CD72 negatively regulates mouse mast cell functions and down-regulates the expression of KIT and FcεRIα

Tatsuki R. Kataoka1, Atsushi Kumanogoh2,3, Nobuyuki Fukuishi4, Chiyuki Ueshima1, Masahiro Hirata1, Koki Moriyoshi1, Tatsuaki Tsuruyama1 and Hironori Haga1

1Department of Diagnostic Pathology, Kyoto University Hospital, Sakyo-ku, Kyoto, Japan
2Department of Respiratory Medicine, Allergy and Rheumatic Diseases, Graduate School of Medicine, Osaka University, Suita, Osaka, Japan
3WPI Immunology Frontier Research Center, Osaka University, Suita, Osaka, Japan
4Faculty of Pharmaceutical Sciences, Tokushima Bunri University, Tokushima, Japan

Correspondence to: T. R. Kataoka; E-mail: trkataoka@yahoo.co.jp

Received 31 August 2013, accepted 11 September 2014

Abstract

CD72 is a transmembrane protein belonging to the C-type lectin family that is expressed by various hematopoietic cells. When bound to its natural ligand, CD100 (semaphorin 4D), CD72 inhibits the KIT-mediated responses of human mast cells, but not IgE/FcεRI-mediated mast cell degranulation. We extended these findings to examine the role of CD72 in mouse mast cells. CD72 expression was detected in mouse bone marrow-derived mast cells (mBMMCs). As for human mast cells, an agonistic antibody against CD72 (K10.6) suppressed the KIT-mediated cell growth of, IL-6 production by and chemotaxis of mBMMCs. However, in contrast to human mast cells, the IgE-triggered degranulation of mBMMCs was suppressed by K10.6. K10.6 did not affect the phosphorylation of SHP-1 in mBMMCs, although SHP-1 mediated the inhibitory effects of CD72 in human mast cells. Administration of K10.6 induced phosphorylation of the ubiquitin ligase Cbl-b and decreased the expression of KIT and FcεRIα on the surface of murine mast cells. We also observed expression of CD72 in a mouse neoplastic cell line, P815, harboring gain-of-function mutations in KIT genes. In addition, we found that K10.6 activated Cbl-b, down-regulated KIT expression and suppressed the mutated KIT-driven growth of these cells. Thus, the mechanism by which CD72 mediates inhibitory effects in mast cells is species-dependent.

Keywords: Cbl, CD72, FcεRIα, KIT, mast cell

Introduction

CD72, or Lyb-2, is a 45-kDa type II transmembrane protein of the C-type lectin family that is expressed in B cells, T cells, NK cells, dendritic cells and macrophages (1, 2). The natural ligand for CD72 has been identified as CD100/semaphorin 4D (3), and the effects of such binding can be mimicked by the addition of agonistic antibodies against CD72 according to cell culture studies (1, 3). CD72 is considered to be a member of the inhibitory receptor family based on the presence of immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in the cytoplasmic region (4). The tyrosine residue within the ITIM consensus sequence (I/V/L/S)XYx(L/V) (x: any residue) becomes phosphorylated following the activation of receptor tyrosine kinases or Src family tyrosine kinases (SFKs) upon receptor ligation and activation (4). This leads to the recruitment of nonreceptor protein phosphatases, Src homology 2 domain-containing tyrosine phosphatase (SHP)-1, SHP-2 or Src homology 2 domain-containing inositol 5-phosphatase (SHIP) 1, which, respectively, dephosphorylate tyrosine-containing signaling molecules and phosphatidylinositol 3,4,5 trisphosphate at the 3′ position (4). As a consequence, signal cascades driven by tyrosine kinases and phosphatidylinositol 3-kinase are terminated (4). Nevertheless, CD72 ligation induces both negative regulation and positive regulation of B cells (1). A previous report demonstrated that CD72 ligation by anti-CD72 agonistic antibodies on a B cell line (K46μm1) and in splenic B cells down-regulated B cell receptor-mediated extracellular signal-regulated kinase (ERK) activation and calcium mobilization (5). In contrast, the stimulation of CD72 positively regulated ERK activation in and the proliferation of B cells (6–9). Different hypotheses have been proposed to explain these conflicting results (1). One hypothesis is that the associated SHP-1, which would
transduce negative signals with CD72, is dependent on the stage of B cell development (1). It has also been suggested that the opposite effects of CD72 on B cell signaling reflect opposing negative and positive signaling regulated by CD72-SHP-1 and -Grb2 complexes (10). In addition, mouse CD72 transduces an inhibitory signal via the ubiquitin ligase Cbl-b (11). Cbl-b activation decreases the expression of signaling molecules, including KIT and high-affinity IgE receptor (FceRI) (12, 13).

Mast cells are hematopoietic cells associated with innate and acquired immune responses (14) that express FceRI and KIT. FceRI induces mast cell degranulation and cytokine production after binding to IgE and subsequent antigen-induced cross-linking of the receptor-bound IgE (15). KIT is activated by its ligand stem cell factor (SCF), also known as steel factor or KIT ligand, and plays an important role in mast cell growth, development and survival (16). SCF also induces mast cell chemotaxis and adhesion through KIT activation (16). Signals initiated by KIT augment the signals downstream of FceRI, resulting in enhanced mast cell degranulation and cytokine production (17). Thus, KIT has the potential to contribute to the exaggerated release of inflammatory mediators from mast cells, associated with the symptoms of atopic diseases and anaphylaxis (17). When the gain-of-function type mutations in KIT genes occur, mast cells show dysregulated proliferation, known as mastocytosis (18). The types of KIT mutations vary among species; almost all human cases of mastocytosis harbor mutations in the catalytic domain of KIT (e.g. D816V (18)), although mutations in the juxtamembrane domain of KIT, like those found in human gastrointestinal stromal tumors, are common in canine mastocytosis (19, 20). Both types of mutation have been observed in mouse mastocytosis; the mouse mastocytosis cell line P815 harbors the D814V mutation in the catalytic domain of KIT, similar to humans (21). In contrast, another mouse mastocytosis cell line, FMA, contains a KIT mutation in the juxtamembrane domain (22).

Mast cells also express various inhibitory receptors (23), in addition to activating receptors such as KIT and FceRI. We recently reported that CD72 is expressed on human mast cells and that CD72 negatively regulates KIT-mediated responses, but not the IgE-triggered degranulation, of human mast cells (24). The negative function of CD72 is associated with the interaction between CD72 and SHP-1 (24). In this study, we examined the expression and function of CD72 in mouse mast cells and compared the results with those obtained in human mast cells.

Methods

Cells

Mouse bone marrow-derived mast cells (mBMMCs) were developed from bone marrow obtained by the lavage of femurs from C57BL/6 mice in RPMI1640 medium supplemented with FBS (10%), l-glutamine (2mM), penicillin (100 U ml⁻¹), streptomycin (100 μg ml⁻¹) and streptomycin (100 μg ml⁻¹). MC/9 and IC/2 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). NIH3T3 cells were cultured in IMDM medium supplemented with FBS (5%), l-glutamine (2mM), penicillin (100 U ml⁻¹), and streptomycin (100 μg ml⁻¹). P815 cells were cultured in RPMI1640 medium supplemented with FBS (10%), l-glutamine (2mM), penicillin (100 U ml⁻¹) and streptomycin (100 μg ml⁻¹). MC/9 and IC/2 cells were cultured in the same medium as mBMMCs.

Antibodies

The anti-mouse CD72 agonistic antibody K10.6 (monoclonal, mouse IgG) and isotype control antibodies were purchased from BD Biosciences (San Jose, CA, USA). The anti-mouse CD72 antibody (H-96, rabbit polyclonal IgG) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phospho-SHP-1 (Tyr 536, rabbit polyclonal IgG) was obtained from ECN Biosciences (Versailles, KY, USA) and anti-phosphotyrosine antibody (4G10, mouse monoclonal IgG) was purchased from Millipore (Billerica, MA, USA). We purchased anti-Cbl-b antibody (rabbit polyclonal IgG) from Cell Signaling Technology (Beverly, MA, USA). Anti-β-actin antibody (mouse monoclonal IgG) was obtained from Sigma-Aldrich (St Louis, MO, USA). The secondary antibodies were peroxidase-labeled anti-rabbit or anti-mouse IgG (Santa Cruz Biotechnology). Anti-KIT-PE and anti-FceRI-PE antibodies were purchased from eBiosciences (San Diego, CA, USA).

RT-PCR

A total of 5 x 10⁶ cells (mBMMCs, NIH3T3, P815, MC/9, IC/2 or freshly collected splenocytes from C57BL/6 mice) were collected by centrifugation and processed with TRIzol (Invitrogen Life Technologies, Carlsbad, CA, USA). Total RNA was isolated from these samples using RNeasy columns according to the manufacturer’s instructions (Invitrogen Life Technologies). Each RNA sample (1 μg) was used for RT-PCR (SuperScript III One-Step RT-PCR System; Invitrogen Life Technologies). Primers were designed as described previously (25): oligo 6 (5′-GGATCTAGCCGTCGGCAAGAC−3′) and oligo 9 (5′-CCTGACACTGGCGCACA−3′). cDNA synthesis and PCR amplification were performed using a DNA Engine PTC-200 cycler (Bio-Rad Laboratories, Hercules, CA, USA), as described previously (25).

Immunoprecipitation and immunoblotting

Cell lysates were prepared and blotted as described previously (26). Anti-Cbl-b antibody (H-96) was used for the detection of CD72. For the immunoprecipitation experiments, cell lysates were prepared with buffer containing 150 mM NaCl, 10 mM Tris–HCl (pH 8.0), 1 mM EDTA, 1 mM NaN₃, 0.5 mM phenylmethylsulphonylfluoride (PMSF), 5 μg ml⁻¹ aprotinin, 5 μg ml⁻¹ leupeptin, complete protease inhibitor cocktail (Roche, Indianapolis, IN, USA) and NP-40 (1%). The lysates were incubated with rabbit IgG-bound protein G-sepharose for 30 min and then incubated with anti-Cbl-b antibody (H-96)-bound protein G-sepharose overnight at 4°C with rotation. The collected proteins were separated by electrophoresis and the gels were probed for 4G10 and anti-Cbl-b antibodies.

Flow cytometry

For the detection of CD72 surface expression, mBMMCs and P815 cells were washed with PBS and fixed with 4% paraformaldehyde (Sigma-Aldrich). The cells were stained.
with anti-mouse CD72 (K10.6) overnight at 4°C, followed by anti-mouse IgG-FITC for 2 h at 4°C. In other experiments, we added anti-mouse CD72 (K10.6) or control IgG (10 μg ml⁻¹ each) to mBMMCs or P815 cells for the indicated times. The cells were fixed using 4% paraformaldehyde and stained with anti-KIT-PE or anti-FcεRIα-PE antibodies for 2 h at 4°C. The stained cells were then analyzed using a FACScan flow cytometer (BD Biosciences). The expression levels of KIT and FceRIα were calculated after K10.6 treatment for 24 h when those after control IgG treatment for 24 h reached 100.

**Cell growth**

mBMMCs were incubated in cytokine-free RPMI medium overnight with K10.6 or control IgG (10 μg ml⁻¹, respectively). Cells (1 × 10⁵ cells) were seeded in 96-well plates and stimulated with or without SCF (10 ng ml⁻¹) or IL-3 (30 ng ml⁻¹). In another assay, P815 cells were incubated in cytokine-free RPMI medium for 24 h with K10.6 or control IgG (10 μg ml⁻¹, respectively), after which the cells (1 × 10⁵ cells) were seeded in 96-well plates without SCF. After 24 h, the proportion of viable cells was assessed using an MTT-based colorimetric assay (Sigma-Aldrich) according to the manufacturer’s protocol (24). Crystalized MTT was dissolved and the absorbance was measured at 570 nm.

**IL-6 secretion**

mBMMCs were incubated in cytokine-free RPMI medium overnight with K10.6 or control IgG (10 μg ml⁻¹, respectively), after which the cells (1 × 10⁵ cells) were seeded in 96-well plates and stimulated with SCF (10 ng ml⁻¹). After 6 h of incubation, the cell-free supernatants were harvested and used for IL-6 ELISA assay (IL-6 DuoSet ELISA system; R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s protocol. In another experiment, 2.5 × 10⁵ mBMMCs were sensitized overnight in cytokine-free medium containing mouse anti-dinitrophenol (DNP) IgE antibody (100 ng ml⁻¹; Sigma-Aldrich) in the presence of K10.6 or control IgG (10 μg ml⁻¹, respectively). Following rinsing in HEPES buffer containing 0.04% BSA, the cells were activated in buffer containing DNP-hSA (100 ng ml⁻¹) for 30 min. In another experiment (Fig. 2G), pre-treated mBMMCs were stimulated with 0.1 μg ml⁻¹ PMA (Sigma-Aldrich) and 1 μg ml⁻¹ ionomycin (Sigma-Aldrich) at 37°C for 30 min in the presence of K10.6 or control IgG (10 μg ml⁻¹, respectively). The above-mentioned reactions were terminated by centrifugation (3000 rpm for 5 min) at 4°C and the cell-free supernatants were aliquoted to 96-well plates for the β-hex assay. The remaining cells were lysed in 0.1% Triton X-100 and analyzed for the β-hex content. Degranulation was calculated as the percentage of the total β-hex content in the supernatants following challenge.

**Statistical analysis**

The data are expressed as means ± SE. Differences between groups were examined for statistical significance using Student’s t-test (Excel; Microsoft, Redmond, WA, USA). A P-value < 0.05 indicated statistical significance.

**Results**

**Mouse mast cells express CD72**

We first examined CD72 expression in mouse mast cells, including mBMMCs, IC/2, MC/9 and P815 cells. Primers were prepared as described previously (25). Freshly isolated splenocytes and NIH3T3 cells were used, respectively, as positive and negative controls (1, 27). As expected, mRNA for CD72 was found in the splenocytes but not in the NIH3T3 cells and P815 cells, with the sizes of the transcripts on the gel being identical to that in splenocytes (Fig. 1A). We could not detect mRNA for CD72 in IC/2 or MC/9 cells (Fig. 1A).

We performed an immunoblot analysis to confirm the protein expression of CD72. These studies revealed that CD72 protein was present in mBMMCs and P815 cells, in addition to splenocytes (Fig. 1B). We could not detect CD72 protein in NIH3T3, MC/9 or IC/2 cells (Fig. 1B). Finally, to confirm that CD72 was expressed at the mouse mast cell surface, we examined surface staining with the anti-mouse CD72 antibody K10.6 using FACS analysis. As can be seen in Fig. 1C, surface CD72 was detected on mBMMCs and P815 cells.
CD72 stimulation suppresses both KIT-mediated and IgE-triggered responses of mBMMCs, and plays a role in mutated KIT-driven growth in P815 cells

We next examined the effects of the agonistic antibody against mouse CD72 and K10.6 (3) on KIT- and IgE-mediated responses in mBMMCs. KIT-mediated responses, cell growth, IL-6 production and chemotaxis (16) were evaluated. All responses were significantly suppressed by treatment with K10.6 compared with the control IgG (Fig. 2A–C). We also evaluated the effect of K10.6 on cell viability in cultures without SCF- and IL-3-induced growth, and found that K10.6 stimulation did not significantly affect viability or IL-3-induced growth (Fig. 2A).

IgE-triggered degranulation was next evaluated by monitoring β-hex release. The inhibitory effect of overnight K10.6 administration on IgE-triggered degranulation was observed at anti-DNP IgE concentrations of 10 and 100 ng ml⁻¹, but not at concentrations of 0, 1 or 5 ng ml⁻¹ (Fig. 2D). Short-term exposure to K10.6 (concurrent addition with DNP-HSA) did not affect degranulation, even at anti-DNP IgE concentrations of 100 ng ml⁻¹ (Fig. 2E). KIT signals are known to augment IgE-triggered degranulation (28); thus, we examined the effect of K10.6 on IgE-triggered degranulation in the presence of SCF. K10.6 treatment suppressed IgE-triggered degranulation, even in the presence of SCF (Fig. 2E). IgE-triggered IL-6 secretion was also evaluated using an IL-6 ELISA. For IgE-triggered degranulation, overnight K10.6 administration suppressed IL-6 secretion significantly, while short-term exposure to K10.6 did not (Fig. 2F). We also evaluated the effect of K10.6 on non-IgE-mediated degranulation.

K10.6 stimulation (including overnight treatment) did not significantly affect PMA/ionomycin-induced degranulation (Fig. 2G).

P815 harbors a gain-of-function mutation (D814V) in KIT (21) that induces dysregulated growth of P815 cells independently of SCF. Thus, we tested the effect of K10.6 on the mutated KIT-induced growth of P815 cells. Using MTT assays, we observed that K10.6 decreased the growth of P815 cells (Fig. 3); this was also observed for KIT-regulated mBMMC growth.

Thus, CD72 suppressed KIT-mediated responses in mouse mast cells, similar to human mast cells. However, CD72 decreased the IgE-triggered degranulation of mouse mast cells, in contrast to our findings in human mast cells.

Fig. 1. mBMMCs and the mouse mast cell line P815 express CD72. (A) RT–PCR. (B) Western blotting. (C) Flow cytometry. We used splenocytes as a positive control and NIH3T3 cells as a negative control.

K10.6 induces the phosphorylation of Cbl-b, but not SHP-1, in mBMMCs and P815 cells

To explore the mechanism by which K10.6 exerted inhibitory effects on mBMMCs and P815 cells, we examined the activation of SHP-1 in mBMMCs and P815 cells because SHP-1 mediates the inhibitory effect of CD72 in human mast cells (24). However, we could not obtain reproducible differences in SHP-1 phosphorylation (Fig. 4A). Therefore, we examined the phosphorylation status of other signaling molecules downstream of KIT and FcεRI, including AKT, STAT3, ERK and JNK, but again observed no detectable change in the phosphorylation status of these molecules as a consequence of the addition of K10.6 (data not shown).

IL-4, IL-10 and TGF-β are known to down-regulate KIT, followed by the down-regulation of mast cell growth (29, 30). We thus examined whether CD72 stimulation induced the secretion of these cytokines using ELISAs, but we were unable to detect the secretion of these cytokines into the culture medium (data not shown). Therefore, CD72-induced KIT down-regulation was not associated with IL-4, IL-10 or TGF-β.

We explored another mechanism of the inhibitory effect of CD72 on mBMMCs and P815 cells. In mouse B cells, CD72 activates Cbl-b, resulting in an inhibitory effect (11). Therefore, we examined the activation of Cbl-b in mBMMCs and P815 cells after CD72 stimulation. Immunoprecipitation assays revealed that the phosphorylation of Cbl-b was induced in mBMMCs and P815 cells (Fig. 4B).

K10.6 down-regulates the expression of KIT and FcεR1α in mBMMCs and KIT in P815 cells

Cbl-b activation induced the ubiquitination and down-regulation of various receptors. Thus, we examined the KIT and FcεR1α expression levels in mBMMCs after K10.6 administration. K10.6 administration decreased the expression of KIT and FcεR1α on mBMMCs in a time-dependent manner compared with the levels after control IgG administration (Fig. 5A and C).

We also examined the expression of mutated KIT on P815 cells after K10.6 administration. K10.6 administration decreased the KIT expression level in P815 cells in a time-dependent manner compared with the levels after control IgG administration (Fig. 5B and D).
Fig. 2. K10.6 stimulation of CD72 suppresses the KIT-mediated and IgE-triggered responses of mBMMCs. (A) MTT assay. mBMMCs (1 × 10^5) treated with control IgG or K10.6 (10 μg ml⁻¹, respectively) were incubated for 24 h in the presence or absence of SCF (10 ng ml⁻¹) or IL-3 (30 ng ml⁻¹). The relative values were indicated when the value of control IgG without either SCF or IL-3 reached 100. *P < 0.05. (B) SCF-induced IL-6 production. mBMMCs (1 × 10^5) with control IgG or K10.6 (10 μg ml⁻¹, respectively) were incubated for 6 h in the presence or absence of SCF (10 ng ml⁻¹) (n = 5). The culture supernatants were used for an ELISA for mouse IL-6. *P < 0.05 compared with SCF + control IgG. (C) Two chamber assay. mBMMCs (1 × 10^5) treated with control IgG or K10.6 (10 μg ml⁻¹, respectively) were incubated in the upper chambers to assess migration to the lower chambers containing SCF (30 ng ml⁻¹) for 4 h (n = 3). After incubation, those cells that migrated to the lower wells were counted using microscopy. *P < 0.05 compared with SCF + control IgG. (D) IgE-triggered degranulation. mBMMCs (1 × 10^5) were added with anti-DNP IgE at the indicated concentrations (0, 1, 5, 10, or 100 ng ml⁻¹) and incubated overnight with control IgG or K10.6 (10 μg ml⁻¹, respectively) without SCF. After washing, DNP-HSA (100 ng ml⁻¹) was added for 30 min in the presence of control IgG (10 μg ml⁻¹). A β-hex assay was then performed. (E) SCF-augmented IgE-triggered degranulation. mBMMCs (1 × 10^5) were added with anti-DNP IgE (100 ng ml⁻¹) and incubated overnight with control IgG or K10.6 (10 μg ml⁻¹, respectively) without SCF. After washing, DNP-HSA (100 ng ml⁻¹) was added for 30 min in the presence of control IgG (10 μg ml⁻¹) and incubated overnight with control IgG or K10.6 (10 μg ml⁻¹, respectively) without SCF. After washing, DNP-HSA (100 ng ml⁻¹) was added for 30 min in the presence of control IgG or K10.6 (10 μg ml⁻¹, respectively) with the indicated concentration of SCF (0, 1, 10, or 100 ng ml⁻¹). The β-hex assay was then performed. (F) IgE-triggered IL-6 production. mBMMCs (2.5 × 10^4) were added with anti-DNP IgE at the indicated concentrations (0, 1, 10, 50, or 100 ng ml⁻¹) and incubated overnight with control IgG or K10.6 (10 μg ml⁻¹, respectively) without SCF. After washing, DNP-HSA (100 ng ml⁻¹) was added for 30 min in the presence of control IgG (10 μg ml⁻¹). A β-hex assay was then performed. (G) PMA/ionomycin-induced degranulation. mBMMCs (1 × 10^5) were incubated overnight with control IgG or K10.6 (10 μg ml⁻¹, respectively) without SCF. After washing, DNP-HSA (100 ng ml⁻¹) was added for 30 min. The culture supernatants were used for an ELISA for mouse IL-6. *P < 0.05 compared with control IgG. (H) PMA/ionomycin-induced degranulation. mBMMCs (1 × 10^5) were incubated overnight with control IgG or K10.6 (10 μg ml⁻¹, respectively) without SCF. mBMMCs were stimulated with 0.1 μg ml⁻¹ PMA and 1 μg ml⁻¹ ionomycin for 30 min. A β-hex assay was then performed.
**CD72 negatively regulates mouse mast cells**

**Discussion**

In this study, we demonstrated that CD72 was expressed on non-neoplastic and neoplastic mouse mast cells (Fig. 1A and B); furthermore, we showed that CD72 had an inhibitory effect on these cells. CD72 plays important roles in B cells by binding to CD100 on T cells (1, 3). CD72 may be important for the interaction between mast cells and T cells (31) in multiple species.

The expression and inhibitory effects of CD72 are common between human and mouse mast cells; but the mechanisms of the inhibitory effect were found to vary between species. The inhibitory effect of CD72 was associated with SHP-1 activation in human mast cells, but not in mouse mast cells (Fig. 4A). The reason for this difference remains unclear, although other molecules have exhibited functional differences between mice and humans (32). However, CD72 has differential effects between mouse and human lymphocytes, dendritic cells and monocytes (33, 34). Mouse CD100 has inhibitory effects on these cells only in the presence of CD40 stimulation, while human CD100 shows efficacy even in the absence of CD40. Human mast cells are known to be deficient in CD40 expression (35), while mouse mast cells express CD40 (36). This difference in the regulation of CD72 between humans and mice may be associated with CD40.

Generally, mast cells express $10^6$ IgE receptors per cell, and about 5% of the receptors are required to generate optimal responses (17, 37). In our current β-hex assay, sensitization with anti-DNP IgE (10 ng ml$^{-1}$) could account for 5% of the IgE receptors on the BMMCs because ≥ 10 ng ml$^{-1}$ anti-DNP IgE seemed to saturate IgE-triggered degranulation (Fig. 2D). In addition, the magnitude of degranulation at an anti-DNP IgE concentration of 5 ng ml$^{-1}$ was about half of that at a concentration of 10 ng ml$^{-1}$ after subtracting the background (Fig. 2D). At the same time, the expression level of FcεRIα at an anti-DNP IgE concentration of 10 ng ml$^{-1}$ after K10.6 treatment was also expected to decrease by approximately 50% compared with control IgG treatment (Fig. 5C). Superficially, the inhibitory effect of K10.6 on IgE-triggered degranulation seemed to be associated with the decrease in FcεRIα levels in our assay system. However, anti-DNP IgE (100 ng ml$^{-1}$) may account for sufficient FcεRIα levels (>5%) and optimal degranulation, even after the FcεRIα level decreased by half after K10.6 treatment. Nevertheless, K10.6 decreased IgE-triggered degranulation at this concentration of anti-DNP IgE. Therefore, a secondary mechanism underlying the inhibitory effect of K10.6 may exist in addition to the decrease in FcεRIα expression level. Certain molecule(s) transducing IgE-triggered signals may be involved in the inhibitory mechanism of K10.6. Cbl-b is reported to down-regulate the levels of such signal molecules, such as syk and src family kinases (38, 39). We speculate that K10.6 stimulation down-regulates such molecule(s), and limits the magnitude of IgE-triggered degranulation even at ≥10 ng ml$^{-1}$ anti-DNP IgE. To determine the precise mechanism by which K10.6 decreases IgE-triggered degranulation will require additional studies.

The expression level of KIT is positively correlated with growth responses through specific receptors (40). AR-42 is thought to be a promising reagent for mastocytosis by down-regulating KIT proteins (41). The current data seemed to suggest K10.6 or another agonistic antibody against CD72.

---

**Fig. 3.** Stimulation of CD72 with K10.6 suppresses the mutated KIT-driven growth of P815 cells. MTT assay. P815 cells treated with control IgG or K10.6 (10 μg ml$^{-1}$, respectively) were incubated for 24 h in the absence of SCF (10 ng ml$^{-1}$). The relative values were indicated when the value of control IgG reached 100. *$P < 0.05$ compared with control IgG.

**Fig. 4.** K10.6 administration induces the phosphorylation of Cbl-b but not SHP-1 in mBMMCs and P815 cells. mBMMCs or P815 cells were incubated for the indicated times with control IgG or K10.6 (10 μg ml$^{-1}$, respectively) in the presence of SCF (10 ng ml$^{-1}$). The levels of phospho-SHP-1 and β-actin were evaluated using immunoblot analysis. (A) mBMMCs. (B) P815. The data are representative of three individual experiments.
Fig. 5. K10.6 administration decreases the expression of KIT and FcεRIα in mBMMCs and mutated KIT in P815 cells. (A) mBMMCs. (B) P815 cells. FACS analysis was used. The data are representative of three individual experiments. (C) Relative expression of KIT and FcεRIα in mBMMCs treated with K10.6 for 24 h when the value of control IgG reached 100. *$P<0.05$ compared with control IgG. (D) Relative expression of KIT in P815 cells treated with K10.6 for 24 h when the value of control IgG reached 100. *$P<0.05$ compared with control IgG.

Fig. 6. Current models.
may be applicable to treat mouse mastocytosis by down-regulating KIT. Here, even when K10.6 was added overnight and expected to decrease the KIT expression level by approximately 30% (Fig. 5C), the degree of augmentation of IgE-triggered degranulation by SCF was similar (Fig. 2E). Specifically, in the presence or absence of K10.6, SCF at 1 ng ml⁻¹ up-regulated KIT expression by 2–3%, while SCF at 10 ng ml⁻¹ up-regulated KIT expression by 7–8%, and SCF at 100 ng ml⁻¹ up-regulated KIT expression by 15–20% compared with in the absence of SCF. This observation suggests that KIT signaling, which augments IgE-triggered degranulation, cannot be suppressed by K10.6 administration, even though K10.6 can decrease KIT expression. K10.6-induced decreases in KIT expression may affect the proliferation of mast cells, but not the augmentation of IgE-triggered degranulation.

Overall, CD72 plays an inhibitory role in mouse mast cells, like in human mast cells; however, the mechanism underlying its inhibitory effects varies among species (Fig. 6).

**Funding**

Ministry of Education, Culture, Sports, Science and Technology, Japan.

**Conflict of interest statement:** The authors declared no conflicts of interest.

**References**


