

Materials and Methods

Animals

The 129SvEv PTP α ^{-/-} mice (29) were backcrossed with C57BL/6 mice for 10 generations. PTP α ^{-/-} and wild type (WT; PTP α ^{+/+}) 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, p38, 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 Fc ϵ RI γ , Fc ϵ RI β , 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 IgG₁-FITC, and rat IgG_{2b}-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 μ g 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 (Sigma-Aldrich). Thirty minutes after Ag challenge, mice were sacrificed and the ears were removed. Extravasated Evans blue dye in the ear tissues was extracted with 1 ml of 0.5% Na₂SO₄ and acetone (3:7) for 24 h, and OD₆₂₀ 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 and ear thickness was measured using a Leica DM4000 B microscope (Leica Microsystems, Deerfield, IL) and Open Laboratory 4.0.2 software (Improvision, Coventry, England).

IgE-mediated passive systemic anaphylaxis

Mice were sensitized by i.v. injection of 2 μ g IgE (anti-DNP Ab, clone SPE-7) in 100 μ l saline or treated with saline alone. After 24 h, the mice were challenged i.v. with 500 mg DNP-HSA in 100 μ l 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 PTP α in PTP α ^{-/-} BMMCs, the bone marrow progenitors were infected with murine stem cell virus (MSCV) carrying MSCVpuro (plasmid control) or MSCV-PTP α 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 and analysis 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 instructions, and activities were defined as a measure of the units of cellular Src (c-Src) kinase activity. To measure Syk kinase activity, immunoprecipitated Syk was assayed using an ELISA-based HTScan Syk Kinase Assay Kit (Cell Signaling Technology) according to the manufacturer's instructions.

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 mixture were separated 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 pelleted cell lysate). To determine the amount of phosphatidylserine 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). Alternatively, supernatants were collected after 1 h of stimulation and assessed for cysteinyl leukotrienes using an EIA kit (Cayman Chemical).

Data analysis

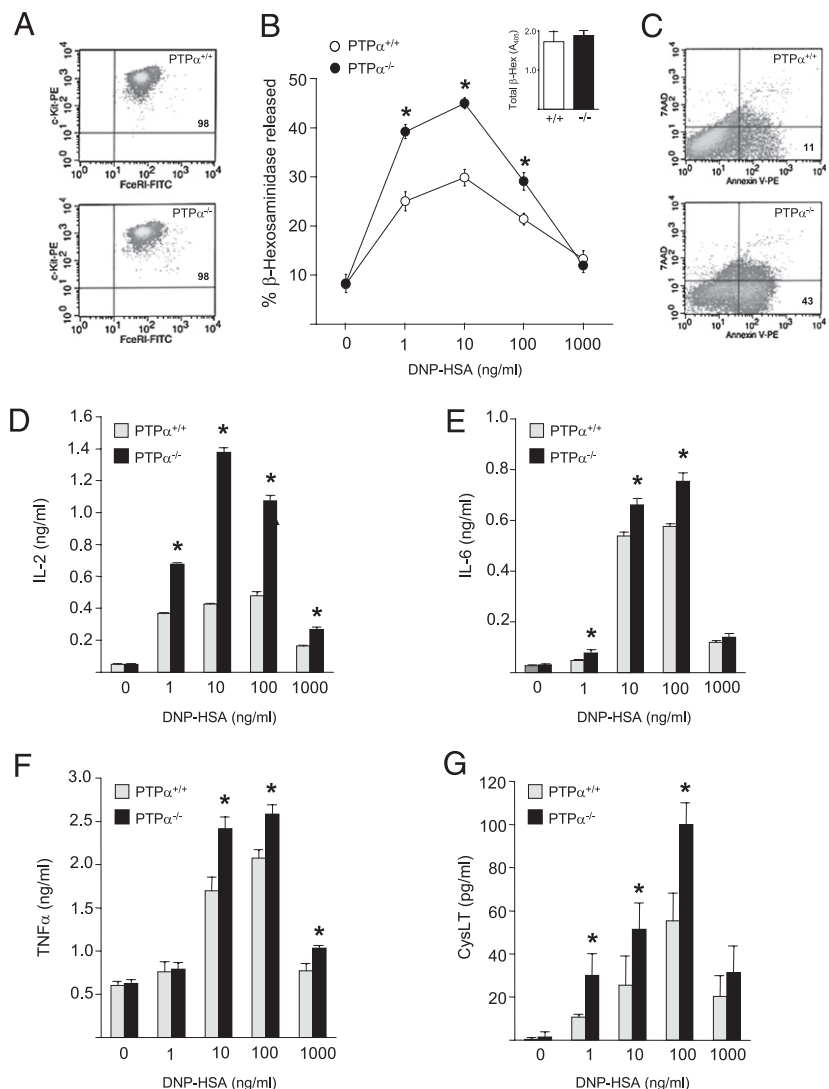
Data were statistically analyzed using the Student *t* test.

Results

PTP α deficiency leads to Fc ϵ RI-dependent hyperdegranulation and augmented cytokine production in BMMCs

To determine whether PTP α regulates Fc ϵ RI-dependent release of bioactive mediators from mast cells, we measured mast cell de-

FIGURE 1. PTP α deficiency leads to enhanced degranulation and cytokine production. **A**, WT (PTP $\alpha^{+/+}$) and PTP $\alpha^{-/-}$ BMMCs (4–6 wk culture) were analyzed for c-Kit and Fc ϵ RI expression by FACS to confirm purity prior to use. Representative FACS graphs are shown. For activation, the cells were sensitized overnight with anti DNP-IgE mAb and challenged with DNP-HSA for 15 min. **B**, Released β -hexosaminidase was measured. The inset shows total (released plus nonreleased) β -hexosaminidase of the cells (WT, $+/+$; PTP $\alpha^{-/-}$, $-/-$) in arbitrary absorbance units. **C**, Cells were stained with annexin V and analyzed by FACS, and the percentage of phosphatidylserine externalization was determined. A representative FACS graph showing annexin V stained scatter for 10 ng/ml DNP-HSA stimulation is shown. Numbers in the bottom right quadrant show the percentage of annexin V-positive cells. **D–G**, Cytokine release was measured from cells after stimulation with DNP-HSA for 15 h; IL-2 (**D**), IL-6 (**E**), and TNF- α (**F**). **G**, Cysteinyl leukotriene release was measured after 1 h of stimulation. Data represent the mean $\pm$ SD from three separate experiments. Asterisks indicate a significant difference ($p < 0.05$) between comparative data from both genotypes.



granulation and cytokine production from BMMCs established from WT and PTP $\alpha^{-/-}$ mice. Both types of BMMCs exhibited equivalent expression of the mature mast cell markers c-Kit and Fc ϵ RI (Fig. 1A). Fc ϵ RI-mediated degranulation of PTP $\alpha^{-/-}$ 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 PTP $\alpha^{-/-}$ mast cells, annexin V-positive phosphatidylserine on the externalized membrane regions of degranulated mast cells was assessed. Degranulated PTP $\alpha^{-/-}$ 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 Fc ϵ RI-mediated cytokine release of WT and PTP $\alpha^{-/-}$ mast cells. Significantly elevated levels of IL-2, IL-6, TNF- α , and cysteinyl leukotrienes were released from PTP α -deficient BMMCs (Fig. 1D–G). Collectively, these results demonstrate that PTP α is a negative regulator of Fc ϵ RI-dependent BMMC degranulation and cytokine release.

To confirm that the observed hyperactivation phenotype of PTP $\alpha^{-/-}$ BMMCs was indeed due to the absence of PTP α , PTP α was re-expressed in the PTP α -null BMMCs by retroviral (i.e., MSCV) infection, to a level that was $86 \pm 11\%$ ($n = 3$) of that in WT BMMCs (Fig. 2A). PTP α 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 PTP α resulted in the loss of the abilities of these mutant cells to release elevated levels of IL-2 and TNF- α (Fig. 2C). PTP $\alpha^{-/-}$ BMMCs infected with MSCV carrying a control (empty) plasmid exhibited degranulation and cytokine release characteristic of the parental PTP $\alpha^{-/-}$ BMMCs (Fig. 2B, 2C).

PTP α -deficient mice show significantly enhanced anaphylactic responses

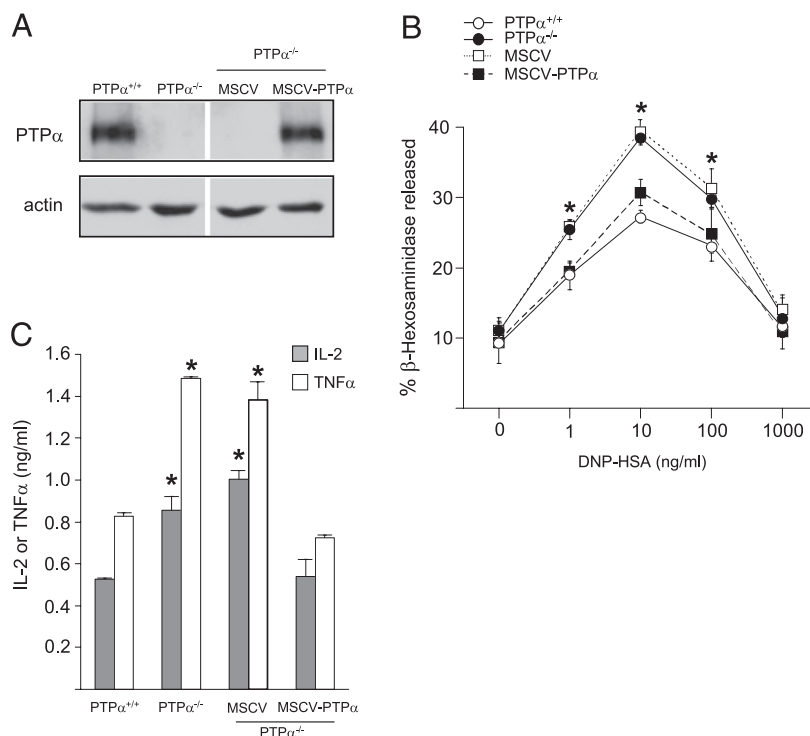
Anaphylaxis is a profound allergic reaction initiated by allergen-induced cross-linking of specific IgE bound to Fc ϵ RI. IgE/Fc ϵ RI-

stimulated mediator release from mast cells is a major determinant of anaphylaxis (2, 39). Because PTP α -deficient mast cells exhibit Fc ϵ RI-dependent hyperdegranulation *ex vivo*, we questioned whether PTP α plays a role in Fc ϵ RI-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 PTP α -deficient mice. As shown in Fig. 3A, mice lacking PTP α 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. PTP $\alpha^{-/-}$ mice generally had thicker inflamed ears than WT mice (knockout, 839 ± 49 μ m; WT, 795 ± 73 μ m; $n = 15$ 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 PTP $\alpha^{-/-}$ 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 PTP $\alpha^{-/-}$ mice (Fig. 3C). Our data demonstrate that PTP α negatively regulates IgE- and Fc ϵ RI-dependent PCA and PSA, consistent with the *ex vivo* hyperdegranulation of cultured BMMCs. PTP $\alpha^{-/-}$ 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.

PTP α differentially regulates Fc ϵ RI-mediated activation of SFKs

PTP α 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 Fc ϵ RI-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 PTP α expression in PTP $\alpha^{-/-}$ BMMCs rescues aberrant activation. WT (PTP $\alpha^{+/+}$), PTP $\alpha^{-/-}$, and PTP $\alpha^{-/-}$ BMMCs that had been infected with MSCV bearing an empty vector (MSCV) or a PTP α expression vector (MSCV-PTP α) were analyzed for (A) PTP α 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 $\pm$ SD from three separate experiments. Asterisks indicate a significant difference ($p < 0.05$) with results from WT BMMCs.



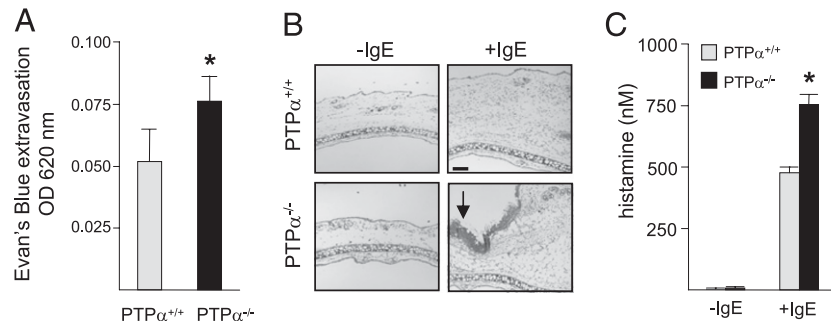


FIGURE 3. Lack of PTP α significantly elevates anaphylaxis in mice. Passive cutaneous anaphylaxis was induced in WT and PTP α ^{-/-} mice. *A*, Thirty minutes after Ag challenge, ears were removed and extravasated Evans blue dye was quantified and normalized for the unit weight ($n = 5$ animals per genotype). *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 PTP α ^{-/-} mouse ears is marked by the arrow. Scale bar, 100 μ m. *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 $\pm$ SD. Asterisks indicate a significant difference ($p < 0.05$) between comparative data from animals of each genotype.

fore investigated the effects of PTP α ablation on Fc ϵ RI-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 PTP α ^{-/-} BMMCs, resulting in significantly reduced Lyn activity for up to 15 min poststimulation in PTP α ^{-/-} BMMCs compared with WT BMMCs (Fig. 4A). We also found a lack of Fyn activation in stimulated PTP α ^{-/-} BMMCs, as indicated by the absence of Fyn Tyr528 dephosphorylation and reduced kinase activity of Fyn in stimulated PTP α ^{-/-} mast cells compared with WT cells (Fig. 4B). In

contrast, Hck activation was evident in IgE cross-linked PTP α ^{-/-} 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 PTP α ^{-/-} cells (Fig. 4C). These analyses reveal unexpected differential abilities of PTP α to regulate Fc ϵ RI-dependent SFK activities, with PTP α positively regulating Fyn and Lyn activation but negatively regulating Hck activation. PTP α functions as an activator of SFKs in multiple signaling systems, indicating that reduced Lyn and Fyn activities in PTP α ^{-/-} BMMCs reflect the loss of PTP α -catalyzed dephosphorylation and activation of these kinases. In contrast, it is

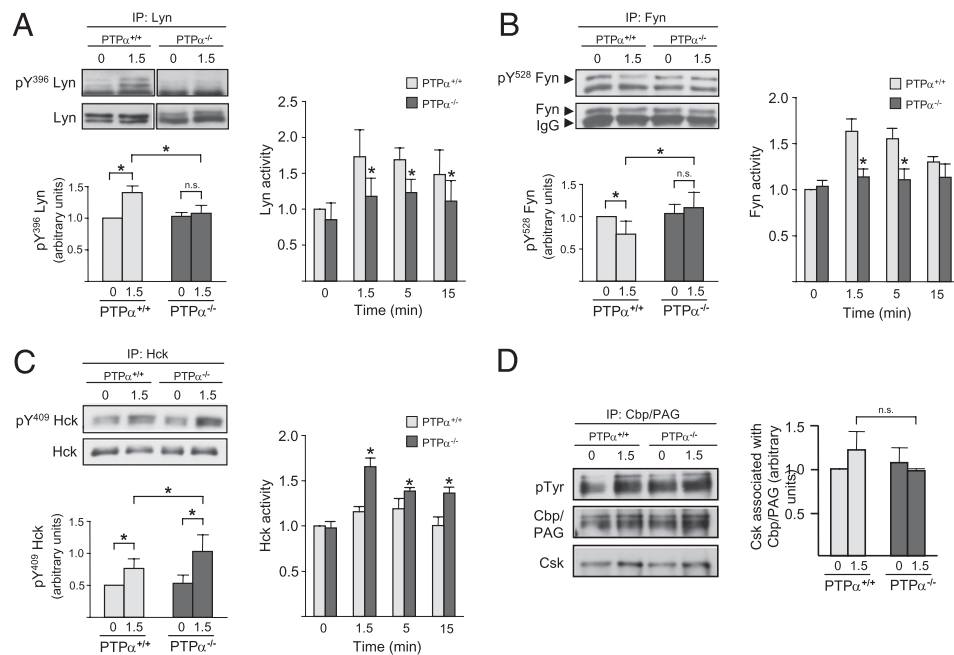


FIGURE 4. Fc ϵ RI-mediated activation of Fyn, Lyn, and Hck are regulated by PTP α . Immunoprecipitates of Lyn (*A*), Fyn (*B*), and Hck (*C*) were prepared from lysates of WT and PTP α ^{-/-} BMMCs that were not stimulated (0) or stimulated with 100 ng/ml DNP-HSA for the indicated times. Immunoprecipitates were immunoblotted with anti-phospho Tyr⁵²⁷-Src Ab 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 $\pm$ 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 $\pm$ 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 $\pm$ 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 PTP α -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 PTP α ^{-/-} BMMCs (Fig. 4D).

PTP α positively regulates Fc ϵ RI receptor phosphorylation and negatively regulates Fc ϵ RI-dependent MAPK and PI3K pathways

Overall protein tyrosine phosphorylation was enhanced in Ag-stimulated PTP α ^{-/-} mast cells (Fig. 5A). To further determine how the absence of PTP α affected specific steps in Fc ϵ RI-mediated signaling to result in mast cell hyperactivation, we examined the early event of Fc ϵ RI 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 Fc ϵ RI- β and Fc ϵ RI- γ subunits to initiate subsequent signaling (3, 7), and Hck can also regulate Fc ϵ RI- β and Fc ϵ RI- γ phosphorylation (9). Fyn does not phosphorylate Fc ϵ RI (11, 18). The Fc ϵ RI- γ subunit was immunoprecipitated from WT and PTP α ^{-/-} BMMCs and probed for tyrosine phosphorylation (Fig. 5A) and associated phosphotyrosyl β subunit (Fig. 4B, 4C). We observed a large decrease ($\sim 70\%$) in Ag-stimulated phosphorylation of the Fc ϵ RI- γ subunit (Fig. 5B) and reduced phosphorylation of the associated β subunit (80% of WT; $n = 2$; Fig 5C), in PTP α -deficient mast cells compared with WT cells. These results

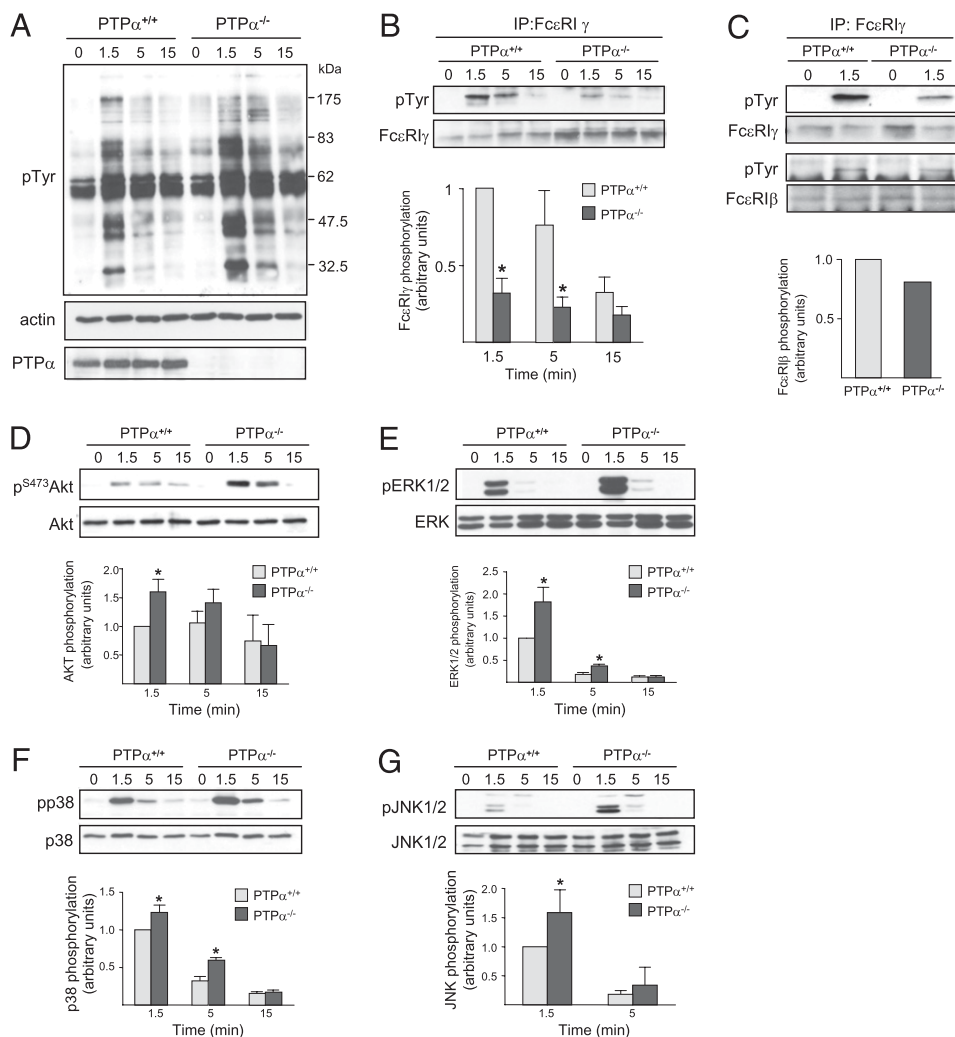
are in accord with the decreased Lyn activity in PTP α -null BMMCs; they also are consistent with the increased Hck activity in PTP α ^{-/-} mast cells, because Hck^{-/-} BMMCs exhibit enhanced Fc ϵ RI γ and β phosphorylation that may be due to the proposed role of Hck as a negative regulator of Lyn and Lyn-mediated phosphorylation events (9).

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

Increased activity of the Fc ϵ RI-interacting kinase Syk in PTP α ^{-/-} mast cells

Lyn-mediated phosphorylation of the γ and β subunits of Fc ϵ RI results in the recruitment of positive and negative regulators of signaling. The tyrosine kinase Syk becomes activated upon interaction with phosphorylated ITAMs of Fc ϵ RI γ , 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 PTP α ^{-/-} BMMCs at 1.5 min after stimulation, and it was equivalent to that in WT BMMCs by 5 and 15 min after stimulation (Fig. 6A). In vitro assays of immunoprecipitated Syk

FIGURE 5. Lack of PTP α leads to reduced phosphorylation of Fc ϵ RI and hyperphosphorylation of Fc ϵ RI downstream molecular targets. WT and PTP α ^{-/-} 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 PTP α as indicated ($n = 3$). Numbers on the right indicate m.w. markers. **B**, Fc ϵ RI γ was immunoprecipitated and probed with anti-4G10 to detect tyrosine phosphorylation (pTyr, top panel). The blot was stripped and probed with anti-Fc ϵ RI γ (bottom panel). Densitometric quantification (mean $\pm$ 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, Fc ϵ RI γ immunoprecipitates were probed as in **B** and reprobed for Fc ϵ RI γ (top panels) and Fc ϵ RI β (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 $\pm$ SD of quantified data from three experiments. Asterisks indicate a significant difference ($p < 0.05$) between comparative data from the two genotypes.



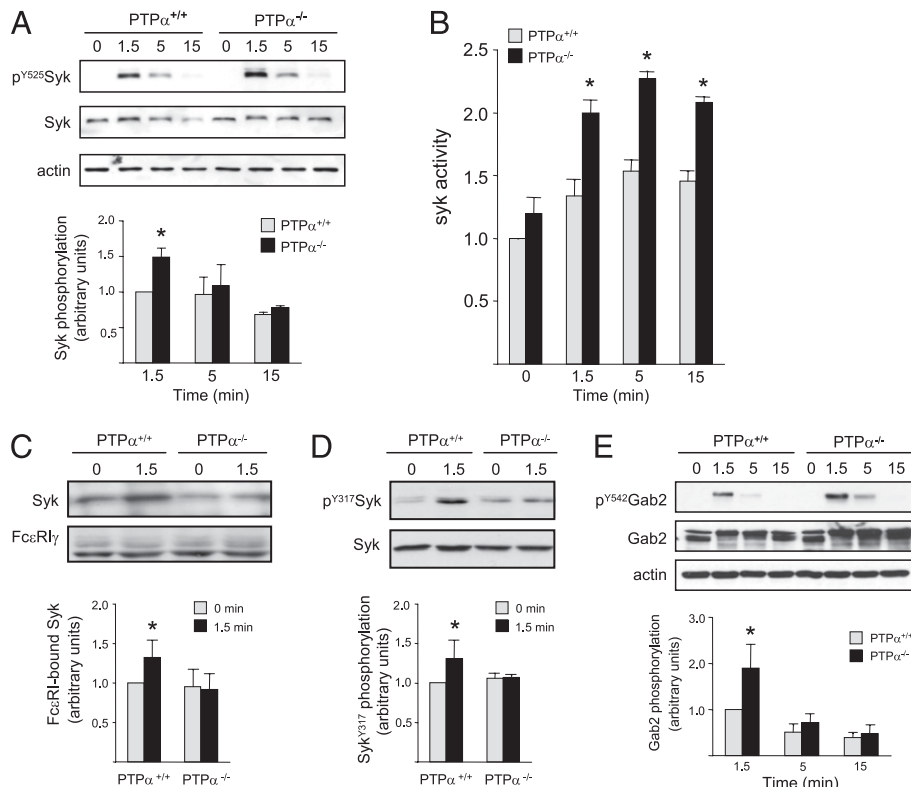


FIGURE 6. FcεRI stimulation-dependent Syk kinase activity, Syk and Gab2 phosphorylation are increased in PTPα-deficient mast cells. WT and PTPα^{-/-} BMMCs were sensitized overnight with anti-DNP-IgE and then left unstimulated (0) or stimulated with 100 ng/ml DNP-HSA for 1.5, 5, and 15 min. *A*, Cell lysates were immunoblotted with phospho-Tyr525/526 Syk (top panel) and then stripped and reprobed for anti-Syk or anti-actin. *B*, Syk immunoprecipitates were analyzed in *in vitro* kinase assays. Kinase activity was calculated relative to the standard kinase activity of c-Src. Data (mean ± SD) represent duplicate measurements from each of three independent experiments. *C*, The FcεRI receptor complex was immunoprecipitated using anti-IgE Ab and then immunoblotted with Syk (top panel) and FcεRIγ (bottom panel) Abs. *D*, As in *A*, but lysates were probed with anti-phosphoTyr317 Syk and anti-Syk Abs. *E*, Cell lysates were immunoblotted with phospho-Tyr542 Gab2 (top panel), stripped, and reprobed for anti-Gab2 and anti-actin as indicated (bottom panels). Representative immunoblots from three independent experiments are shown. Densitometric quantification (mean ± SD) of immunoblots is shown in the graphs. Asterisks indicate a significant difference ($p < 0.05$) between comparative data from each genotype.

demonstrated significantly elevated Syk activity in PTPα^{-/-} BMMCs, compared with WT BMMCs, that was evident for up to 15 min after stimulation (Fig. 6*B*). 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-FcεRIγ is required for Syk activation. Because stimulated PTPα^{-/-} BMMCs have reduced FcεRIγ phosphorylation, but have increased Syk activity, we investigated the interaction of Syk with FcεRIγ in PTPα^{-/-} and WT cells. Immunoprecipitations of FcεRIγ 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 FcεRI and then probed for FcεRIγ and associated Syk. Although Syk association with FcεRI increased upon Ag stimulation of WT BMMCs, no Ag-dependent increase was observed in PTPα^{-/-} BMMCs (Fig. 6*C*). This finding suggests that Syk is hyperactivated in PTPα-null mast cells via a mechanism other than that involving enhanced Syk binding to FcεRI.

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. 6*D*). 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 FcεRI-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. 6*E*). Therefore, PTPα functions as a negative regulator of FcεRI-dependent activation of Syk and Gab2.

Regulation of FcεRI-associated phosphatases by PTPα

Lyn is associated with and phosphorylates the FcεRI-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. 7*A*, 7*B*).

Discussion

We have used PTPα-deficient BMMCs and mice to define roles of PTPα as a negative regulator of FcεRI-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 FcεRI signaling. In PTPα-null BMMCs, Ag-stimulated activation of the SFKs Lyn and Fyn is

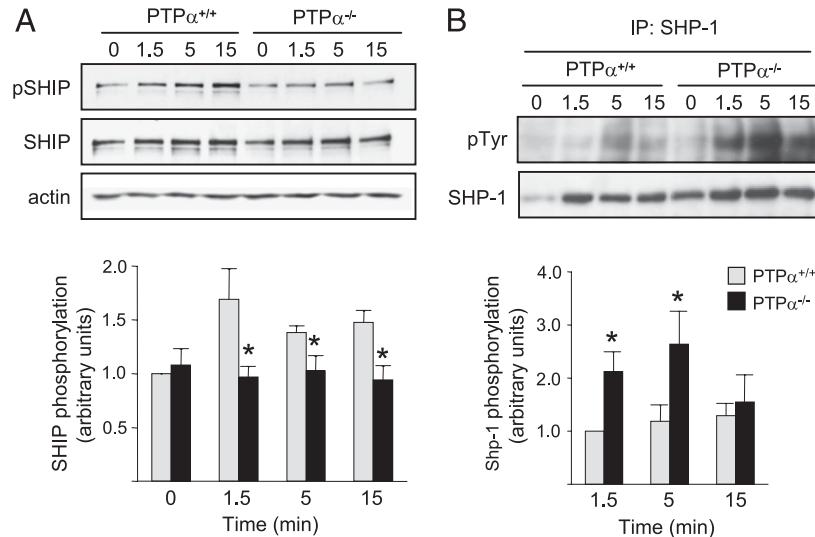


FIGURE 7. PTP α positively regulates SHIP phosphorylation and negatively regulates SHP-1 phosphorylation in Fc ϵ RI-stimulated BMMCs. WT and PTP $\alpha^{-/-}$ BMMCs were sensitized overnight with anti-DNP-IgE and then left unstimulated (0) or stimulated for 1.5, 5, and 15 min with DNP-HSA. **A**, Cell lysates were immunoblotted with phospho-Tyr1020 SHIP (*top panel*) and then stripped and reprobed with SHIP and actin Abs (*bottom panels*). **B**, SHP-1 immunoprecipitates were immunoblotted with anti-phosphotyrosine (pTyr, *top panel*) and then stripped and reprobed for anti-SHP-1. A representative immunoblot from three independent experiments is shown. Densitometric quantification (mean $\pm$ SD) of three immunoblots is shown in the graphs. Asterisks indicate a significant difference ($p < 0.05$) between comparative data from each genotype.

abrogated, whereas Hck activation is enhanced. In conjunction with the well-established actions of PTP α to activate SFKs in multiple signaling systems and its demonstrated phosphatase activity toward Fyn (25, 47), these findings indicate that PTP α acts as an Ag-stimulated Lyn-Fyn phosphatase and a critical positive regulator of these SFKs in Fc ϵ RI signaling, in addition to acting as a negative regulator of Hck (Fig. 8).

The resemblance of the situation of enhanced Hck activity and reduced Lyn activity in PTP $\alpha^{-/-}$ BMMCs to the enhanced Fyn activity in Lyn $^{-/-}$ BMMCs (12, 13) supports the likelihood that

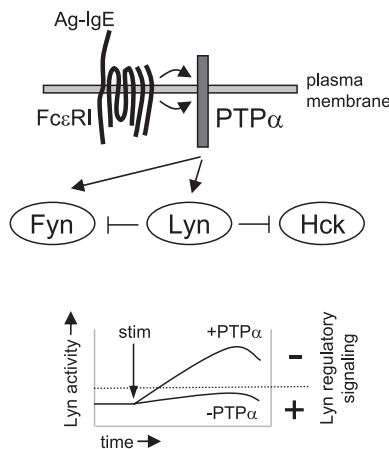


FIGURE 8. Model of PTP α -regulated SFK activities and Lyn regulatory signaling. In the top schematic, Ag-IgE-engaged Fc ϵ RI promotes PTP α -mediated activation of Lyn and Fyn. Activated Lyn negatively regulates Fyn and Hck activation, countering PTP α -mediated Fyn activation to an unknown extent. In the absence of PTP α , 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 PTP α . PTP α promotes Lyn activation and Lyn negative and positive regulatory signaling (+PTP α). In the absence of PTP α (-PTP α), 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 PTP α -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 ^{WeeB} 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), PTP α may additionally promote Lyn-mediated inhibition of Fyn, although apparently the direct activation of Fyn by PTP α 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 PTP α (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 PTP $\alpha^{-/-}$ 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 PTP $\alpha^{-/-}$ 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 $PTP\alpha^{-/-}$ BMMCs at least partially contributes to $PTP\alpha^{-/-}$ 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 $PTP\alpha$, because reduced phosphorylation of SHIP and enhanced phosphorylation of the PI3K target Akt occur in $PTP\alpha^{-/-}$ BMMCs. Like SHIP, the PTP SHP-1 undergoes Fc ϵ RI-mediated and Lyn-dependent phosphorylation and activation (7, 46, 48). SHP-1 is reported to dephosphorylate various signaling molecules, including Fc ϵ RI β/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 $PTP\alpha^{-/-}$ BMMCs. This finding suggests that SHP-1 catalytic activity and its regulatory actions are unexpectedly enhanced in the absence of $PTP\alpha$. Nevertheless, this enhancement is insufficient to exert a net inhibition of $PTP\alpha^{-/-}$ BMMC activation. However, SHP-1 targets remain to be clarified, and because a distinct positive role of SHP-1 in Fc ϵ RI signaling may involve its function as an adaptor that facilitates SLP-76 and PLC γ association (50), the significance of enhanced SHP-1 phosphorylation in $PTP\alpha$ -null cells is difficult to interpret. SHP-1 autodephosphorylation has been reported in RBL-2H3 basophilic leukemia cells (48). An additional mode of regulation is indicated by our finding that $PTP\alpha$ directly or indirectly limits Ag-stimulated SHP-1 phosphorylation.

A key positive action of Lyn in Fc ϵ RI-mediated signaling is the phosphorylation of ITAMs in Fc ϵ RI- 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^{WeeB} mast cells, very low Lyn 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 $PTP\alpha^{-/-}$ BMMCs, Ag-stimulated Lyn activity and phosphorylation of Fc ϵ RI- 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 Fc ϵ RI- 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 $PTP\alpha$ -dependent signaling, certainly that involving Lyn activation, is manifested through the loss of negative but not positive regulation of Syk.

A recent study of Lyn^{WeeB} 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 $PTP\alpha^{-/-}$ mast cells with WT Lyn expression and basal activity, but with minimal Lyn activation. We also identify $PTP\alpha$ as a critical upstream initiator of the majority of Ag- and Fc ϵ RI-induced Lyn activation that drives negative regulatory signaling. Furthermore, the ex vivo hyperactive phenotype of $PTP\alpha^{-/-}$ mast cells is reflected by enhanced PSA and PCA in mice, indicating that $PTP\alpha$ limits Fc ϵ RI-initiated anaphylactic allergic reactions. Further investigation of the mechanisms by which Ag engagement of Fc ϵ RI promotes $PTP\alpha$ to

function in what appears to be one of the earliest enzymatic reactions that triggers subsequent signaling, and of whether $PTP\alpha$ targets a discrete population of Lyn while another is inaccessible to $PTP\alpha$, will be required to explore the utility of therapeutically manipulating $PTP\alpha$ to control allergic disease.

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Disclosures

The authors have no financial conflicts of interest.

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