

Protein Tyrosine Phosphatase ϵ is a Negative Regulator of Fc ϵ RI-mediated Mast Cell Responses

M. Akimoto^{*,†}, K. Mishra^{*}, K.-T. Lim^{*}, N. Tani[‡], S.-i. Hisanaga[†], T. Katagiri[‡], A. Elson[§], K. Mizuno^{*} & H. Yakura^{*,†}

^{*}Department of Immunology and Signal Transduction, Tokyo Metropolitan Institute for Neuroscience, Tokyo Metropolitan Organization for Medical Research, Fuchu, Tokyo; [†]Graduate School of Science, Tokyo Metropolitan University, Hachioji, Tokyo; [‡]Department of Biology, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Toyama, Japan; and [§]Department of Molecular Genetics, Weizmann Institute of Science, Rehovot, Israel

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Correspondence to: H. Yakura, L'IHPST, Université Paris 1 Panthéon-Sorbonne, 13, rue du Four, 75006 Paris, France.
E-mail: hide.yakura@orange.fr

Abstract

Modulation of mast-cell activation may provide novel ways to control allergic diseases. Here, we show that protein tyrosine phosphatase ϵ (PTP ϵ ; *Ptpre*) plays key regulatory roles during mast-cell activation mediated by the high-affinity IgE receptor (Fc ϵ RI). Bone marrow-derived mast cells (BMMC) from *Ptpre*^{-/-} mice exhibited enhanced Fc ϵ RI-induced Ca²⁺ mobilization and mitogen-activated protein kinase (MAPK) (JNK and p38) activation, and showed corresponding enhancement of evoked degranulation and cytokine production, but not leukotriene production. Examination of proteins linking tyrosine kinase activation and Ca²⁺ mobilization revealed that the absence of PTP ϵ leads to increased phosphorylation of the linker for activation of T cells and SH2 domain-containing leucocyte phosphoproteins of 76 kDa, but not Grb2-associated binder-2 (Gab2). Because Gab2 is considered to be situated downstream of Fyn kinase, we reasoned that Fyn may not be a target of PTP ϵ . In the event, Syk but not Lyn was hyperphosphorylated in PTP ϵ -deficient BMMC. Thus, PTP ϵ most likely exerts its effects at the level of Syk, inhibiting downstream events including phosphorylation of SLP-76 and linker of activated T cells and mobilization of Ca²⁺. Consistent with the *in vitro* data, antigen- and IgE-mediated passive systemic anaphylactic reactions were augmented in *Ptpre*^{-/-} mice. Given that the number of mast cells is unchanged in these mice, this observation most likely reflects alterations of mast cell-autonomous signalling events. These data suggest that PTP ϵ negatively regulates Fc ϵ RI-mediated signalling pathways and thus constitutes a novel target for ameliorating allergic conditions.

Introduction

Antigen (Ag)-induced ligation of the high affinity IgE receptor (Fc ϵ RI) on mast cells triggers a cascade of signalling events that ultimately leads to a wide variety of effector functions: release of granules containing histamine, serotonin and β -hexosaminidase; production of leukotrienes and prostaglandins; and transcription of cytokine genes and secretion of their products [1–3]. All of these factors contribute to the development of allergic responses, and one of the earliest signalling events in that process is the activation of protein tyrosine kinases (PTK) [4–6].

A consensus view holds that, upon Fc ϵ RI aggregation, the Src-family PTK Lyn is activated and phosphorylates immunoreceptor tyrosine-based activation motifs of the β

and γ subunits of the Fc ϵ RI complex [4, 7–9], which in turn recruit and activate Syk PTK [6, 10, 11]. Activated PTKs subsequently catalyze the phosphorylation of a variety of signalling molecules, including phospholipase C (PLC) γ [7, 12], Bruton's tyrosine kinase [13, 14] and adaptor proteins such as linker of activated T cells (LAT) [15, 16], non-T cell activation linker (NTAL)/linker for activation of B cells (LAB) [17, 18], and SH2 domain-containing leucocyte phosphoproteins of 76 kDa (SLP-76) [19, 20]. PI3K [21, 22], which catalyzes the synthesis of PI 3,4-bisphosphate and PI 3,4,5-trisphosphate, also plays a critical role by contributing to the recruitment of pleckstrin homology domain-containing molecules, such as Akt and PLC γ , to the plasma membrane. As an alternative proximal pathway, the Src-family PTK Fyn plays an essential role in initiating Fc ϵ RI signalling by

phosphorylating Grb2-associated binder-2 (Gab2) [23, 24], which recruits and activates PI3K. These signals are then transduced to Ca²⁺ mobilization and activation of mitogen-activated protein kinases (MAPK) [25–27], effects which are ultimately integrated to modulate mast cell function.

Originally cloned from human placenta, protein tyrosine phosphatase ϵ (PTP ϵ) is a ubiquitously expressed enzyme with two PTP domains [28]. There are at least four isoforms generated from a single gene: a transmembrane form (RPTP ϵ) and three cytoplasmic isoforms (cyt-PTP ϵ , p67 and p65) [29–33]. The cytoplasmic forms of p67 and p65 are subject to translational and post-translational regulation [31, 33], and given the differences in their subcellular localization, each isoform likely has distinct cellular functions. For example, RPTP ϵ contributes to the maintenance of the transformed phenotype of *neu*-induced mouse mammary tumour cells [34, 35], which appears to be mediated by activating Src *in vivo* [36]. In addition, insulin receptor signalling *in vitro* is downregulated by RPTP ϵ [37] and cyt-PTP ϵ [38]. The cyt-PTP ϵ dephosphorylates and inhibits delayed-rectifier, voltage-gated potassium (Kv) channels in Schwann cells, and young Ptpre^{-/-} mice exhibit hypomyelination of sciatic nerve axons [39]. PTP ϵ is also involved in other aspects of physiological functions: the negative regulation of MAPK activity [40, 41], inhibition of IL-6- and IL-10-induced JAK-STAT signalling in myeloid cells [42, 43], suppression of tumourigenesis [44], regulation of macrophage function [45] and positive regulation of osteoclast function [46].

In this study, we examined the role of PTP ϵ in Fc ϵ RI-mediated mast cell function. Using bone marrow-derived mast cells (BMMC) from Ptpre^{-/-} and Ptpre^{+/+} mice, we found that PTP ϵ -deficient BMMC exhibited enhanced calcium mobilization and activation of MAPK (JNK and p38), leading to greater degranulation and cytokine secretion without significant effects on leukotriene production. Proximal events such as phosphorylation of Syk, LAT, SLP-76, but not Lyn or Gab2, were negatively regulated by PTP ϵ . It is thus reasonable to speculate that PTP ϵ acts on Syk, downstream of Lyn, thereby inhibiting the pathways leading to phosphorylation of SLP-76 and LAT, calcium mobilization and MAPK activation. Furthermore, passive systemic anaphylaxis (PSA) induced by IgE and Ag was also significantly elevated in Ptpre^{-/-} mice. Given that the numbers of mast cells at various locations were not significantly altered in Ptpre^{-/-} mice, the effect on PSA exerted by the absence of PTP ϵ is caused by alteration of mast cell function *per se* and PTP ϵ is minimally involved in the development and maturation of mast cells *in vivo*. Collectively, our findings suggest that PTP ϵ negatively regulates Fc ϵ RI-mediated signalling pathways and may serve as a useful target for medical interventions in allergic conditions.

Materials and methods

Mice. Ptpre^{+/+} and Ptpre^{-/-} mice [39] were used at 6–12 weeks of age in accordance with Guideline for the Care and Use of Animals (Tokyo Metropolitan Institute for Neuroscience, 2000).

Antibodies and reagents. Anti-DNP IgE MoAb (SPE-7) and DNP-conjugated human serum albumin (DNP-HSA) (30–40 mol DNP/mol albumin) were purchased from Sigma-Aldrich (St Louis, MO, USA). Anti-PTP ϵ antibody was prepared by immunizing a rabbit with keyhole limpet haemocyanin-coupled peptide NKEENREKN-RYPNI (amino acids 154–167), to which a Cys residue was added at the amino-terminal [34]. Anti-phosphotyrosine MoAb (PY20) and rabbit anti-mouse Abs against Lyn, Syk, ERK, JNK and p38 were all from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-mouse phospho-specific p38 Ab and phospho-specific JNK Ab were from Cell Signalling Technology (Beverly, MA, USA), and rabbit anti-mouse phospho-specific ERK Ab was from Promega (Madison, WI, USA). Anti-Gab2 Abs were provided by Dr Benjamin Neel (Ontario Cancer Institute, Toronto, Canada) or purchased from Upstate Biotechnology (UBI; Lake Placid, NY, USA). Anti-LAT Ab was from UBI. Anti-SLP-76 Ab was a gift from Dr Gary Koretzky (University of Pennsylvania, Philadelphia, PA, USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG was from Tago (Burlingame, CA, USA). FITC-conjugated anti-mouse Igs was from Southern Biotechnology Associates (Birmingham, AL, USA). FITC-conjugated anti-CD117 (c-Kit), anti-TNF- α and anti-IL-4 MoAb were from BD Pharmingen (San Diego, CA, USA). IL-3 and stem cell factor (SCF) were purchased from PeproTech (London, UK). Initial experiments were performed with IL-3 generously provided by Dr Hiroshi Matsuda (Tokyo University of Agriculture & Technology, Fuchu, Tokyo, Japan). Ionomycin was from Sigma-Aldrich.

Generation of BMMC. BMMC were generated from the femoral bone marrow cells by a slightly modified protocol of the original report by Razin *et al.* [47]. Briefly, cells were cultured in the Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum, 100 μ M 2-ME, 100 mM sodium pyruvate, non-essential amino acids, 100 μ g/ml streptomycin, 100 U/ml penicillin for 3 weeks in the presence of 10 ng/ml IL-3 and then with 10 ng/ml IL-3 and 10 ng/ml SCF for the rest of the culture period. The culture medium was replaced once a week. Cells were used at 5–8 weeks and fluorescence-activated cell sorter (FACS) analysis showed >97% of the cells to be positive for Fc ϵ RI and CD117 (c-kit) (Fig. 1A).

Flow cytometric analysis. Flow cytometry was carried out as reported previously [48]. BMMC were incubated with FITC-conjugated anti-CD117 (c-Kit) or with

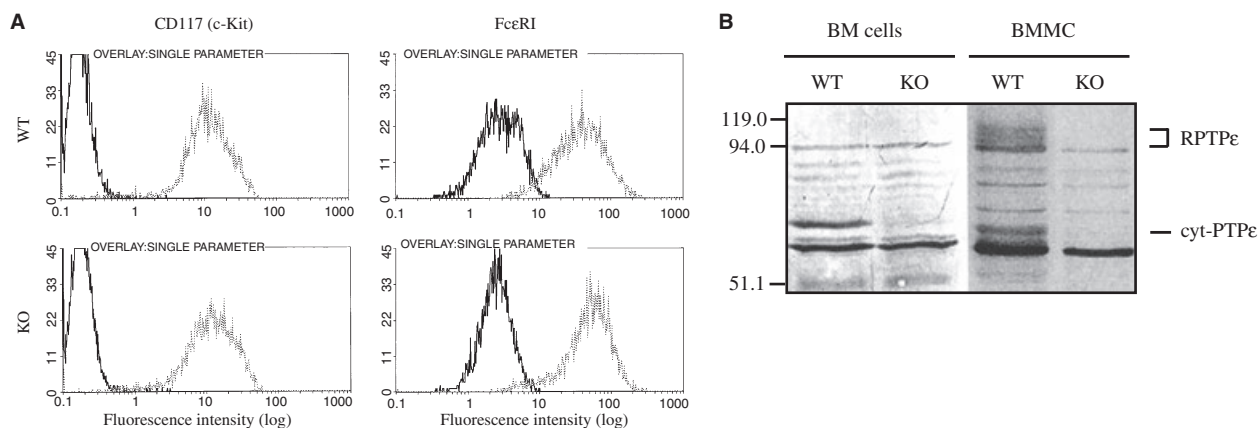


Figure 1 Characterization of BMMC from WT and *Ptpre*^{-/-} (KO) mice. (A) BMMC from WT and *Ptpre*^{-/-} mice were incubated with FITC-conjugated anti-CD117 (c-Kit) (dotted line) or with anti-DNP IgE followed by FITC-conjugated anti-mouse Igs (dotted line). Controls (solid line) were incubated with buffer or with FITC-conjugated anti-mouse Igs alone. (B) Expression of RPTP ϵ and cyt-PTP ϵ in BMMC. Total cell lysates from bone marrow (BM) cells or BMMC prepared from WT and *Ptpre*^{-/-} mice were subjected to Western blotting with anti-PTP ϵ Ab. Numbers at the left indicate mol wt (kDa).

anti-DNP IgE followed by FITC-conjugated anti-mouse Igs. Cells were then subjected to cytometric analysis using a Beckman-Coulter ELITE. The quantification of the surface area under each curve was performed with IMAGEJ (National Institutes of Health, Bethesda, MD, USA).

Cell stimulation. Cells were incubated with DMEM medium containing 100 ng/ml anti-DNP IgE MoAb overnight at 37 °C in a CO₂ incubator. After the excess MoAb was removed, the cells were stimulated by incubation with 10–1000 ng/ml DNP-HSA at 37 °C for the indicated periods of times. The cells were then subjected to functional assays.

Measurement of intracellular free Ca²⁺ concentration ([Ca²⁺]_i). [Ca²⁺]_i was assayed as previously described [25]. Briefly, cells preincubated with anti-DNP IgE MoAb as above were loaded with the acetoxymethyl ester of Fluo-3 (Fluo-3AM) (Molecular Probes, Eugene, OR, USA) for 30 min at 37 °C in Tyrode's buffer (10 mM HEPES, pH 7.4, 130 mM NaCl, 5 mM KCl, 1.4 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose, and 0.1% BSA). After loading, the Fluo-3-containing cells were washed and stimulated with DNP-HSA (10–1000 ng/ml). As a control, the cells loaded with Fluo-3AM were stimulated with 4 μg/ml ionomycin. Fluctuations in [Ca²⁺]_i were measured over a 5-min period using a flow cytometer; mean fluorescence intensity was determined using the MULTITIME program (Phoenix Flow Systems, San Diego, CA, USA).

Degranulation assay. The degree of degranulation was determined by measuring the release of β-hexosaminidase. Cells (2 × 10⁶/ml) were first preincubated overnight with anti-DNP IgE (100 ng/ml) in medium. To measure β-hexosaminidase release, the sensitized cells were stimulated for 20 min with DNP-HSA in Tyrode's buffer. The enzymatic activities of β-hexosaminidase in the supernatants and cell pellets solubilized with 0.5%

Triton X-100 in Tyrode's buffer were measured using *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide in 0.1 M sodium citrate (pH 4.5) as a substrate. The reaction was allowed to run for 30 min at 37 °C and then stopped by adding of 0.2 M glycine (pH 10.7). Release of the product, 4-*p*-nitrophenol, was detected based on the absorbance at 405 nm. The extent of degranulation was calculated by dividing 4-*p*-nitrophenol absorbance in the supernatants by the sum of the absorbance in the supernatants and detergent-solubilized cell pellets.

ELISA for cytokine secretion and leukotrienes production. BMMC were treated with anti-DNP IgE and then stimulated with DNP-HSA for 1–4 h, after which the supernatants were assayed for TNF-α and IL-6 using an ELISA kit (BioSource International, Camarillo, CA, USA), and leukotrienes C₄/D₄/E₄ were assayed using an ELISA (GE Healthcare, Piscataway, NJ, USA). Both assays were used according to the manufacturer's instructions.

Western blot analysis. Western blots were performed as previously described [25]. Briefly, cells were stimulated as described above and solubilized in lysis buffer (1% NP-40, 20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM Na₃VO₄, 2 mM EDTA) supplemented with protease inhibitor cocktail (Boehringer Mannheim GmbH, Mannheim, Germany). The lysates were centrifuged at 10,000 g at 4 °C for 30 min, after which the proteins in the NP-40-soluble supernatant were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was then incubated overnight at 4 °C with optimal concentrations of various antibodies in 10 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween-20. The resultant blots were visualized by incubation with HRP-conjugated goat anti-rabbit IgG and then with ECL Western Blotting Detection Reagent (Amersham Pharmacia Biotech Ltd, Buckinghamshire, UK). The intensity of the protein

bands was measured using a Bio-Rad Imaging Densitometer (Bio-Rad, Richmond, CA, USA).

Passive systemic anaphylaxis. *Ptpr^{e+/+}* and *Ptpr^{e-/-}* mice were either left unsensitized or sensitized by i.v. injection of 9 μ g of anti-DNP IgE. Twenty-four hours later, both groups were challenged with a 200- μ l i.v. injection containing 500 μ g of DNP-HSA. After challenge for 90 s, blood was immediately collected, and the plasma histamine concentration was determined using a competitive histamine immunoassay kit (Immuno-Biological Laboratories, Hamburg, Germany) according to the manufacturer's instructions.

Histological analysis. Organs were harvested from *Ptpr^{e+/+}* and *Ptpr^{e-/-}* mice and fixed in 10% formalin. Tissue specimens were then embedded in paraffin, cut into 5- μ m sections and stained with toluidine blue. To obtain mast cell numbers, >10 high-power fields (40 \times objective) were examined in each tissue from three mice of each genotype.

Results

Characterization of BMMC

To explore the role of PTP ϵ in mast cells, BMMC from PTP ϵ -deficient (KO: *Ptpr^{e-/-}*) and wild-type (WT: *Ptpr^{e+/+}*) mice were obtained by culturing the respective BM cells in the presence of IL-3 for 3 weeks and then with IL-3 plus SCF for 3–5 weeks. FACS analysis showed that BM cells thus generated were 99% positive for CD117 (c-kit) and >97% positive for Fc ϵ RI in both WT and KO (Fig. 1A). The surface area under each curve was also similar between WT and KO cells. The KO:WT ratios of each area were as follows: 1 for CD117 staining (background staining, 0.93) and 1.05 for Fc ϵ RI staining (background, 0.95). The expression of specific PTP ϵ isoforms was then examined in biochemical analyses. Fig. 1B shows that bulk WT BM cells predominantly expressed cyt-PTP ϵ which was not detected in KO BM cells (left panel; in this experiment, non-specific bands were used as loading controls) and that BMMCs expressed both cyt-PTP ϵ and RPTP ϵ isoforms KO BMMCs expressed neither cyt-PTP ϵ nor RPTP ϵ (right panel).

Fc ϵ RI-induced tyrosine phosphorylation of total proteins in PTP ϵ -deficient BMMC

To detect the biochemical changes caused by PTP ϵ deficiency in BMMC, we first examined the tyrosine phosphorylation of total cellular proteins initiated by Fc ϵ RI cross-linking. BMMC from *Ptpr^{e+/+}* and *Ptpr^{e-/-}* mice were either left unsensitized or sensitized by incubation overnight with 100 ng/ml anti-DNP IgE MoAb and then stimulated with 100 ng/ml DNP-HSA for 2, 5, 15 or 30 min, after which the total cell lysates were sub-

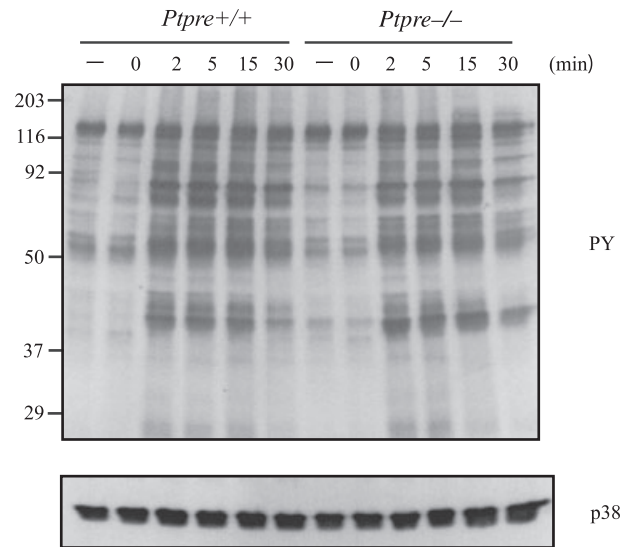


Figure 2 Fc ϵ RI-mediated tyrosine phosphorylation of total cellular protein in PTP ϵ -deficient and WT BMMC. BMMC from *Ptpr^{e+/+}* and *Ptpr^{e-/-}* mice were either sensitized overnight with anti-DNP IgE MoAb or left unsensitized (–) and then stimulated with DNP-HSA for the times indicated. Cell lysates were then subjected to Western blot analysis with anti-PY MoAb. Protein loading was evaluated by blotting with anti-p38 MAPK Ab (lower panel). The results are representative of three separate experiments. Numbers at the left indicate molecular weight (kDa).

jected to Western blotting with anti-PY MoAb or anti-p38 MAPK Ab (control). As shown in Fig. 2, the profiles of the overall tyrosine phosphorylation were similar, and no reproducible difference between WT and PTP ϵ -deficient cells was detected.

Fc ϵ RI-induced Ca²⁺ mobilization is enhanced in PTP ϵ -deficient BMMC

Fc ϵ RI cross-linking induces synthesis of IP₃ via activation of PLC γ , which in turn leads to the release of Ca²⁺ from intracellular stores. This initial release is reportedly coupled to influx of extracellular Ca²⁺ through store-operated Ca²⁺ channels in the plasma membrane [49, 50], and these events are vital for the release of preformed mediators such as histamine, serotonin, and the initial release of TNF- α [51, 52]. Calcium signalling events are also important for de novo cytokine production in mast cells [53, 54]. To evaluate the influence of PTP ϵ on this process, BMMC were sensitized as above, and Ca²⁺ mobilization was assayed for 5 min after stimulation with 10, 100 or 1000 ng/ml DNP-HSA. To examine the effect of PTP ϵ on the Ca²⁺ mobilization machinery *per se*, cells were stimulated with 4 μ g/ml ionomycin. As shown in Fig. 3, Ca²⁺ mobilization was enhanced in PTP ϵ -deficient cells at all Ag concentrations tested, with the most prominent differences being observed at 100–1000 ng/ml DNP-HSA. By contrast, the Ca²⁺ response induced by ionomycin was

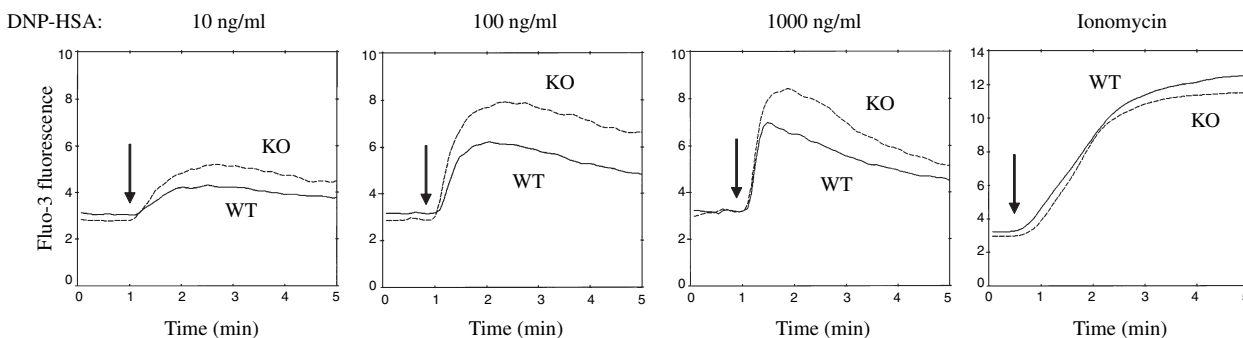


Figure 3 Ca^{2+} mobilization is enhanced in PTP ϵ -deficient BMMC. WT (solid line) and PTP ϵ -deficient (KO: dashed line) BMMC were incubated overnight with 100 ng/ml anti-DNP IgE, washed with Tyrode's buffer and loaded with Fluo-3. The cells were then stimulated with 10, 100 or 1000 ng/ml DNP-HSA. As a control, WT and KO BMMC were stimulated with 4 $\mu\text{g}/\text{ml}$ ionomycin. Fluctuations in Fluo-3 fluorescence were then measured for 5 min using a flow cytometer. Addition of DNP-HSA or ionomycin is indicated by the arrows. The results are representative of three experiments.

similar in the two groups (Fig. 3), suggesting that the machinery underlying Ca^{2+} mobilization is not, itself, affected by the absence of PTP ϵ . Instead it appears that the signalling pathway leading from Fc ϵ RI ligation to Ca^{2+} mobilization is negatively regulated by PTP ϵ .

Fc ϵ RI-mediated phosphorylation of Lyn, Syk, LAT, SLP-76 and Gab2 in PTP ϵ -deficient and WT BMMC

To pin down the molecule(s) that mediates the observed enhancement of Ca^{2+} mobilization in PTP ϵ -deficient BMMC, we next assessed the tyrosine phosphorylation of molecules thought to exert positive regulatory effects on Ca^{2+} responses. Upon tyrosine phosphorylation, several adaptor proteins, including LAT, SLP-76 and Gab2, recruit such signalling molecules as PLC γ and Grb2, positively regulating degranulation and cytokine production. WT and PTP ϵ -deficient BMMC sensitized as above were stimulated with 100 ng/ml DNP-HSA for 2 or 5 min, and the immunoprecipitates were immunoblotted with anti-PY MoAb or an Ab against the respective protein.

Figure 4A shows that tyrosine phosphorylation of Lyn seemed to be only marginally reduced at 5 min of stimulation in WT but not in PTP ϵ -deficient BMMC. Because the reduction was meager and was not reproducibly observed, we consider it does not represent a significant effect. The phosphorylation of the C-terminal negative regulatory tyrosine residue of Lyn was not significantly changed between WT and KO cells (data not shown), suggesting that Lyn activity is not affected by PTP ϵ . On the other hand, phosphorylation of Syk was strongly enhanced in the absence of PTP ϵ (Fig. 4B). The tyrosine phosphorylation status of Gab2 was similar in WT and PTP ϵ -deficient BMMC (Fig. 4C); the observed shift in molecular weight of Gab2 following cross-linking with Ag was most likely because of serine phosphorylation [55] and did not significantly differ in WT and PTP ϵ -deficient cells. As shown in Fig. 4D,E, however, LAT

and SLP-76 were hyperphosphorylated upon Fc ϵ RI ligation in PTP ϵ -deficient BMMC. Taken together, these results suggest that PTP ϵ primarily exerts its effect on Syk- and LAT-/SLP-76-mediated signalling pathways rather than on Fyn-/Gab2-mediated pathways.

Fc ϵ RI-mediated activation of JNK and p38 MAPK is enhanced in PTP ϵ -deficient BMMC

To assess the role of PTP ϵ in MAPK pathways, WT and PTP ϵ -deficient BMMC were sensitized as above and then stimulated with 100 ng/ml DNP-HSA for 5, 15 or 30 min. The activation states of ERK, JNK and p38 MAPK were then assessed by Western blot analysis using Abs against the activated forms of each MAPK. Fig. 5A shows representative results of four separate experiments. Although the phosphorylation/activation status of ERK was unchanged in PTP ϵ -deficient BMMC, phosphorylation/activation of JNK1 was strongly increased 15 min and, albeit to a lesser extent, 30 min after cross-linking. Moreover, the kinetics of p38 activation was slightly shifted to the right (maintained until 15 min after cross-linking) in PTP ϵ -deficient cells. As shown in Fig. 5B, this type of enhancement in JNK and p38 activation was consistently observed and statistically significant. These results suggest that PTP ϵ is principally involved in the regulation of JNK and p38 activation without affecting the ERK pathway.

Fc ϵ RI-mediated degranulation is enhanced in PTP ϵ -deficient BMMC

To examine the effect of PTP ϵ deficiency on the capacity to release mediators upon Fc ϵ RI cross-linking with Ag, WT and PTP ϵ -deficient BMMC sensitized as above were incubated for 20 min with graded concentrations of DNP-HSA, after which release of β -hexosaminidase was assayed. We found that Fc ϵ RI-mediated β -hexosaminidase release

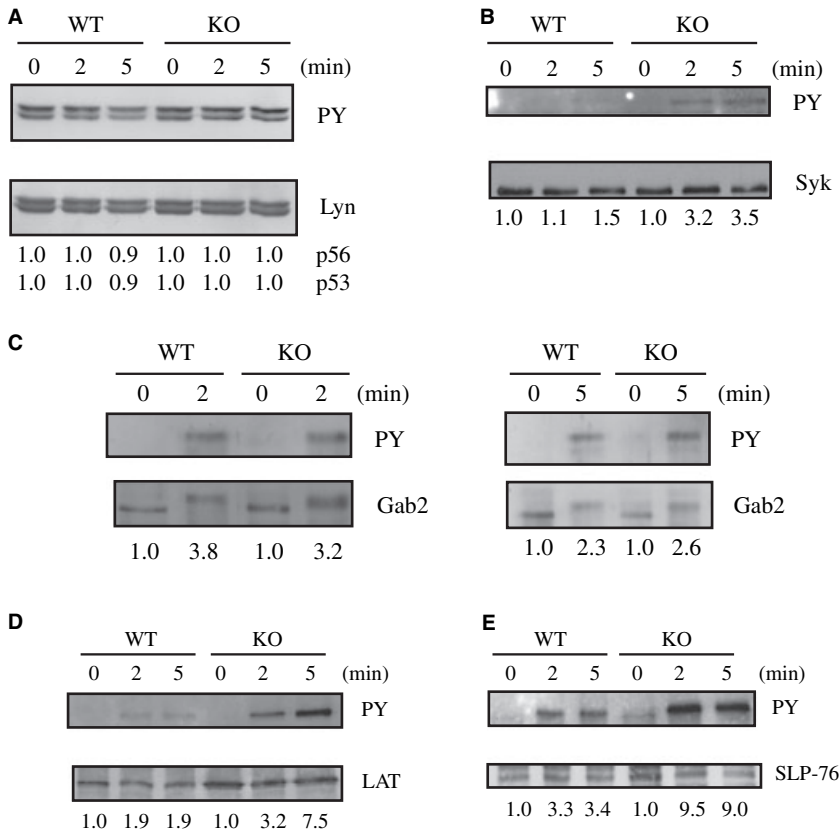


Figure 4 Phosphorylation state of Lyn (A), Syk (B), Gab2 (C), LAT (D), and SLP-76 (E) in WT and PTP ϵ -deficient (KO) BMMC. BMMC were incubated overnight with 100 ng/ml anti-DNP IgE, and then stimulated with 100 ng/ml DNP-HSA for the indicated times. The cell lysates were immunoprecipitated with Abs against the respective proteins and then subjected to immunoblotting with anti-PY MoAb or with Abs against respective proteins. The results are representative of three separate experiments. The intensity of phosphorylated bands was normalized to the level of each protein. The numbers at the bottom of each panel are fold increases in the intensity of each phosphorylated band, as compared with that of unstimulated samples, which were assigned a value of 1.

from PTP ϵ -deficient BMMC elicited by 100 and 1000 ng/ml DNP-HSA ($38 \pm 4\%$ and $26 \pm 2\%$, respectively) was significantly increased as compared with WT cells ($19 \pm 2\%$ and $12 \pm 2\%$, respectively), while ionomycin-induced degranulation was similar in the two groups (Fig. 6A). Thus, the degranulation process *per se* was not affected by the absence of PTP ϵ . Instead, PTP ϵ appears to negatively regulate one or more of the signalling pathways downstream of Syk leading to degranulation.

Fc ϵ RI-mediated cytokine production, but not leukotriene production, is enhanced in PTP ϵ -deficient BMMC

We also assessed the role of PTP ϵ in evoked cytokine production. BMMC sensitized as above were stimulated for 4 h with various concentrations of DNP-HSA, after which the amount of cytokine secreted into the culture supernatant was measured using an ELISA. As shown in Fig. 6B, secretion of TNF- α in PTP ϵ -deficient BMMC was significantly enhanced at 12.5 and 25 ng/ml DNP-HSA (288 ± 10 pg/ml and 264 ± 3 pg/ml, respectively) as compared with WT BMMC (108 ± 8 pg/ml and 128 ± 10 pg/ml, respectively). Similarly, IL-6 production was significantly higher at 12.5 and 25 ng/ml DNP-HSA in PTP ϵ -deficient BMMC (1000 ± 35 pg/ml and 862 ± 11 pg/ml, respectively) than WT cells (350 ± 35 pg/ml and 430 ± 4 pg/ml, respectively). By contrast, production of leukotrienes C₄/D₄/E₄ was unaf-

ected by the absence of PTP ϵ as revealed by ELISA assays (Fig. 6D). Thus, PTP ϵ appears to negatively regulate Fc ϵ RI-mediated cytokine production (at least TNF α and IL-6), without affecting the pathway leading to leukotriene production.

Enhancement of PSA in PTP ϵ -targeted mice

It is well established that passive systemic immunization with mouse IgE followed by i.v. injection of the corresponding Ag leads to activation of mast cells by Fc ϵ RI cross-linking, triggering anaphylactic responses [56, 57]. To determine the extent to which PTP ϵ contributes to Fc ϵ RI-mediated mast cell activation *in vivo*, we next assessed Fc ϵ RI-mediated PSA. WT and Ptpre^{-/-} mice were left untreated or treated with an i.v. administration of 9 μ g anti-DNP mouse IgE, and 24 h later both groups were intravenously administered 500 μ g of DNP-HSA. After 1.5 min, the mice were killed and plasma histamine levels were measured by ELISA assays. As shown in Fig. 7, plasma histamine levels were significantly higher following *in vivo* Fc ϵ RI cross-linking in Ptpre^{-/-} mice than in Ptpre^{+/+} mice. Moreover, histamine levels after Ag injection alone or left untreated (not shown) were similar in the two groups. These findings suggest that as shown in BMMC, PTP ϵ exerts a negative regulatory effect on IgE-dependent mast cell activation *in vivo*.

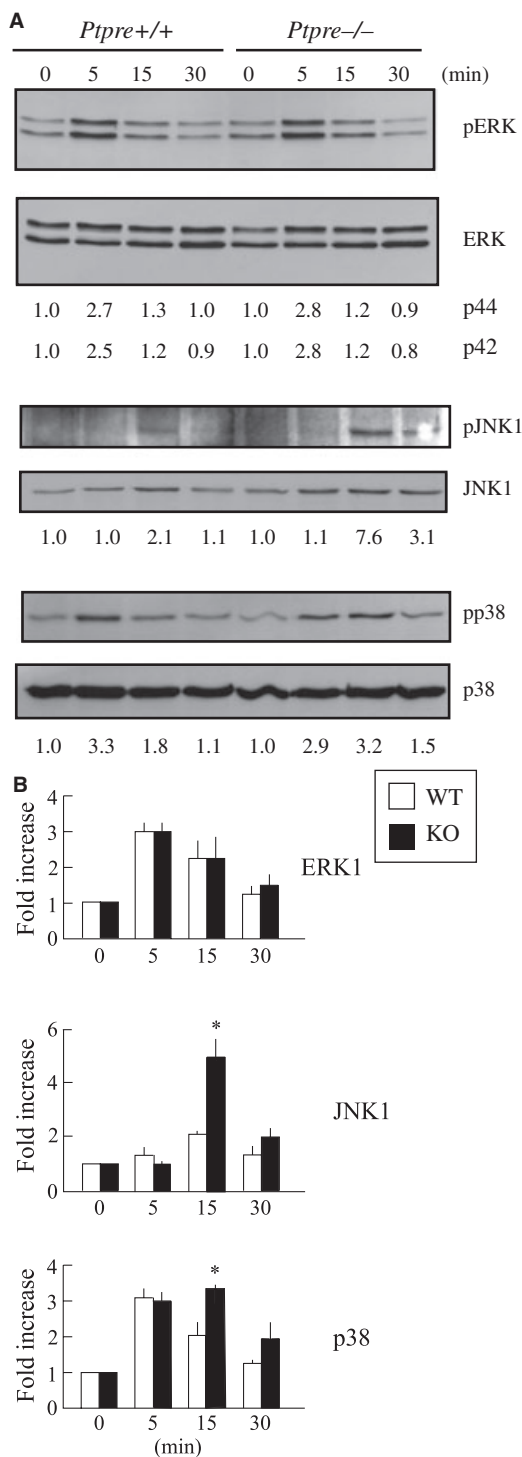


Figure 5 Activation of MAPK in WT and PTPε-deficient BMMC. (A) WT and PTPε-deficient BMMC were incubated overnight with 100 ng/ml anti-DNP IgE and then stimulated with 100 ng/ml DNP-HSA for the times indicated. The cell lysates were then subjected to immunoblotting with Abs against the activated forms of ERK (pERK), JNK (pJNK) and p38 (pp38), and with anti-ERK, anti-JNK and anti-p38 Abs. The results are representative of four separate experiments. The intensity of the phosphorylated bands was normalized to the level of each MAPK. The numbers at the bottom of the ERK, JNK and p38 blots indicate fold increases in the intensity of each phosphorylated band, as compared with that of the unstimulated samples, which was assigned a value of 1. (B) Summary of four experiments. Student's *t*-test indicates that activation of JNK1 and p38 activation at 15 min of stimulation was significantly enhanced in KO BMMC (solid columns) as compared with WT BMMC (open columns). **P* < 0.05.

blue. The results summarized in Table 1 show that there were some slight differences in the numbers of toluidine blue-stained cells in the various tissues from WT and *Ptpre*^{-/-} mice, but the changes were not statistically significant as assessed by Student's *t*-test. It is thus highly unlikely that the enhanced PSA observed in *Ptpre*^{-/-} mice reflects a difference in number of mast cells *in situ*. It is more likely that the signalling machinery within mast cells is altered by the absence of PTPε. The results also suggest that PTPε plays only a minimal role in the development and maturation of mast cells at local sites.

Discussion

In this study, we used BMMC from *Ptpre*^{-/-} mice to examine the role of PTPε in mast cell activation triggered by FcεRI. Our findings suggest that PTPε exerts a negative regulatory effect on FcεRI-mediated BMMC activation both *in vitro* and *in vivo*. First, PTPε appears to downregulate degranulation and production of TNF-α and IL-6, but not secretion of leukotrienes (Fig. 6). Secondly, Ca²⁺ mobilization (Fig. 3) and activation of JNK and p38 MAPKs, but not ERK (Fig. 5), were also inhibited by PTPε. This prompted us to search for a target of PTPε. Significantly, phosphorylation of Syk, but not Lyn, was concordantly increased (Fig. 4). Among the molecules linking Syk activation to MAPK activation or Ca²⁺ mobilization, a family of adaptor proteins is implicated in mast cell activation. For example, LAT and SLP-76 are crucial positive regulators of IgE/Ag-induced mast cell activation [15, 16, 20], and Gab2 exerts positive effects on Akt/PI3 kinase pathway in a Fyn-dependent manner [23, 24]. Gab2 is also required for signalling via the c-Kit/KitL pathway [58]. By contrast, the recently identified adaptor LAB/NTAL is involved primarily in negative regulation of IgE/Ag-mediated signalling and effector functions in the mouse mast cells [17, 18]. Our findings show that PTPε appears to downregulate phosphorylation of LAT and SLP-76, but not Gab2 (Fig. 4).

Mast cell numbers are not affected in *Ptpre*^{-/-} mice

One possible explanation for the enhanced PSA reaction seen in *Ptpre*^{-/-} mice is that it reflects a difference in the number of mast cells *in situ*. To rule out this possibility, we measured the number of mast cells by staining various tissues from WT and *Ptpre*^{-/-} mice with toluidine

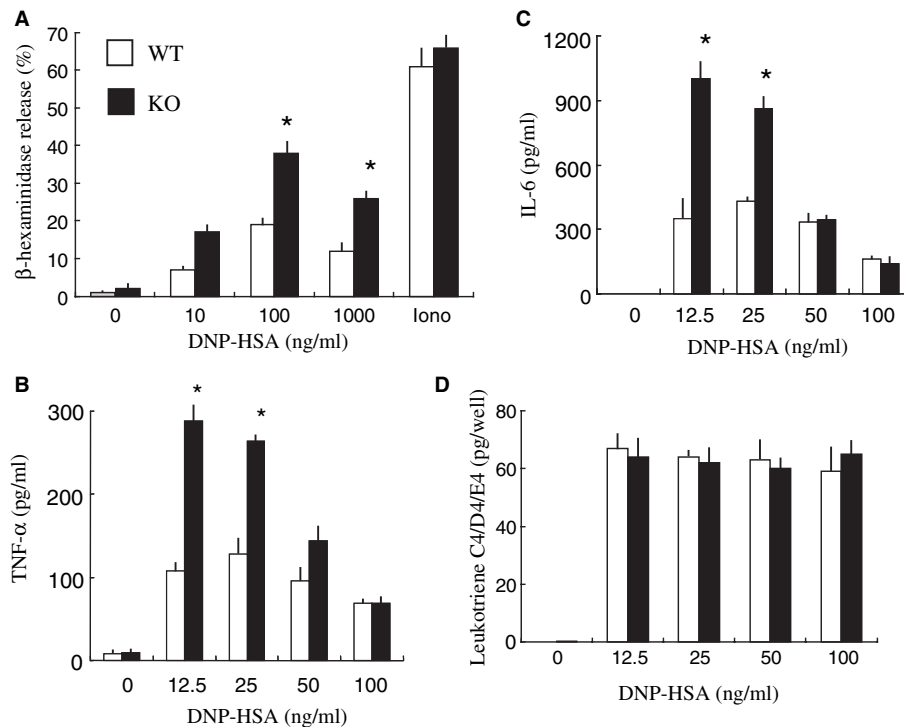


Figure 6 (A) FcεRI-mediated release of β-hexosaminidase is enhanced in PTPε-deficient BMMC. WT (open columns) and PTPε-deficient (solid columns) BMMC were incubated overnight with 100 ng/ml anti-DNP IgE and then either left unstimulated or stimulated for 20 min with 10, 100 or 1000 ng/ml DNP-HSA. As a control, BMMC were stimulated with 4 μg/ml ionomycin (Iono) for 20 min. Degranulation was measured as a function of β-hexosaminidase release. The results are shown as mean % β-hexosaminidase release ± SEM of three separate experiments. Statistical significance of differences in responses between WT and KO BMMC was assessed by Student's *t*-test: **P* < 0.05. (B, C) FcεRI-mediated cytokine expression was enhanced in PTPε-deficient BMMC. WT (open columns) and PTPε-deficient (solid columns) BMMC sensitized overnight as above with 100 ng/ml anti-DNP IgE were left unstimulated or stimulated with 12.5, 25, 50 or 100 ng/ml DNP-HSA for 4 h. The resultant supernatants were assayed for TNF-α (B) and IL-6 (C) using respective ELISAs. The results are shown as mean concentration (pg/ml) of each cytokine ± SEM of three separate experiments. Statistical significance was determined as in (A). **P* < 0.05. (D) Leukotriene production is not affected by the absence of PTPε. The supernatants were prepared as in (B), and production of leukotrienes C₄/D₄/E₄ were assessed by ELISA. The results are shown as mean leukotriene production (pg/well) ± SEM of three separate experiments.

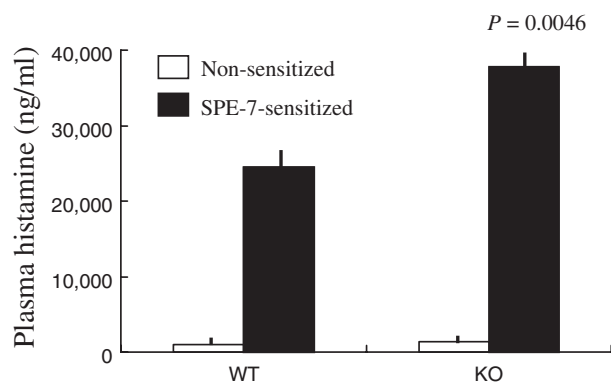


Figure 7 IgE-mediated PSA was enhanced in *Ptpre*^{-/-} mice. WT and *Ptpre*^{-/-} (KO) mice were either left unsensitized (*n* = 4) or sensitized by i.v. injection of 9 μg anti-DNP mouse IgE (*n* = 5). Twenty-four hours later, mice were challenged systemically with 500 μg DNP-HAS. After 1.5 min, the mice were killed and blood was collected for determination of plasma histamine concentrations. The results are expressed as mean histamine concentration in plasma (ng/ml) ± SEM in WT and *Ptpre*^{-/-} mice with and without sensitization. Differences between means were evaluated using Student's *t*-test.

Table 1 Numbers of mast cells in *Ptpre*^{+/+} and *Ptpre*^{-/-} mice.

Organ	<i>Ptpre</i> ^{+/+}	<i>Ptpre</i> ^{-/-}
Ear	11.35 ± 0.38	14.00 ± 1.33
Skin (dorsal)	6.17 ± 0.32	8.90 ± 0.44
Skin (ventral)	8.33 ± 1.14	7.60 ± 0.96
Tongue	3.53 ± 0.64	4.67 ± 0.41
Trachea	3.15 ± 0.25	3.00 ± 0.28
Heart	0.77 ± 0.32	0.90 ± 0.55
Stomach	3.38 ± 1.47	3.83 ± 0.14
Lung	0.08 ± 0.01	0.10 ± 0.03
Mesentery	0.58 ± 0.08	0.38 ± 0.10
Footpad	13.17 ± 0.58	12.85 ± 0.71

5–10 high power fields (HPFs) (×40 objective) were examined on each tissue from three mice per group. Results are expressed as mean numbers of toluidine blue-positive cells ± SEM per HPF. Differences between *Ptpre*^{+/+} and *Ptpre*^{-/-} mice were not statistically significant by Student's *t*-test.

In spite of hyperphosphorylation of LAT, ERK was not significantly affected (Fig. 5). In the original report of LAT-deficient BMMC, ERK activation was completely

abrogated [15], while a recent study showed that the absence of LAT abrogated activation of JNK and p38, but not ERK, in BMMC [17]. It may be possible that the contribution of LAT to mast cell activation is less than in T cells and that the enhancement of LAT phosphorylation observed in this study may not have been sufficient for exerting effects on ERK activation. Furthermore, LAT plays both positive and negative roles in Fc ϵ RI-mediated signalling and the intricate balance between the two determines the downstream events [59]. The dual function of LAT may also contribute to our results.

The most proximal event affected in PTP ϵ -deficient BMMC was thus phosphorylation of Syk, which makes Syk the likely target of PTP ϵ in BMMC. Whether LAT and SLP-76 also serve as direct targets for PTP ϵ remains to be seen. It has been proposed that two interdependent pathways are involved in mast cell degranulation: Lyn-dependent pathway that involves LAT and SLP-76 and Fyn-dependent, Gab2- and PI3K-mediated pathway [24]. Our findings suggest PTP ϵ regulates primarily the Lyn-dependent pathway, most likely at the level of Syk phosphorylation.

Consistent with these *in vitro* observations, PTP ϵ also mitigates PSA induced by IgE and Ag, as indicated by diminished histamine release (Fig. 7). That the numbers of mast cells in diverse tissues in Ptpre^{-/-} mice did not significantly differ from those in WT mice (Table 1) suggests the *in vivo* effect of PTP ϵ reflected by mitigation of challenge-induced increases in plasma histamine levels was not the result of differences in mast cell numbers, but to alterations in cell-autonomous signalling events. Furthermore, PTP ϵ may not be involved in differentiation and maturation of mast cells into tissue-resident mast cells. Because IgE-mediated anaphylaxis is dependent on Fc ϵ RI [56], these results suggest that PTP ϵ is indeed critically involved in the Fc ϵ RI signalling pathways. As both cyt-PTP ϵ and RPTP ϵ are expressed in BMMC (Fig. 1B), it would be good to know how the functions of these two major isoforms are coordinated to regulate mast cell activation. Given that RPTP ϵ and cyt-PTP ϵ likely act on distinct substrates and that the cytosolic forms can change their localization during the course of cellular activation, regulation exerted by PTP ϵ could be dynamic and complex.

PTP ϵ is a critical regulator of various cellular processes, including neuronal, myeloid and osteoclast functions [39, 42, 43]. Previous studies suggest that expression of either RPTP ϵ or cyt-PTP ϵ inhibits phosphorylation/activation of ERK1/2 or the downstream transcriptional activities of AP-1, Elk1 and serum response element [40, 41]. In addition, PTP ϵ inhibits c-Jun- and C/EBP binding protein-mediated transcriptional activation [41], suggesting PTP ϵ could potentially regulate a broad array of MAPK family members. More recently, PTP ϵ was shown to reduce association of Shc

with Grb2 by dephosphorylating Shc, inhibiting the downstream ERK activation [60]. The fact that PTP ϵ did not affect ERK activation in BMMC may imply that the spectrum and magnitude of PTP ϵ action may differ with the cellular context. It has also been reported that PTP ϵ regulates the morphologic phenotypes during *neu*-induced transformation by activating Src via dephosphorylation of the inhibitory C-terminal Tyr residues [36]. Furthermore, PTP ϵ forms a stable complex with the Fyn and Yes PTKs and mediates their activation in *neu*-induced mammary tumour cells [61]. Our finding that in BMMC, PTP ϵ does not affect total tyrosine phosphorylation (Fig. 2) nor Lyn phosphorylation (Fig. 4A) or Fyn activation, shown by the unaffected phosphorylation of the downstream Gab2 (Fig. 4C), highlights the notion that the spectrum and intensity of PTP ϵ activity varies with the cellular context.

Our findings also indicate that PTP ϵ negatively regulates Fc ϵ RI-mediated mast cell activation leading to degranulation and cytokine production. The most likely scenario is that PTP ϵ acts on Syk, downstream of Lyn, to affect SLP-76, LAT, and Ca²⁺ mobilization, all of which are critically involved in degranulation and cytokine responses. In general, PTP activity is regulated by at least four mechanisms: change in spatio-temporal localization, dimerization, phosphorylation, and reversible oxidation. It has been proposed that cyt-PTP ϵ activity is regulated by dimerization, oxidation and spatio-temporal regulation [62, 63]. cyt-PTP ϵ readily dimerizes via the distal catalytic domain 2 (D2), inhibiting its catalytic activity, presumably because of interference with the wedge domain of D1 [62]. At present, there is no information available on the dimerization of PTP ϵ during Fc ϵ RI ligation in mast cells. However, its negative role in Fc ϵ RI-mediated processes suggests enhancement of PTP ϵ activity may be an effective means of dampening unwanted mast cell activation. Notably, suramin derivatives reportedly serve as specific activators of PTP α , a member of the family to which PTP ϵ also belongs [64]. In addition, it was recently demonstrated that PTP ϵ activity is regulated by its EGF-dependent association with microtubules *in vivo* [63]. Although it remains unknown whether Fc ϵ RI-initiated signalling induces the association of PTP ϵ with microtubules, such spatio-temporal regulation could be a target for modulation of PTP ϵ activity in mast cells.

In conclusion, we have clearly shown that PTP ϵ is a negative regulator of Fc ϵ RI-mediated signal transduction leading to cytokine production and degranulation, most likely by acting at the level of Syk to affect downstream events such as phosphorylation of SLP-76 and LAT and mobilization of Ca²⁺. Given the negative role played by PTP ϵ during Fc ϵ RI-mediated mast cell activation, enhancement of its activity may provide a potential means to control allergic reactions.

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