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Blockage by SP600125 of Fcε Receptor-Induced Degranulation and Cytokine Gene Expression in Mast Cells is Mediated Through Inhibition of Phosphatidylinositol 3-Kinase Signalling Pathway

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SP600125 is used as a specific inhibitor of c-Jun *N*-terminal kinase (JNK). We initially aimed to examine physiological roles of JNK in mast cells that play a central role in inflammatory and immediate allergic responses. We found that Fc receptor for IgE (FcεRI)-induced degranulation (serotonin release) and cytokine gene expression [interleukin (IL)-6, tumour necrosis factor-α and IL-13] in bone marrow-derived mast cells, were almost completely inhibited by SP600125. However, the time course of FcεRI-induced JNK activation did not correlate with that of serotonin release. Furthermore, FcεRI-induced degranulation and cytokine gene expression were not impaired in a JNK activator, MKK7-deficient mast cells, in which JNK activation was lost. These results indicate that the inhibitory effects by SP600125 are not due to impaired JNK activation. Instead, we found that SP600125 markedly inhibited the FcεRI-induced activation of phosphatidylinositol 3-kinase (PI3K) and Akt, the same as a PI3K inhibitor, wortmannin. Finally, we found that SP600125 specifically inhibits delta form of p110 catalytic subunit (p110δ) of PI3K. Thus, SP600125 exerts its influence on mast cell functions by inhibiting the kinase activity of PI3K, but not JNK.

Key words: IgE, JNK, mast cell, PI3K, SP600125.

Abbreviations: Ab, antibody; BMMC, bone-marrow derived mast cell; ERK, extracellular signal-regulated kinase; Gab2, Grb2-associated binder 2; HA, hemagglutinin; IL, interleukin; JNK, c-Jun N-terminal kinase; LPA, lysophophatidic acid; mAb, monoclonal Ab; MKK, MAPK kinase; pAb, polyclonal Ab; PIP3, phosphatidylinositol-3,4,5-triphosphate; SAPK, stress-activated protein kinase; SEK, stress-activated protein kinase/extracellular signal-regulated kinase kinase; TNFα, tumor necrosis factor α.

INTRODUCTION

Mast cells are known to be the main effector cells in the elicitation of the IgE-mediated allergic response. The initiation of an allergic response requires the activation of mast cells and is primarily a consequence of allergen-induced aggregation of the IgE-occupied high-affinity Fc receptor for IgE (FcεRI). Activated mast cells secrete granules, which contain chemical mediators such as proteases, histamine and serotonin, and generate cytokines such as interleukin (IL)-6, IL-13 and tumour necrosis factor (TNF)-α (*1*, *2*). Aggregation of the FcεRI triggers the activation of different signal transduction pathways (*3–6*). Activation of protein tyrosine kinases (PTKs), including Syk, Fyn and Lyn is one of the earliest signalling events induced by aggregation of the FcεRI on mast cells. PTK activation is thought to be proximal to the activation of phosphatidylinositol 3-kinase (PI3K), PKC and calcium signal and appears to be essential for mast cell degranulation (*7*), since PTK inhibitors prevent the liberation of inositol triphosphate and histamine release (*8*, *9*). Both PKC activation and increased intracellular calcium are required for and sufficient for maximal degranulation response.

Another important intermediate is PI3K, which catalyses phosphatidylinositol-3,4,5-triphosphate [PtdIns (3,4,5) P_3] synthesis. PI3K inhibitors abrogate the mast cell calcium response required for degranulation (*10–12*) and impair the activation of mitogen-activated protein kinases (MAPKs) needed for cytokine gene induction (*13*, *14*). Src family tyrosine kinase, Fyn and scaffolding adaptor protein Grb2-associated binder 2 (Gab2) are important molecules to relay the signal from FcεRI to PI3K in mast cells. Fyn binds and phosphorylates Gab2 on FcεRI aggregation and phosphorylated Gab2 binds and activates PI3Ks. Since mast cells lacking Fyn or Gab2 are severely impaired in degranulation and cytokine production, the Fyn-Gab2-PI3K pathway is

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a necessary signal for mast cell function. PI3Ks activated by the FcεRI include the heterodimeric class IA PI3Ks, which consist of a p110α, p110β or p110δ catalytic subunit bound to either one of five regulatory proteins (p85α, p55α, p50α, p85β or p55γ) that link the p110 subunits to tyrosine kinase signalling pathways. However, the specific role for each of the three class IA PI3K catalytic subunits has not been determined.

Aggregation of the FcεRI also activates MAPKs including extracellular signal-regulated kinase (ERK), p38 MAPK and c-Jun N-terminal kinase (JNK)/stress-activated protein kinase (SAPK). JNK is activated not only by many types of cellular stresses including UV irradiation, heat shock, but also by certain cytokines and mitogens. In hematopoietic cells, JNK is thought to play an important role for cytokine production through their regulation of transcription. The activated JNK phosphorylates a number of substrates including c-Jun, a component of the activator protein-1 (AP-1) transcription factor, to regulate gene expression for the stress responses and immune responses. JNK also regulates various cellular functions independent of transcription regulation (*15–17*). In MAP kinase family, ERK and p38 were reported to be important in cytokine expression and degranulation by the inhibitor study in FcεRI-activated mast cells (*18–21*). MAPK kinase (MKK) 7 directly phosphorylates and activates JNK. We previously showed MKK7-deficient mast cells hyperproliferate in response to growth factor stimulation, suggesting that JNK signalling plays an important role in mast cell homeostasis (*22*). In contrast, physiological role of FcεRI-induced JNK activation in mast cells is still unclear. Recently, many researchers use a specific inhibitor, SP600125 (*15*, *23*, *24*). Under this situation, we thought to use both pharmacological approach using SP600125 and genetical approach using *mkk7*-deficient mast cells to reveal the role of JNK activation in FcεRI-activated mast cells.

In this report, we found that SP600125 abrogates Fcε-induced degranulation and cytokine gene expression. Unexpectedly, SP600125 exerts its influence on mast cell function by inhibiting the kinase activity of PI3K but not JNK.

MATERIALS AND METHODS

Materials—The anti-JNK1 (C-17), anti-JNK1 (FL), anti-JNK2 (FL), anti-Fyn (FYN3), anti-Fyn (15), anti-Lyn (44), anti-Lyn (H-6), anti-Akt1/2 (N-19), anti-Akt1 (C-20) and anti-p110α (c-17) Abs were purchased from Santa Cruz Biotechnology, Inc. The anti-Phosphotyrosine (4G10) was from Upstate Biotechnology, Inc. Anti-phospho-SAPK/JNK (9251), anti-phospho-ERK (9106) and anti-phospho-Akt (9271) Abs were purchased from Cell Signaling Technology, Inc. Anti-Shc antibody was from BD Transduction Laboratories. Anti-FLAG (M2) agarose, anti-DNP IgE Ab (SPE-7), DNP–HSA, adenosine, A23187, anisomycin and Thapsigargin were purchased from Sigma-Aldrich Co. PD98059, LY294002 and PP2 were purchased from Calbiochem. SP600125 and wortmannin were from BIOMOL International, L.P. and Wako Pure Chemical Industries, Ltd, respectively. Horseradish peroxidase-labelled anti-rabbit IgG was from Jackson ImmunoResearch Laboratories, Inc. All other reagents were analytical grade and were obtained from commercial sources.

Mast Cell Preparation—Bone marrow cells were flushed from femurs of mice. Cells were washed twice with PBS and resuspended at 5×10^5/ml in culture medium (OptiMEM medium supplemented with 10% FCS, 50 μM β-mercaptoethanol, antibiotics (GIBCO/BRL) and 2 ng/ml IL-3 (Genzyme). FcεRI$^+$ c-Kit$^+$ Bone marrow-derived mast cells (BMMCs) were prepared by continuous transfer of cells growing in suspension as described (*22*). On the other hand, MKK7-deficient FcεRI$^+$ c-Kit$^+$ mast cells were prepared from fetal liver cells of *mkk7*$^{-/-}$ mice (25). *mkk7*$^{-/-}$ fetal liver cells at embryonic date 11.5 (E11.5) were cultured in the presence of IL-3 the same as BMMCs.

Flow Cytometry—For the measurement of FcεRI and c-Kit, Cells were incubated with 1 μg/ml anti-TNP IgE (BD PharMingen) for 50 min, washed with culture medium and then incubated with FITC-conjugated anti-mouse IgE (BD PharMingen) and PE-conjugated anti-c-Kit mAb (BD PharMingen) for 30 min. Flow cytometric analysis of the stained cells was performed with FACScan or FACSCalibur (Becton–Dickinson) equipped with CellQuest software.

Degranulation Assay—The degree of degranulation was determined by measuring the release of serotonin. Cells (1×10^6 cells/ml) were sensitized for 3 h at CO_2 incubator with anti-DNP IgE (1 μg/ml) and [^{3}H] serotonin in culture medium without IL-3. Sensitized cells were washed twice with starvation medium (OptiMEM medium supplemented with 50 μM β-mercaptoethanol and 0.1% BSA) and then resuspended at 5×10^6 cells/200 μl. Cells were preincubated for 15 min with inhibitor and then stimulated with DNP–HSA for 15 min. Samples were put on ice and centrifuged 8.8×10^3 r.p.m. for 2 min. Release of [^{3}H] serotonin to supernatant was measured in triplicate by liquid scintillation and results are described as the percent of degranulation.

Northern Blot Analysis—Cells (6×10^6 cells/ml) were sensitized for over night at CO_2 incubator with anti-DNP IgE (1 μg/ml) in starvation medium. Sensitized cells were washed twice with starvation medium and then resuspended at 1.2×10^7 cells/4.8 ml. Cells were preincubated for 15 min with inhibitor and then stimulated with DNP–HSA for indicated time. Total RNA from BMMCs was prepared using Trizol reagent (Sigma-Aldrich), separated by formamide agarose gel, and transferred to a Hybond-XL (Amersham Biosciences). IL-6, IL-13, TNF-α and β-actin were detected by Northern blotting using the specific DNA probes. The cDNA fragments corresponding to mouse IL-6, IL-13, TNF-α and β-actin were amplified using the following primers: IL-6, 5′-ATG AAG TTC CTC TCT GCA AGA GAC T-3′ and 5′-CAC TAG GTT TGC CGA GTA GAT CTC-3′, IL-13, 5′-AAT GAA TTC ATG GCG CTC TGG GTG -3′ and 5′-ATA GTC GAC TTA GAA GGG GCC G-3′, TNF-α, 5′-GGC AGG TCT ACT TTG GAG TCA TTG C-3′ and 5′-ACA TTC GAG GCT CCA GTG AAT TCG G-3′, β-actin, 5′-CAT CAC TAT TGG CAA CGA GC-3′ and 5′-ACG CAG CTC AGT AAC AGT CC-3′.

Immunoblotting and Immunoprecipitation—Cells (1×10^6 to 6×10^6 in 1 ml) were sensitized for 3 h with anti-DNP IgE (1 μg/ml) in culture medium (without IL-3). Sensitized cells (2.5×10^6 to 10×10^6 in 1 ml) were preincubated with inhibitor and stimulated with DNP–HSA (10 ng/ml) for indicated time. Cells were pelleted by centrifugation and resuspended in ice cold lysis buffer (1% NP-40, 20 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.05% 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and 100 μM Na_3VO_4, 4 μg/ml aprotinin). The detergents used for Shc immunoprecipitation were 1% NP-40, 0.5% deoxicholate, and 0.1% SDS. Abs used for immunoprecipitation were anti-Shc, anti-Fyn (FYN3), anti-Lyn (44), anti-p110α (c-17), anti-Akt1 (c-20) and anti-JNK (c-17). Immunoprecipitations and immunoblotting were carried out by using standard procedures (*26*).

JNK Kinase Assay—For detection of JNK kinase activity, total JNKs were immunoprecipitated (2 h, 4°C) from BMMC lysates using polyclonal rabbit anti-JNK IgG reactive against all JNK isoforms. Immune complexes were harvested on protein A-Sepharose beads. And the *in vitro* kinase assay was performed with enolase as a substrate by the method described previously (*26*).

Akt Kinase Assay—For detection of Akt kinase activity, total Akts were immunoprecipitated (2 h, 4°C) from BMMC lysates using polyclonal rabbit anti-Akt Ab (C-20; Santa Cruz Biotechnology, Inc.). Immune complexes were harvested on protein G-Sepharose beads. For kinase assays, immune complexes were washed three times with TBS 0.1% NP-40. The beads were resuspended in 20 μl kinase buffer (10 mM $MgCl_2$, 50 mM HEPES, 0.01% NP-40, 1 mM DTT) and Akt activity was assayed at 30°C for 20 min in the presence of 4 μCi [γ-^{32}P] ATP using 1 μg of GST-GSK3β as the *in vitro* substrate. The reaction was stopped by the addition of 4× SDS sample buffer. GST-GSK3β phosphorylation was visualized by autoradiography as described previously (*26*).

Src Family Kinase Assay—293T cells were transfected with pME/Flag-Fyn or pME/Lyn using LipofectAMINE 2000 (Invitrogen). Cell lysates were immunoprecipitated (2 h, 4°C) using polyclonal rabbit anti-Lyn or Fyn (Santa Cruz Biotechnology, Inc.). Immune complexes were harvested on protein A-Sepharose beads. And the *in vitro* kinase assay was performed with enolase as a substrate as described previously (*27*).

PI3K Kinase Assay—The cDNA encoding mouse p110 α or p110 δ were cloned into mammalian expression vector pCMV5-Flag. 293T cells were transfected with pCMV5-Flag p110 α or p110 δ using LipofectAMINE 2000 (Invitrogen). PI3K activity was measured described previously (*22*). Briefly, the immunoprecipitates containing PI3K were washed with TBS and suspended in 25 μl of a kinase buffer consisting of 40 mM Tris–HCl (pH 7.4), 1 mM EGTA (pH 7.5), 0.2 mM phosphatidylinositol 4, 5-bisphosphate and 0.2 mM phosphatidylserine and incubated with or without 1 μM wortmannin at 30°C for 15 min. After addition of 10 μl ATP solution (25 mM $MgCl_2$, 0.5 mM ATP, and 10 μCi of [γ-^{32}P]ATP), the immunoprecipitates were further incubated for 15 min. The reaction was terminated by adding 470 μl of chloroform/methanol/8% $HClO_4$ (30:60:4) and mixed with 50 μl of water and 150 μl each of chloroform and 8% $HClO_4$. The lipid phase separated by centrifugation was washed twice with chloroform-saturated 0.5 M NaCl containing 1% $HClO_4$ and dried. The extracted lipid was dissolved in 20 μl of chloroform/methanol (95:5) and spotted on a TLC plate (Silica gel 60, Merck). Prior to spotting, the plate was once developed with methanol/water (2:3) containing 1.2% potassium oxalate and pre-activated by heating at 110°C for 20 min. The plate was developed in chloroform/acetone/methanol/acetic acid/water (80:30:26:24:14), and radioactivity was visualized with the bioimaging analyzer as described previously (*28*).

Assays for PIP3 Production—PIP3 production was measured according to the method described by Momose *et al*. (*28*). Cells (1×10^6 cells/ml) were over night sensitized at CO_2 incubator with anti-DNP IgE (1 mg/ml) in culture medium without IL-3.The sensitized BMMCs were suspended at the density of 4×10^7 cells/ml in a labeling medium consisting of 10 mM HEPES–NaOH (pH 7.4), 136 mM NaCl, 4.9 mM KCl and 5.5 mM glucose, and incubated at 37°C for 30 min with 500 mCi/ml of ^{32}Pi. The radiolabelled cells were washed twice with the same medium and resuspended at 5×10^6 cells/ml in the medium containing 1 mM $CaCl_2$. Aliquots (400 ml, 2×10^6 cells/ml) were preincubated for 5 min and further incubated with the indicated reagents. The reaction was terminated by the addition of 1.55 ml of chloroform/methanol/8% $HClO_4$ (50:100:5). Then 0.5 ml each of chloroform and 8% $HClO_4$ were added to the mixture, and the lipid phase was separated by centrifugation. The lipid phase was washed with chloroform-saturated 1 M NaCl containing 1% $HClO_4$ and dried. The procedure after this step was performed by the method described in *PI3K kinase assay* (*28*).

RESULTS

SP600125 Inhibits FcεRI-Induced Degranulation in BMMC—We first examined the effect of SP600125 on FcεRI-induced degranulation. After BMMCs were sensitized by the incubation with IgE for 3 h, they were pretreated with inhibitors for 15 min and stimulated with DNP–HSA for 15 min, and finally measured a serotonin release as the extent of degranulation. As expected, a PI3K inhibitor, wortmannin completely inhibited FcεRI-induced serotonin release in a dose-dependent manner (Fig. 1A). An ERK kinase (MEK) inhibitor, PD98059 and a p38 kinase inhibitor, SB203580 partially inhibited the serotonin release (Fig. 1B and C). In contrast, a JNK inhibitor, SP600125 almost completely inhibited FcεRI-induced serotonin release (Fig. 1D). These results clearly show that SP600125 inhibits FcεRI-induced degranulation in BMMC the same as PI3K inhibitor, wortmannin.

SP600125 Inhibits FcεRI-Induced Cytokine Gene Expression in BMMC—We next examined the effect of SP600125 on FcεRI-induced cytokine gene expression. Sensitized BMMCs were pretreated with inhibitors for 15 min and stimulated with DNP–HSA for 60 min. Total RNAs were extracted from cells and were analysed by Northern blotting. Wortmannin completely inhibited

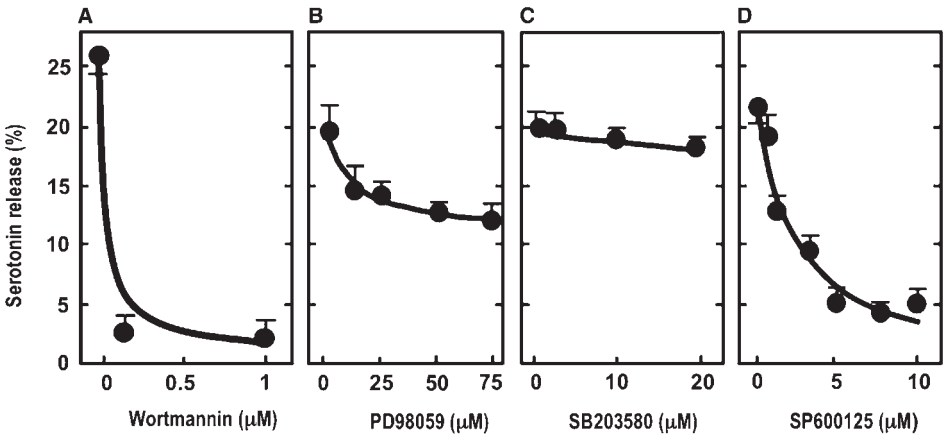

Fig. 1. **Inhibition of FcεRI-induced serotonin release by SP600125 in BMMCs.** BMMCs were sensitized with IgE for 3 h and were preincubated in the presence of indicated concentrations of (A) Wortmannin, (B) PD98059, (C) SB203580, (D) SP600125 and stimulated with 10 ng/ml of DNP–HSA for 15 min. Serotonin release was measured as described in MATERIALS AND METHODS.

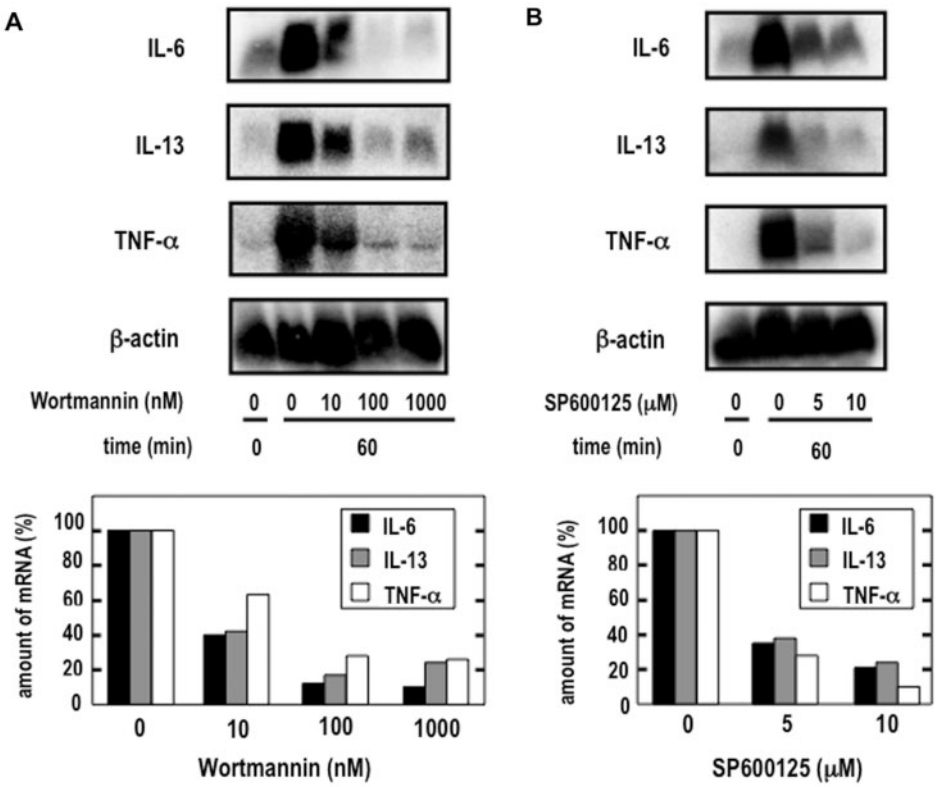

Fig. 2. **Inhibition of FcεRI-induced cytokine gene expression by SP600125 in BMMCs.** BMMCs were sensitized over night with IgE. Sensitized BMMCs were preincubated for 15 min in the presence of (A) wortmannin or (B) SP600125 and stimulated with DNP–HSA for 60 min. mRNA was purified from cells and cytokine mRNAs were detected by northern blotting. Upper panel, autoradiography; lower panel, quantitation of specific cytokines.

gene expression of IL-6, IL-13 and TNF-α in a dose-dependent manner (Fig. 2A). SP600125 also inhibited cytokine gene expression the same as wortmannin (Fig. 2B). These results clearly show that SP600125 inhibits FcεRI-induced cytokine gene expression in BMMC the same as PI3K inhibitor, wortmannin.

FcεRI-Induced Degranulation Occurs Before JNK Activation in BMMC—As SP600125 inhibited FcεRI-induced mast cell function severely, we further investigated a relationship between FcεRI-induced degranulation and JNK activation. Sensitized BMMCs were stimulated with DNP–HSA for indicated time and JNK

activity was measured (Fig. 3A). Unexpectedly, FcεRI-induced JNK activation started at 8 min after stimulation. A time-course of FcεRI-induced degranulation was much faster than that of JNK activation (Fig. 3B).

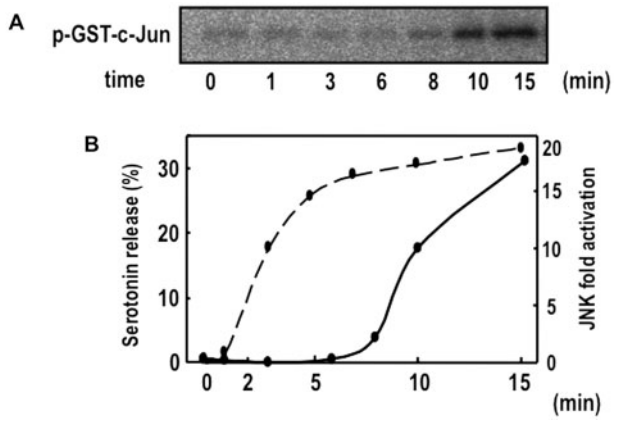

Fig. 3. **A time course of FcεRI-induced JNK activation and serotonin release.** (A) Time course of FcεRI-induced JNK activation. BMMCs were sensitized with anti-DNP IgE and stimulated with DNP–HSA for indicated time periods. Lysates from these cells were immunoprecipitated with an anti- JNK (C-17) Ab. Immunoprecipitated JNK activity in the precipitated fractions were measured using GST-c-Jun as a substrate in the presence of [γ-^{32}P] ATP as described in MATERIALS AND METHODS. (B) Comparison of FcεRI-induced JNK activation and serotonin release. Solid line shows a time course of quantified JNK activation in (A) and dotted line shows time course of FcεRI-induced serotonin release.

These results indicates that FcεRI-induced degranulation is independent of JNK activation.

SP600125 Inhibits FcεRI-Induced Degranulation in MKK7-Deficient Mast Cells—To address the relationship between FcεRI-induced mast cell function and JNK activation, we prepared MKK7-deficient mast cells, which expressed both FcεRI and c-Kit (FcεRI$^+$ c-Kit$^+$). Fetal liver derived mast cells (FLMCs) were prepared from *mkk7*$^{-/-}$ embryos at E11.5. Both wild-type (*mkk7*$^{+/+}$) and *mkk7*$^{-/-}$ FLMCs expressed FcεRI and c-Kit (Fig. 4A). Sensitized FLMCs were stimulated with DNP–HSA and cell lysates were subjected to Western blot analysis. FcεRI-induced ERK activation was equivalent in both wild-type and *mkk7*$^{-/-}$ FLMCs (Fig. 4B). In contrast, FcεRI-induced JNK activation was completely lost in *mkk7*$^{-/-}$ FLMCs (Fig. 4B). So, we used these cells to examine relationship between FcεRI-induced mast cell function and JNK activation. Wild-type and *mkk7*$^{-/-}$ FLMCs were stimulated with DNP–HSA for 15 min and serotonin release was measured. FcεRI-induced serotonin release was comparable in wild-type and *mkk7*$^{-/-}$ FLMCs (Fig. 4C). Wild-type and *mkk7*$^{-/-}$ FLMCs were sensitized with IgE and stimulated with DNP–HSA for indicated time, and then RNAs from these cells were subjected to Northern blot analysis. Gene expression of IL-6, IL-13 and TNFα was almost the same in wild-type and *mkk7*$^{-/-}$ FLMCs (Fig. 4E). Interestingly, SP600125 inhibits FcεRI-induced serotonin release in MKK7-deficient mast cells (Fig. 4D). These results clearly show that the inhibitory effect of SP600125 on FcεRI-induced mast cell function is independent of

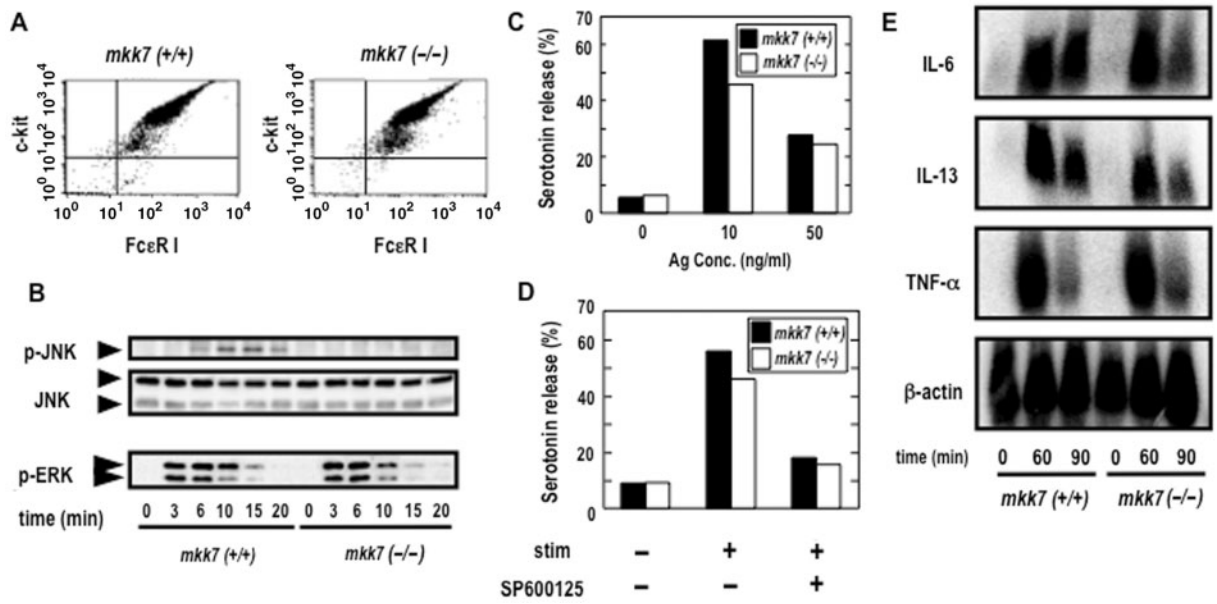

Fig. 4. **FcεRI-induced mast cell function in wild-type and *mkk7*$^{-/-}$ mast cells.** A: Cell surface expression of the high-affinity IgE receptor (FcεRI) and c-Kit in FLMCs were analysed by FACS. *mkk7*$^{+/+}$ and *mkk7*$^{-/-}$ FLMCs were incubated with anti-TNP IgE mAb and then incubated with FITC-anti-mouse IgE and PE-anti-Kit Abs followed by flow cytometry. B: *mkk7*$^{+/+}$ and *mkk7*$^{-/-}$ FLMCs were sensitized with anti-DNP IgE (1 μg/ml) for 3 h and then stimulated with DNP–HSA (10 ng/ml) for the indicated time period. Total lysates from these cells were resolved on SDS–PAGE, transferred to PVDF membrane filters, and then immunoblotted with anti-phospho-SAPK/JNK Ab, anti-JNK Ab and anti-phospho-ERK Ab. C and D: FLMCs were sensitized for 3 h with anti-DNP IgE and then stimulated with DNP–HAS. Serotonin release was measured. E: FLMCs were sensitized over night with anti-DNP IgE and then stimulated with DNP–HSA. mRNA was purified from cells and cytokine mRNAs were detected by Northern blotting.

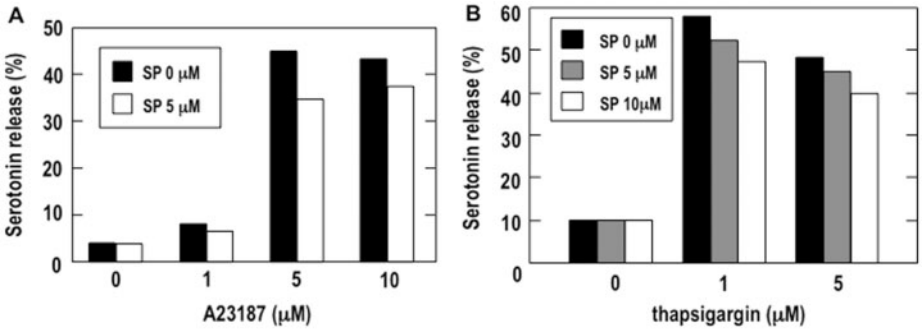

Fig. 5. **Effects of SP600125 on A23187- or thapsigargin-induced serotonin release in BMMCs.** IgE sensitized BMMCs were preincubated in the presence of indicated concentrations of SP600125 for 15 min and stimulated with (A) A23187 or (B) thapsigargin for 15 min. Serotonin release was determined as described in MATERIALS AND METHODS.

JNK activation. Thus, SP600125 has another target molecule(s) other than JNK in mast cells.

The Target Molecule of SP600125 is Located Upstream of Calcium Signalling in FcεRI-Induced Signalling Pathway—To address the target molecule(s), we examined whether the target of SP600125 was located upstream or downstream of Calcium signalling. BMMCs were treated with 5 μM of SP600125 or DMSO for 15 min and stimulated with various concentrations of Calcium ionophore A23187 or Thapsigargin, which elevates cytosolic Calcium concentration by inhibiting the Calcium channel in ER, for 15 min. SP600125 could not inhibit both A23187-induced and thapsigargin-induced serotonin release in BMMCs (Fig. 5). These results indicate that the target molecule(s) of SP600125 is located upstream of Calcium signalling in mast cells.

Syk and Src Family Tyrosine Kinases are Not Target Molecules of SP600125—Like all immunoreceptor family members, FcεRI lacks intrinsic tyrosine kinase activity. Agtigen-induced crosslinking of FcεRI initiates a complex series of phosphate transfer events via the activation of Syk and Src family protein tyrosine kinases and deficiency of these kinases lead to severe defect in mast cell function. To examine the effect of SP600125 on Syk, we checked the phosphorylation of Shc, which is phosphorylated by Syk in response to FcεRI-induce stimulation (Fig. 6A). Sensitized BMMCs were pretreated with various concentrations of SP600125 or DMSO and stimulated with DNP–HSA. Lysate prepared from BMMCs was immnoprecipitated by anti-Shc Ab, and immunoblotted with anti-phospho-Tyr antibody. There were almost no different FcεRI-induced Shc phosphorylation between SP600125- and DMSO-treated BMMCs. These results indicate that Syk is not a target molecule of SP600125 in FcεRI-induced signalling pathway.

To examine the effect of SP600125 on Src family, Fyn and Lyn, we directly measured kinase activities of Fyn and Lyn using enolase as a phosphorylation substrate. Recombinant Fyn and Lyn were immunoprecipitated from 293T cells and subjected to *in vitro* kinase assay (Fig. 6B). A Src faimly kinase inhibitor, PP2 efficiently inhibited phosphorylation of enolase. However, SP600125 could not inhibit phosphorylation of enolase by Lyn and Fyn. Inhibitory specificities of SP600125 and PP2 were confirmed by the *in vitro* JNK kinase assay (Fig. 6C). These results indicate that Src family members, Fyn and Lyn are not direct target molecules of SP600125.

SP600125 Inhibits FcεRI-Induced PI3K Activation in BMMCs—We examined the effect of SP600125 on PI3K activation in two ways. First, sensitized BMMCs were pretreated with SP600125 or DMSO control and stimulated with DNP–HSA for indicated time. Total lysates prepared from BMMCs were subjected to Western blot anlysis using anti-phospho-Akt Ab. As shown in Fig. 7A, Akt was Tyr phosphorelated in control cells, however, the Tyr phoshorylation of Akt was completely diminished in SP600125-treated cells. Secondly, we measured the intracellular accumulation of PIP_3 in FcεRI-activated BMMCs by TLC chromatography. As shown in Fig. 7B, PIP_3 production was detected in control (DMSO) cells, however, was not detected in cells treated with SP600125 or wortmannin. These results clearly show that SP600125 inhibits FcεRI-induced PI3K activation.

SP600125 Specifically Inhibits IgE Receptor but Not Adenosine Receptor-Mediated PIK3 Signalling Pathway in BMMCs—It is well known that mast cells have adenosine receptor on its cell surface and produces PIP3 through PI3Kγ in response to adenosine stimulation. To examine specificities of PI3K isoforms, we examined the effect of SP600125 on FcεRI- or adenosine-induced PI3K activation in BMMCs (Fig. 8). We used Akt kinase assay to evaluate the activation of PI3K quantitatively. BMMCs were pretreated with various concentrations of SP600125 or DMSO and stimulated with DNP–HSA or adenosine. Lysates prepared from BMMCs were immnoprecipitated by anti-Akt Ab and were subjected to Akt kinase assay. Interestingly, adenosine-induced Akt activation was affected partially by SP600125 pretreatment, however, FcεRI-induced Akt activation was greatly impaired. These results indicate that SP600125 inhibits a specific isoform(s) of PI3K family members.

SP600125 Specifically Inhibits Delta Type of PI3K Isoform—Finally, we investigate whether the SP600125 directly inhibits PI3K the same as wortmannin and LY294002. We cloned p110α isoform, which express ubiquitously, and p110δ isoform, which is primarily found in leucocytes, from mast cell cDNA library. Flag tagged p110α or p110δ transfected to 293T cells were immunoprecipitated by anti-Flag Ab and subjected to

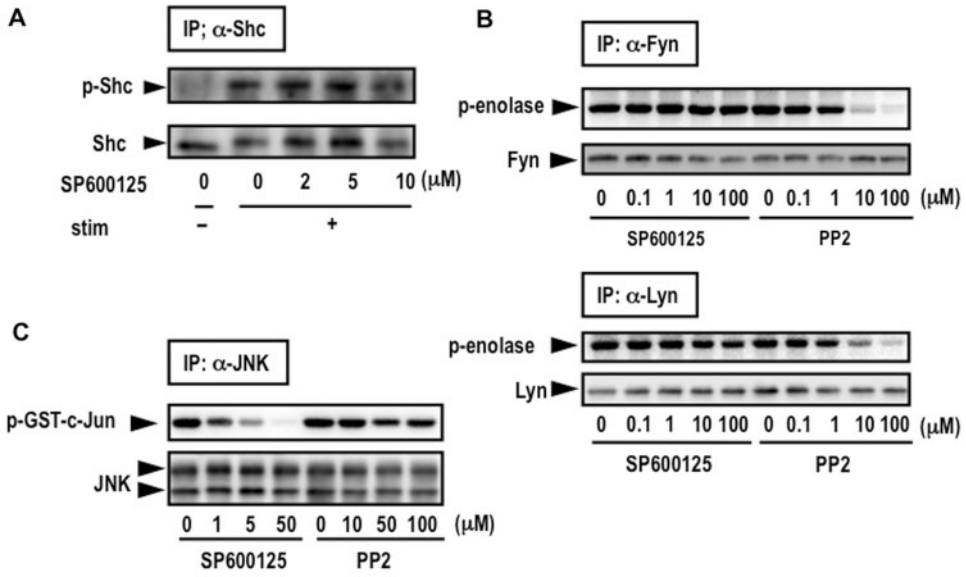

Fig. 6. **Effects of SP600125 on Shc phosphorylation and kinase activities of Src family tyrosine kinases.** A: IgE-sensitized BMMCs were preincubated in the presence of indicated concentrations of SP600125 and stimulated with DNP–HSA for indicated time periods. Lysates from these cells were immunoprecipitated with anti-Shc Ab. Immunoprecipitated, proteins were resolved on SDS–PAGE, transferred to PVDF membrane filters, and then immunoblotted with anti-phosphotyrosine Ab (upper lanes) and anti-Shc Ab (lower lanes). B: 293T cells were transfected with pME/Flag-Fyn or pME/Lyn. The transfected 293T cells, after being cultured for 48 h, were lysed and Fyn and Lyn were immunoprecipitated with anti-Fyn and anti-Lyn Abs. Inhibitory effect of SP600125 was measured by kinase activities of immunoprecipitated Fyn and Lyn with acid-treated enolase as a substrate in the presence of inhibitor and [γ-^{32}P] ATP. The insets show the ^{32}P-phosphorylated enolase (upper lanes) and precipitated kinases (lower lanes). C: Hela cells were stimulated with anisomycin (10 μg/ml) for 30 min. Cell lysates were prepared from the stimulated cells and immnoprecipitated with an anti-SAPK/JNK (C-17) Ab. Inhibitory effect of SP600125 was assessed by measuring immunoprecipitated SAPK/JNK activity with GST-c-Jun as a substrate in the presence of inhibitor and [γ-^{32}P] ATP. The insets of panels show the ^{32}P-phosphorylated GST-c-Jun (upper lanes) and precipitated kinases (lower lanes).

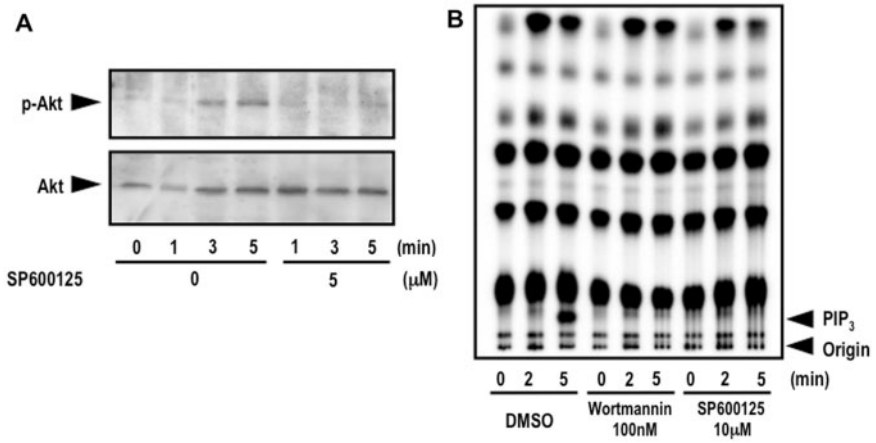

Fig. 7. **Effects of SP600125 on FcεRI-induced PI3K activation and PIP_3 production.** A: IgE sensitized BMMCs were preincubated in the presence of indicated concentrations of SP600125 and stimulated with DNP–HSA for indicated time periods. Lysates from these cells were resolved on SDS–PAGE, transferred to PVDF membrane filters, and then immunoblotted with anti-phospho-Akt Ab (upper lanes) and anti-Akt Abs (lower lanes). B: Sensitized BMMCs were labelled with ^{32}P-orthophosphate. Then, these cells were pretreated with wortmannin or SP600125 for 15 min and stimulated with DNP–HSA for indicated time periods. Lipids were extracted from these cells and separated by thin-layer chromatography.

in vitro kinase assay. As shown in Fig. 9, PIP3 production in Flag-p110α immunoprecipitants were not affected by SP600125. In contrast, SP600125 severely inhibited PIP3 production in the Flag-p110δ immunoprecipitants. This result indicates that SP600125 specifically inhibits p110δ rather than p110α.

DISCUSSION

In this study, we found that FcεRI-induced degranulation and cytokine gene expression including IL-6, TNF-α and IL-13 were almost completely inhibited by a well-known JNK inhibitor, SP600125 in BMMCs (Figs 1 and 2).

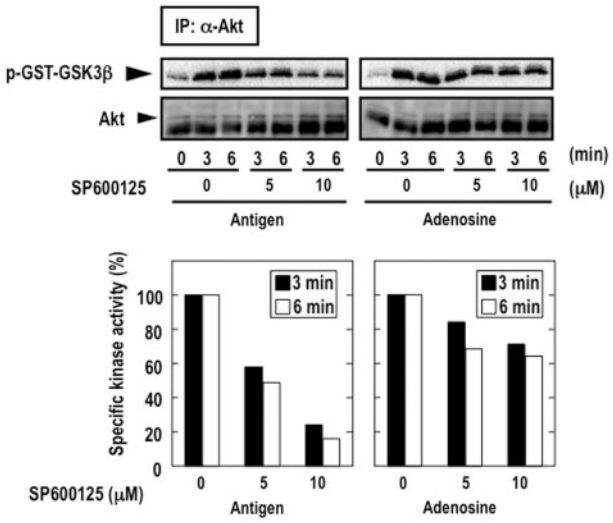

Fig. 8. **Effects of SP600125 on FcεRI- or adenosine-induced Akt activation.** BMMCs were pretreated with various concentrations of SP600125 and then stimulated with FcεRI or adenosine for indicated time periods. Lysates from these cells were immunoprecipitated with anti-Akt Ab. Kinase activities of immunoprecipitated Akt in the precipitated fractions were measured with GST-GSK3β as a substrate in the presence of [γ-^{32}P] ATP. The insets of panels show the ^{32}P-phosphorylated GST-GSK3β (upper lanes) and precipitated Akt (lower lanes). Lower panels show the Akt kinase activity, which expressed as the percentage compared with the control level observed in 0 μM of SP600125 in each time period.

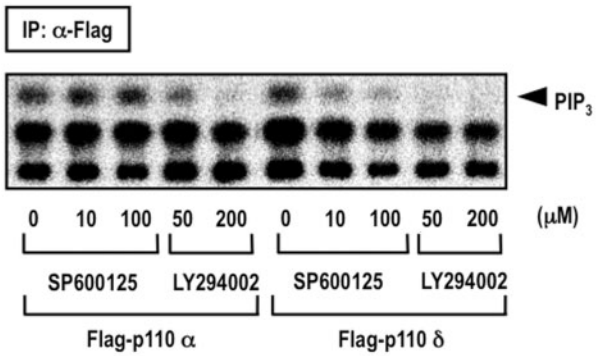

Fig. 9. **Effects of SP600125 on PI3Ks, p110α and p110δ *in vitro*.** 293T cells were transfected with pCMV/Flag-p110α or pCMV/Flag-p110δ. The transfected 293T cells, after being cultured for 48 h, were lysed and immunoprecipitated with anti-FLAG (M2) agarose. Inhibitory effects of SP600125 were assessed by measuring immunoprecipitated p110 α/δ activity with PIP2 as a substrate in the presence of inhibitor and [γ-^{32}P] ATP as described in MATERIALS AND METHODS.

However, the time course of JNK activation did not correlate with that of Fcε-induced degranulation (Fig. 3). FcεRI-induced degranulation and cytokine gene expression were not impaired in MKK7-deficient mast cells, which lost JNK activation (Fig. 4). Furthermore, SP600125 inhibited FcεRI-induced degranulation in MKK7-deficient mast cells (Fig. 4). These results clearly show that the impaired phenotypes observed by SP600125 treatment are not due to impaired JNK activation. Instead, we found that SP600125 markedly inhibited the activation of PI3K and Akt (Fig. 7), but did not inhibit Syk and Src family tyrosine kinases (Fig. 6). Finally, we found that SP600125 specifically inhibit delta isoform of PI3K, which is recently revealed to be an important molecule in FcεRI-signalling in mast cells (Fig. 9).

We have shown that MKK7 is essential for JNK activation and that MKK7 negatively regulate the proliferation of mast cells using MKK7-deficient mast cells (*22*). Therefore, we examined the role of MKK7-JNK signalling in FcεRI-induced mast cell function. Indeed, FcεRI-induced JNK activation in MKK7-deficient mast cells was lost (Fig. 4B). However, there were no differences of mast cell function between wild-type and MKK7-deficient mast cells.

SP600125 was originally reported as a specific and reversible ATP-competitive inhibitor for JNK and has been used to study JNK signal in cellular context. Bain *et al*. (*29*) previously reported that SP600125 inhibits several kinds of kinases *in vitro*. In the present work, we also showed that SP600125 inhibit p110δ isoform of class IA PI3K besides JNK and impaired mast cell function. Unlike the ubiquitously expressed p110α and p110β, the p110δ isoform is primarily expressed in leucocytes. Therefore, it would be better to take care of using SP600125 as a specific JNK inhibitor in leucocytes. Other JNK inhibitors, such as CEP1347 and small peptide inhibitor I-JIP, may be useful for studying JNK function in leucocytes.

Among the different isoforms of PI3K, the class I variant is activated after FcεRI aggregation. Class I PI3Ks are further subdivided into two groups. Class IA PI3Ks consist of p110α, β or δ catalytic subunit and link to the family of receptor tyrosine kinases. Class IB PI3K consists of p110γ catalytic subunit and link to the family of G-protein-coupled receptors. We examined the effect of SP600125 on adenosine-receptor-induced PI3K activation, because adenosine receptor is a G-protein-coupled receptor (GPCR) and activates class IB PI3Kγ. However, we found that SP600125 did not inhibit adenosine-induced activation of PI3K (Fig. 8). We also examined the effect of SP600125 on IL-3 and SCF, and found that SP600125 severely inhibited IL-3 or SCF-induced PI3K activation the same as FcεRI (data not shown). IL-3 and SCF receptors belong to the family of receptor tyrosine kinase but not GPCR family. Thus, SP600125 inhibit Class IA type PI3K, p110δ but not class IB type PI3Kγ. Recent study indicated that p110δ isoform is the main component of the PI3K-dependent antigen-IgE signalling cascade leading to degranulation and cytokine production. Although wortmannin and LY294002 are well used and are powerful inhibitors of PI3K, there is no specificity among the four members of the class I PI3K. Therefore, we show the possibility that SP600125 and its derivatives may be useful as specific inhibitor of p110δ isoform.

SP600125 was reported to suppress bronchoalveolar accumulation of eosinophils and lymphocytes in animals subjected to repeated allergen exposure (*24*). Cytokines, proteases, biogenic amines and lipid mediators secreted

from mast cells were known to induce basophils and eosinophils to inflammatory tissues. So, FcεRI-activated mast cells contribute to leucocyte infiltration. Therefore, the effect of SP600125 in model animal, in part, may be explained by impaired PI3K signalling in mast cells.

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CONFLICT OF INTEREST

None declared.

REFERENCES

1. Wedemeyer, J., Tsai, M., and Galli, S. J. (2000) Roles of mast cells and basophils in innate and acquired immunity. *Curr. Opin. Immunol.* **12**, 624–631
2. Marone, G., Galli, S. J., and Kitamura, Y. (2002) Probing the roles of mast cells and basophils in natural and acquired immunity, physiology and disease. *Trends Immunol.* **23**, 425–427
3. Kinet, J. P. (1999) The high-affinity IgE receptor (Fc epsilon RI): from physiology to pathology. *Annu. Rev. Immunol.* **17**, 931–972
4. Kawakami, T. and Galli, S. J. (2002) Regulation of mast-cell and basophil function and survival by IgE. *Nat. Rev. Immunol.* **2**, 773–786
5. Siraganian, R. P. (2003) Mast cell signal transduction from the high-affinity IgE receptor. *Curr. Opin. Immunol.* **15**, 639–646
6. Galli, S. J., Kalesnikoff, J., Grimbaldeston, M. A., Piliponsky, A. M., Williams, C. M. M., and Tsai, M. (2005) Mast cells as "tunable" effector and immunoregulatory cells: recent advances. *Annu. Rev. Immunol.* **23**, 749–786
7. Ozawa, K., Szallasi, Z., Kazanietz, M. G., Blumberg, P. M., Mischak, H., Mushinski, J. F., and Beaven, M. A. (1993) Ca(2+)-dependent and Ca(2+)-independent isozymes of protein kinase C mediate exocytosis in antigen-stimulated rat basophilic RBL-2H3 cells. Reconstitution of secretory responses with Ca2+ and purified isozymes in washed permeabilized cells. *J. Biol. Chem.* **268**, 1749–1756
8. Kawakami, T., Inagaki, N., Takei, M., Fukamachi, H., Coggeshall, K. M., Ishizaka, K., and Ishizaka, T. (1992) Tyrosine phosphorylation is required for mast cell activation by Fc epsilon RI cross-linking. *J. Immunol.* **148**, 3513–3519
9. Oliver, J. M., Burg, D. L., Wilson, B. S., McLaughlin, J. L., and Geahlen, R. L. (1994) Inhibition of mast cell Fc epsilon R1-mediated signaling and effector function by the Syk-selective inhibitor, piceatannol. *J. Biol. Chem.* **269**, 29697–29703
10. Nakanishi, S., Yano, H., and Matsuda, Y. (1995) Novel functions of phosphatidylinositol 3-kinase in terminally differentiated cells. *Cell Signal.* **7**, 545–557
11. Barker, S. A., Caldwell, K. K., Hall, A., Martinez, A. M., Pfeiffer, J. R., Oliver, J. M., and Wilson, B. S. (1995) Wortmannin blocks lipid and protein kinase activities associated with PI 3-kinase and inhibits a subset of responses induced by Fc epsilon R1 cross-linking. *Mol. Biol. Cell* **6**, 1145–1158
12. Barker, S. A., Lujan, D., and Wilson, B. S. (1999) Multiple roles for PI 3-kinase in the regulation of PLCgamma activity and Ca2+ mobilization in antigen-stimulated mast cells. *J. Leukoc. Biol.* **65**, 321–329
13. Kawakami, Y., Hartman, S. E., Holland, P. M., Cooper, J. A., and Kawakami, T. (1998) Multiple signaling pathways for the activation of JNK in mast cells: involvement of Bruton's tyrosine kinase, protein kinase C, and JNK kinases, SEK1 and MKK7. *J. Immunol.* **161**, 1795–1802
14. Ishizuka, T., Chayama, K., Takeda, K., Hamelmann, E., Terada, N., Keller, G. M., Johnson, G. L., and Gelfand, E. W. (1999) Mitogen-activated protein kinase activation through Fc epsilon receptor I and stem cell factor receptor is differentially regulated by phosphatidylinositol 3-kinase and calcineurin in mouse bone marrow-derived mast cells. *J. Immunol.* **162**, 2087–2094
15. Davis, R. J. (2000) Signal transduction by the JNK group of MAP kinases. *Cell* **103**, 239–252
16. Chang, L. and Karin, M. (2001) Mammalian MAP kinase signalling cascades. *Nature* **410**, 37–40
17. Manning, A. M. and Davis, R. J. (2003) Targeting JNK for therapeutic benefit: from junk to gold? *Nat. Rev. Drug Disc.* **2**, 554–565
18. Xu, R., Seger, R., and Pecht, I. (1999) Cutting edge: extracellular signal-regulated kinase activates syk: a new potential feedback regulation of Fc epsilon receptor signaling. *J. Immunol.* **163**, 1110–1114
19. Ishizuka, T., Terada, N., Gerwins, P., Hamelmann, E., Oshiba, A., Fanger, G. R., Johnson, G. L., and Gelfand, E. W. (1997) Mast cell tumor necrosis factor alpha production is regulated by MEK kinases. *Proc. Natl Acad. Sci. USA* **94**, 6358–6363
20. Zhang, C., Baumgartner, R. A., Yamada, K., and Beaven, M. A. (1997) Mitogen-activated protein (MAP) kinase regulates production of tumor necrosis factor-alpha and release of arachidonic acid in mast cells. Indications of communication between p38 and p42 MAP kinases. *J. Biol. Chem.* **272**, 13397–13402
21. Hirasawa, N., Sato, Y., Fujita, Y., and Ohuchi, K. (2000) Involvement of a phosphatidylinositol 3-kinase-p38 mitogen activated protein kinase pathway in antigen-induced IL-4 production in mast cells. *Biochem. Biophys. Acta* **1456**, 45–55
22. Sasaki, T., Wada, T., Kishimoto, H., Irie-Sasaki, J., Matsumoto, G., Goto, T., Yao, Z., Wakeham, A., Mak, T. W., Suzuki, A., Katada, T., Nishina, H., and Penninger, J. M. (2001) The stress kinase mitogen-activated protein kinase kinase (MKK)7 is a negative regulator of antigen receptor and growth factor receptor-induced proliferation in hematopoietic cells. *J. Exp. Med.* **194**, 757–768
23. Bennett, B. L., Sasaki, D. T., Murray, B. W., O'Leary, E. C., Sakata, S. T., Xu, W., Leisten, J. C., Motiwala, A., Pierce, S., Satoh, Y., Bhagwat, S. S., Manning, A. M., and Anderson, D. W. (2001) SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. *Proc. Natl Acad. Sci. USA* **98**, 13681–13686
24. Ura, S., Nishina, H., Gotoh, Y., and Katada, T. (2007) Activation of the c-Jun N-terminal kinase pathway by MST1 is essential and sufficient for the induction of chromatin condensation during apoptosis. *Mol. Cell Biol.* **27**, 5514–5522
25. Wada, T., Joza, N., Cheng, H. M., Sasaki, T., Kozieradzki, I., Bachmaier, K., Katada, T., Schreiber, M., Wagner, E. F., Nishina, H., and Penninger, J. M. (2004) MKK7 couples stress signalling to G2/M cell-cycle progression and cellular senescence. *Nat. Cell Biol.* **6**, 215–226
26. Kishimoto, H., Nakagawa, K., Watanabe, T., Kitagawa, D., Momose, H., Seo, J., Nishitai, G., Shimizu, N., Ohata, S., Tanemura, S., Asaka, S., Goto, T., Fukushi, H., Yoshida, H.,

Suzuki, A., Sasaki, T., Wada, T., Penninger, J. M., Nishina, H., and Katada, T. (2003) Different properties of SEK1 and MKK7 in dual phosphorylation of stress-induced activated protein kinase SAPK/JNK in embryonic stem cells. *J. Biol. Chem.* **278**, 16595–16601

27. Parravicini, V., Gadina, M., Kovarova, M., Odom, S., Gonzalez-Espinosa, C., Furumoto, Y., Saitoh, S., Samelson, L. E., O'Shea, J. J., and Rivera, J. (2002) Fyn kinase initiates complementary signals required for IgE-dependent mast cell degranulation. *Nat. Immunol.* **3**, 741–748
28. Momose, H., Kurosu, H., Tsujimoto, N., Kontani, K., Tsujita, K., Nishina, H., and Katada, T. (2003) Dual phosphorylation of phosphoinositide 3-kinase adaptor Grb2-associated binder 2 is responsible for superoxide formation synergistically stimulated by Fc gamma and formyl-methionyl-leucyl-phenylalanine receptors in differentiated THP-1 cells. *J. Immunol.* **171**, 4227–4234
29. Bain, J., McLauchlan, H., Elliott, M., and Cohen, P. (2003) The specificities of protein kinase inhibitors: an update. *Biochem. J.* **371**, 199–204