Cellular Immunology 289 (2014) 128–134

Contents lists available at ScienceDirect

Cellular Immunology

journal homepage: www.elsevier.com/locate/ycimm

Protein tyrosine phosphatase-PEST (PTP-PEST) regulates mast cell-activating signals in PTP activity-dependent and -independent manners

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ARTICLE INFO

Article history:
Received 5 February 2014
Accepted 4 April 2014
Available online 13 April 2014

Keywords:
Allergy
Fc receptors
Protein tyrosine phosphatase
Signal transduction

ABSTRACT

Aggregation of the high-affinity IgE receptor (FcεRI) in mast cells leads to degranulation and production of numerous cytokines and lipid mediators that promote allergic inflammation. Tyrosine phosphorylation of proteins in response to FcεRI aggregation has been implicated in mast cell activation. Here, we determined the role of PTP-PEST (encoded by *PTPN12*) in the regulation of mast cell activation using the RBL-2H3 rat basophilic leukemia cell line as a model. PTP-PEST expression was significantly induced upon FcεRI-crosslinking, and aggregation of FcεRI induced the phosphorylation of PTP-PEST at Ser39, thus resulting in the suppression of PTP activity. By overexpressing a phosphatase-dead mutant (PTP-PEST CS) and a constitutively active mutant (PTP-PEST SA) in RBL-2H3 cells, we showed that PTP-PEST decreased degranulation and enhanced IL-4 and IL-13 transcription in FcεRI-crosslinked RBL-2H3 cells, but PTP activity of PTP-PEST was not necessary for this regulation. However, FcεRI-induced TNF-α transcription was increased by the overexpression of PTP-PEST SA and suppressed by the overexpression of PTP-PEST CS. Taken together, these results suggest that PTP-PEST is involved in the regulation of FcεRI-mediated mast cell activation through at least two different processes represented by PTP activity-dependent and -independent pathways.

1. Introduction

The binding of allergen/IgE complex to the high-affinity IgE receptor (FcεRI) on mast cells triggers a cascade of signaling events that ultimately lead to a wide variety of effector functions. These effector functions include release of histamine, serotonin, and β-hexosaminidase by granule exocytosis, production of leukotrienes and prostaglandins, and transcription of Th2-type cytokine genes and secretion of their products; these together contribute to the development of allergic responses. Tyrosine phosphorylation of cellular proteins is one of the earliest signaling events induced by crosslinking of the FcεRI on mast cells or basophils. It is widely considered that upon FcεRI aggregation, the Src-type protein tyrosine kinase (PTK) Lyn is activated to induce phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) on the β and γ subunits of the FcεRI complex, which in turn recruit and activate Syk PTK. These PTKs subsequently phosphorylate and activate downstream signal molecules [1]. Thus, protein tyrosine phosphorylation is an essential aspect of the regulation of mast cell activation. The net tyrosine phosphorylation state of cellular proteins is under the control of PTKs and protein tyrosine phosphatases (PTPs), which suggests equally important roles for both PTKs and PTPs in the control of signaling events regulated by tyrosine phosphorylation. At present, with few exceptions, e.g., SHP-1 and -2 [2–5], PTP-MEG2 [6], HePTP [7], PTP α [8], and PTP ε [9], the functions of PTPs in mast cell activation remain unclear. Therefore, to better understand the potential contribution of PTP to FcεRI-mediated signaling, we focused on PTP-PEST (*PTPN12*) using the RBL-2H3 rat basophilic leukemia cell line as a model.

PTP-PEST is a ubiquitously expressed non-receptor-type PTP with a molecular weight of 120 kDa that belongs to the PEST subfamily of PTP, which includes PEP/Lyp and PTP-HSCF [10]. It contains a conserved PTP domain at its NH_2-terminus and a long COOH-terminal tail comprising several signaling motifs, including protein interacting motifs. The cellular function of PTP-PEST has

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http://dx.doi.org/10.1016/j.cellimm.2014.04.003
0008-8749/

been characterized by studies on adherent cells, which have shown that PTP-PEST acts as a regulator of integrin-mediated signal transduction involved in cell adhesion, spreading, and migration [11–14]. Recent studies have also emphasized the importance of PTP-PEST in osteoclasts and lymphocytes. In osteoclasts, inhibition of PTP-PEST was accompanied by an increase in tyrosine phosphorylation of WASP and other associated signaling molecules. These experiments indicate the involvement of PTP-PEST in sealing-ring formation and bone resorption [15]. In the B cell line A20, PTP-PEST is an efficient negative regulator of antigen-receptor signaling as it reduces the phosphorylation of Shc, Pyk2, Fak, and p130Cas [16]. In T cells, PTP-PEST was found to dephosphorylate the Lck kinase WASP at its activation loop site Y394 and Pyk2, resulting in the suppression of WASP-driven actin polymerization, synapse formation, and TCR signaling [17–19]. It was also reported that phosphorylation/dephosphorylation of PTP-PEST at Ser39 acts as a switch to regulate PTP-PEST activity [20]. However, the physiological role of PTP-PEST in mast cell activation has not been elucidated.

In this study, expression of PTP-PEST was elevated in FcεRI-crosslinked RBL-2H3 cells, and phosphorylation of PTP-PEST at Ser39 was induced by FcεRI crosslinking. The alteration of expression and regulation of PTP activity by Ser39 phosphorylation indicate the complexity of PTP-PEST function within mast cell activation. We then introduced a PTP-PEST phosphatase-dead mutant (PTP-PEST CS) or a constitutively active mutant (PTP-PEST SA) into RBL-2H3 cells and assayed the functional consequences. We determined that the overexpression of PTP-PEST in RBL-2H3 cells decreased degranulation and increased IL-4 and IL-13 expression in FcεRI-crosslinked RBL-2H3 cells, and PTP activity of PTP-PEST was not necessary for PTP-PEST-mediated regulation of degranulation and IL-4 and IL-13 transcription. In contrast, FcεRI-induced TNF-α transcription was upregulated by the overexpression of PTP-PEST SA and suppressed by the overexpression of PTP-PEST CS.

2. Materials and methods

2.1. Antibodies

Anti-dinitrophenol (DNP) IgE mAb (SPE-7), DNP-conjugated human serum albumin (DNP-HSA), anti-p38 MAP kinase Ab, and anti-diphosphorylated ERK-1 & 2 mAb were purchased from Sigma–Aldrich (St. Louis, USA). The anti-ERK1/2 mAb, anti-SAPK/JNK mAb, phospho-p38 MAP kinase Ab, and phospho-SAPK/JNK Ab were purchased from Cell Signaling Technology (Beverly, MA). The anti-Myc and anti-β-actin mAbs were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The anti-PTP-PEST and anti-phospho-Ser39 PTP-PEST Abs were previously reported [21]. The alkaline phosphatase (AP)-conjugated anti-rabbit IgG and AP-conjugated anti-mouse IgG Abs were obtained from Jackson Immunoresearch Laboratories (West Grove, PA).

2.2. Cells and stimulation

Human embryonic kidney (HEK) 293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated FCS, 50 μg/ml penicillin, 50 μg/ml streptomycin, and 100 μg/ml neomycin at 37 °C in a CO_2 incubator. The rat basophilic leukemia cell line, RBL-2H3, was obtained from Human Science Research Resource Bank (Osaka, Japan). RBL-2H3 cells were cultured in Eagle's minimum essential medium (MEM) supplemented with 10% heat-inactivated FCS, 50 μg/ml penicillin, 50 μg/ml streptomycin, and 100 μg/ml neomycin at 37 °C in a CO_2 incubator. RBL-2H3 cells were incubated with MEM containing 50 ng/ml SPE-7 overnight at 37 °C. After the excess SPE-7 was removed, Tyrode's solution (10 mM HEPES buffer, pH 7.4, 130 mM NaCl, 5 mM KCl, 1.4 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, and 0.1% bovine serum albumin) containing 1–100 ng/ml DNP-HSA was added to the dish, and the cells were further incubated at 37 °C for varying time periods; after that they were subjected to RT-PCR, immunoblot analysis, and other functional assays.

2.3. Plasmid construction and transfection

WT Myc/His-tagged PTP-PET and its catalytic region (amino acids 1–300) were previously cloned in pcDNA3.1/Myc-His plasmids [21]. Two PTP-PEST mutants, C231S (PTP-PEST CS) and S39A (PTP-PEST SA), were constructed using PCR, after which the mutations were confirmed by DNA sequencing on a Genetic Analyzer 3130 xi using Big Dye Terminator technology (Applied Biosystems, Foster City, CA). To construct plasmids replacing the cytomegalovirus (CMV) promoter region of pcDNA3.1-derived plasmids with the EF1 promoter, the *Hin*d III (fill-in blunt end)/*Xba* I EF1 promoter fragment of the pEF-Flag plasmid was ligated to a *Bgl* II (fill-in blunt end)/*Xba* I pcDNA3.1-derived plasmid. HEK 293 cells were suspended in Cytomix electroporation buffer (120 mM KCl, 0.15 mM $CaCl_2$, 10 mM K_2HPO_4/KH_2PO_4, 2 mM EGTA, 5 mM $MgCl_2$, 5 mM reduced glutathione, 2 mM ATP, and 25 mM HEPES; pH 7.6), and the cells (100 μl) were electroporated with 10 μg of plasmid using the Bio-Rad Gene Pulser Xcell system (Bio-Rad, Hercules, CA) with 0.2-cm cuvettes and the 293 preset protocol (square wave, 110 V, and 25 ms). Following electroporation, the cells were used within 48 h for each experiment. RBL-2H3 cells were suspended in Cytomix electroporation buffer, and the cells (100 μl) were mixed with 10 μg of plasmid and electroporated for 20 ms at 190 V in the time constant mode using the Gene Pulser Xcell system. The transfected cells were used within 24 h for each experiment.

2.4. Degranulation assay

The degree of degranulation was determined by measuring the release of β-hexosominidase. The cells were preincubated with 50 ng/ml of SPE-7 in medium overnight. To measure β-hexosaminidase release, the sensitized cells were stimulated with DNP-HAS in Tyrode's solution. The enzymatic activity of β-hexosaminidase in the supernatants and cell pellets solubilized with 0.5% Triton X-100 in Tyrode's buffer was measured using *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide as a substrate in 0.1 M sodium citrate buffer (pH 4.5). The reaction was allowed to run for 60 min at 37 °C and then stopped by adding 0.1 M carbonate buffer (pH 11.0). The reaction product, *p*-nitrophenol, was determined by measuring the absorbance at 405 nm. The extent of degranulation was calculated by dividing the *p*-nitrophenol absorbance in the supernatant by the sum of the absorbance values in the supernatants and Triton X-100-solubilized cell pellets.

2.5. Immunoblot analysis

RBL-2H3 cells were harvested and collected by centrifugation at 1000×*g* for 5 min, and then lysed in the lysis buffer (10 mM Tris–HCl buffer, pH 7.5, 1% Nonidet P-40, 150 mM NaCl, 10 mM NaF, 2 mM Na_3VO_4, 10 mM $Na_4P_2O_7$, and 25 μg/ml benzylsulfonyl fluoride). Insoluble materials were removed by centrifugation for 20 min at 16,000×*g*, and the resultant lysate was fractionated by SDS–PAGE and transferred to a PVDF membrane where the membrane-bound proteins were detected by immunoblot analysis with the appropriate antibodies. The fluorogenic substrate DDAO phosphate (9H-(1,3-dichloro-9,9-dimethylacridin-2-one-7-yl phosphate) (Molecular Probes, Eugene, OR) was used for detection via