

Distinct Roles of Small GTPases Rac1 and Rac2 in Histamine H₄ Receptor–Mediated Chemotaxis of Mast Cells

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ABSTRACT

Histamine induces chemotaxis of mast cells through the H₄ receptor. However, little is known about the precise intracellular signaling pathway that mediates this process. In this study, we identified small GTPases Rac1 and Rac2 as intracellular binding partners of the H₄ receptor and characterized their roles in H₄ receptor signaling. We showed that histamine induced Rac GTPase activation via the H₄ receptor. A Rac inhibitor NSC23766 attenuated chemotaxis of mast cells toward histamine, as well as histamine-induced calcium mobilization and extracellular signal-regulated kinase (ERK) activation. Histamine-induced migration of mast cells was also sensitive to PD98059, an inhibitor of the mitogen-activated protein kinase kinase, indicating that the Rac-ERK pathway

was involved in chemotaxis through the H₄ receptor. Inhibition of phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) by LY294002 suppressed the histamine-induced chemotaxis and activation of Rac GTPases, suggesting that PI3K regulates chemotaxis upstream of Rac activation. Specific knockdown of Rac1 and Rac2 by short-hairpin RNA revealed that both Rac GTPases are necessary for histamine-induced migration. Downregulation of Rac1 and Rac2 led to attenuated response in calcium mobilization and ERK activation, respectively. These observations suggested that Rac1 and Rac2 have distinct and essential roles in intracellular signaling downstream of H₄ receptor-PI3K in histamine-induced chemotaxis of mast cells.

Introduction

Directed migration or chemotaxis of mast cells is an important process for their recruitment to target tissues in various pathophysiological conditions such as inflammation and allergy. Many chemoattractants including eicosanoids, antigens, growth factors, chemokines, and others are known to induce chemotaxis of mast cells (Halova et al., 2012). Additionally, mast cells themselves produce and release various attractants such as histamine to attract other mast cells and/or their progenitor cells, resulting in mast cell accumulation in local tissues (Hofstra et al., 2003).

The histamine H₄ receptor is a G protein-coupled receptor (GPCR) that is predominantly expressed in immune cells such as mast cells, eosinophils, dendritic cells, monocytes, and T lymphocytes, and plays various roles including migration, shape change, actin polymerization, expression of surface molecules, and regulation of cytokine production (Zampeli and Tiligada, 2009; Thurmond, 2015). In mouse bone marrow–derived mast cells (BMMCs), H₄ receptor stimulation induces calcium mobilization from intracellular

storage and chemotaxis toward histamine, both of which are dependent on pertussis toxin–sensitive G protein and phospholipase C (PLC) (Hofstra et al., 2003). Histamine also induces interleukin (IL)-6 production in mast cells, which requires both the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) and extracellular signal-regulated kinase (ERK) pathways downstream of the H₄ receptor (Desai and Thurmond, 2011). Although many H₄ receptor–related functions have been reported in various cell types, little is known about the functional relevance of the intracellular signaling pathway downstream of H₄ receptor activation.

Rac GTPases belong to the Rho subfamily of the Ras small G protein superfamily. While Rac1 is widely expressed, Rac2 expression is restricted to hematopoietic cells. Rac2-deficient mice show functional abnormality in various cell types including mast cells, neutrophils, macrophages, and lymphocytes, suggesting that Rac1 and Rac2 have distinct roles (Roberts et al., 1999; Yang et al., 2000; Croker et al., 2002; Pradip et al., 2003). In mast cells, activation of Rac GTPases is involved in various cell functions such as migration, polarity, adhesion, cell cycle, and transcriptional regulation (Massol et al., 1998; Timokhina et al., 1998; Yang et al., 2000; Gu et al., 2002; Samayawardhena et al., 2007). Typically, Rac activation in these events occurs downstream of the cytokine or immunoglobulin E receptor. However,

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ABBREVIATIONS: BMMC, bone marrow–derived mast cell; BSA, bovine serum albumin; DTT, dithiothreitol; ERK, extracellular signal-regulated kinase; GEF, guanine nucleotide exchange factor; GPCR, G protein-coupled receptor; GST, glutathione S-transferase; IL, interleukin; IP₃, 1,4,5-triphosphate; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MBP, maltose binding protein; MEK, mitogen-activated protein kinase kinase; mH4RCT, mouse H₄ receptor carboxy-terminal tail; PAK1, p21-activated kinase; PBD, p21 binding domain; PH, pleckstrin homology; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; PLC, phospholipase C; shRNA, short-hairpin RNA.

little is known about Rac GTPase activation downstream of GPCRs such as the histamine H₄ receptor in mast cells.

Accumulating evidence has indicated that the carboxy-terminal cytoplasmic domain of the GPCR interacts with various intracellular proteins that may have important functions, such as receptor targeting, clustering of receptor with various effector molecules, and modulation of signaling efficiency (Bockaert et al., 2003). Our previous work also showed proteins that interact with the carboxy-terminal domain of histamine H₂ and H₃ receptors have essential roles in receptor trafficking and signal transduction (Maeda et al., 2008; Xu et al., 2008).

In this study, to further characterize H₄ receptor signaling, we determined proteins that interacted with the carboxy-terminal domain of the H₄ receptor. We showed that Rac1 and Rac2 GTPases physically associated with the H₄ receptor. Furthermore, these proteins were also functionally coupled with H₄ receptor regulation of histamine-induced chemotaxis of mast cells. Thus, our results indicated that Rac1 and Rac2 have distinct intracellular roles: Rac1 regulates histamine-induced calcium mobilization and Rac2 controls ERK activation.

Materials and Methods

Reagents and Antibodies. Histamine dihydrochloride, pyrilamine maleate, and cimetidine were obtained from Sigma (St. Louis, MO). JNJ7777120 and NSC23766 were obtained from Tocris Bioscience (Bristol, United Kingdom). PD98059, LY294002, and phospho-p44/42 mitogen-activated protein kinase (MAPK) (ERK1/2) (Thr202/Tyr204) antibody were obtained from Cell Signaling Technology (Tokyo, Japan). SP600125 was obtained from Abcam (Tokyo, Japan). Fluo-3 AM was obtained from Dojindo (Kumamoto, Japan). Anti-Rac1 mouse monoclonal antibody (clone 23A8) and anti-Rac2 rabbit polyclonal antibody were obtained from Millipore (Billerica, MA). Anti-maltose binding protein (MBP) monoclonal antibody was obtained from New England Biolabs (Ipswich, MA). ERK1/2 polyclonal antibody was obtained from Enzo Life Sciences (Farmingdale, NY). PE-Cy7 anti-mouse CD117 (c-Kit) antibody (clone 2B8) and PE anti-mouse FcεRIα antibody (clone MAR-1) were obtained from BioLegend (San Diego, CA). Peroxidase-conjugated anti-glyceraldehyde-3-phosphate dehydrogenase monoclonal antibody was obtained from Wako (Osaka, Japan). Horseradish peroxidase-conjugated anti-rabbit IgG was obtained from Jackson Immunoresearch (West Grove, PA). Peroxidase-labeled anti-mouse IgG (H+L) antibody was obtained from KPL (Gaithersburg, MD).

Plasmids. Plasmids encoding glutathione *S*-transferase (GST)-fused mouse histamine H₄ receptor and MBP-fused Rac were constructed from pGEX-5X2 (GE Healthcare, Tokyo, Japan) and pMAL-c2x (New England Biolabs), respectively. The coding sequence for the full carboxy terminus (amino acids 363–391), the proximal half of the carboxy terminus (amino acids 363–376), and the third intracellular loop (amino acids 195–306) of the mouse H₄ receptor, and the full-length mouse Rac1 and Rac2 were amplified by polymerase chain reaction. The cDNA for the p21-activated kinase 1 (PAK1) p21 binding domain (PBD) (amino acids 199–450) was cloned into pGEX-5X2 to generate the GST-PAK1-PBD expression plasmid.

BMMC Culture and Flow Cytometry. Bone marrow cells were isolated from the femur bones of a female 4- to 6-week-old C57BL/6 mouse. The use of animals was carried out in accordance with the Guide for the Care and Use of Laboratory Animals (<https://www.ncbi.nlm.nih.gov/books/NBK54050/>) as adopted and promulgated by the U.S. National Institutes of Health, and approved by the Institutional Animal Care and Use Committee of Tohoku University and Yamaguchi University. Cells were suspended in RPMI1640 medium supplemented with 10% fetal bovine serum, 100 U/ml

penicillin-100 μg/ml streptomycin, and 5 ng/ml recombinant mouse IL-3 (BioLegend). After 24 hours of culture, floating cells were transferred to a new dish, and cell medium was replaced every 5–7 days. Differentiation into mast cells was confirmed by the expression of FcεRI and CD117 (c-kit) as assessed by flow cytometry. After 4–12 weeks in culture, the BMNCs were used for the experiments.

Affinity Purification and Nano-Liquid Chromatography-Tandem Mass Spectrometry. Cleared lysate of *Escherichia coli* expressing GST or GST-fused mouse H₄ receptor carboxy-terminal tail [(mH4RCT), i.e., GST-mH4RCT] was bound to Glutathione Sepharose 4B beads (GE Healthcare). BMNCs (6 × 10⁷ cells) were lysed in lysis buffer [50 mM Tris-HCl pH 7.4, 100 mM NaCl, 10% glycerol, 1 mM dithiothreitol (DTT), 1 mM EGTA, 1 mM EDTA, 0.5% Triton X-100, with Roche Complete protease inhibitor cocktail (Roche, Basel, Switzerland)] at 4°C on a rotator overnight and centrifuged at 15,000g for 15 minutes at 4°C. The supernatant was applied to the GST column and the flow-through was then applied to the GST-mH4RCT column. Both columns were washed extensively and bound proteins were eluted with elution buffer (50 mM Tris-HCl pH 7.4, 1 M NaCl, 10% glycerol, 1 mM DTT, 1 mM EGTA, 1 mM EDTA, 0.5% Triton X-100). The proteins were precipitated with ice-cold trichloroacetic acid (final concentration of 10%) and centrifuged at 15,000g for 10 minutes at 4°C. The pellet was washed with ether/ethanol (1:1) and dissolved in SDS-PAGE sample buffer. The samples were resolved on a 10%–20% gradient SDS-PAGE gel and stained using the silver stain mass spectrometry kit (Wako). Excised gel bands were reduced with 100 mM DTT and alkylated with 100 mM iodoacetamide. After washing, the gel pieces were incubated with trypsin overnight at 30°C. Recovered peptides were desalted with Ziptip c18 (Millipore). Samples were analyzed by nano-liquid chromatography-tandem mass spectrometry systems (DiNa HPLC; KYA TECH Corporation, Tokyo, Japan) and QSTAR XL (Applied Biosystems, Foster City, CA). Mass data acquisitions were piloted by the Mascot software (Kanno et al., 2007).

In Vitro Pull-Down Experiment. Cleared lysates from *E. coli* expressing GST- or MBP-fusion proteins were mixed together and incubated for 1 hour. The mixture was further incubated with Glutathione Sepharose 4B for 1 hour, and proteins bound to the beads were separated on SDS-PAGE and subjected to immunoblot with anti-MBP antibody. Signals were visualized by horseradish peroxidase-conjugated secondary antibody and Chemi-Lumi One L (Nacalai Tesque, Kyoto, Japan).

Rac Activation Assay. BMNCs (3 × 10⁷ cells) were starved of IL-3 for at least 24 hours. The cells were resuspended in Hanks' balanced salt buffer with calcium and magnesium (Wako) containing 0.1% bovine serum albumin [(BSA); Sigma Aldrich (St. Louis, MO)], and preincubated for 10 minutes in the presence of an inhibitor or antagonist at the indicated concentrations at 37°C. The BMNCs were stimulated with 10 μM histamine for 5 minutes and immediately placed in an ice-water bath. The cells were washed once with ice-cold Tris-buffered saline containing 0.1% BSA and lysed with lysis buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 1% NP-40, 1 mM DTT, 5% glycerol, 1.5× Roche Complete protease inhibitor cocktail) for 5 minutes at 4°C. The cleared lysate was incubated with GST-PAK1-PBD-bound beads at 4°C for 1 hour. The beads were washed and bound proteins were extracted with Laemmli sample buffer (Sigma Aldrich). A portion of total lysate was mixed with SDS sample buffer to detect the total Rac input. The samples were separated on 12% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Rac GTPases were detected by immunoblotting using mouse Rac1- and Rac2-specific antibodies.

Chemotaxis Assay. Chemotaxis cell well inserts with a pore size of 5 μm (KURABO, Osaka, Japan) were coated with 10 μg/ml of human plasma fibronectin (Roche) for 30 minutes at 37°C. After removing the fibronectin solution, cells (1 × 10⁵) in an assay medium (0.1% BSA in RPMI1640) were added into the Chemotaxis cell inserts in a 200 μl volume with or without inhibitor. After incubation for 30 minutes at 37°C 600 μl of assay medium with 10 μM histamine was added into the lower chamber. The plate was further incubated for 1 hour at 37°C and

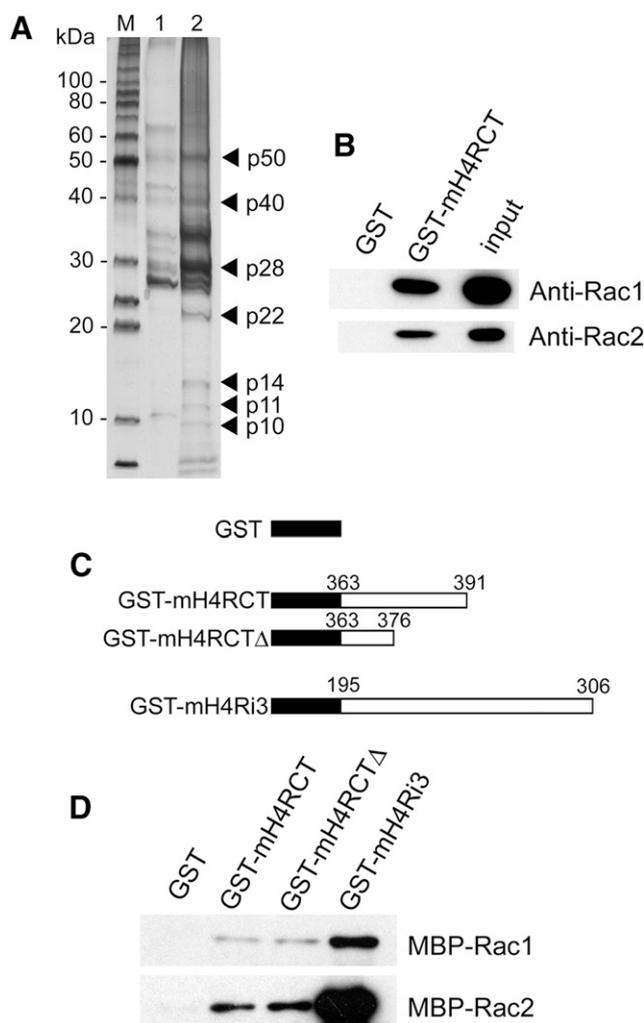


Fig. 1. Rac1 and Rac2 physically associate with the H₄ receptor. (A) Silver staining of affinity-purified H₄ receptor-binding proteins separated on SDS-PAGE. BMMC lysate was first negatively purified with GST-bound beads and the flow-through was further purified with GST-mH4RCT-bound beads. Seven bands indicated by arrowheads were isolated and subjected to liquid chromatography–tandem mass spectrometry (LC-MS/MS). M, marker; lane 1, proteins bound to GST; lane 2, proteins bound to GST-mH4RCT. Numbers on the left indicate molecular weight in kilodalton. (B) Affinity-purified proteins were separated on SDS-PAGE and immunoblotted with anti-Rac1 and anti-Rac2 antibodies to confirm the LC-MS/MS result. (C) Schematic illustration of GST-fused full carboxy-terminus (GST-mH4RCT), proximal half of the carboxy-terminus (GST-mH4RΔ), and third intracellular loop (GST-mH4Ri3) of mouse H₄ receptor. Numbers indicate the amino acid position. (D) Rac1 and Rac2 bind directly to the third intracellular loop and carboxy-terminus of the histamine H₄ receptor. Bacterially expressed GST fusion proteins were incubated with MBP-fused Rac1 or Rac2 in vitro and pulled down with glutathione beads. Proteins were separated on SDS-PAGE and immunoblotted with anti-MBP antibody.

the number of migrated cells in the lower chamber was counted by flow cytometry. A suspension of fluorospheres of known counts (Flow-Count; Beckman Coulter, Brea, CA) was included to determine the absolute cell number.

ERK Activation Assay. BMMCs (1×10^5 cells) were cultured in RPMI1640 containing 0.1% BSA for at least 24 hours. The cells were preincubated for 5 minutes in the presence of an inhibitor or antagonist at the indicated concentrations at 37°C. The BMMCs were stimulated with 10 μM histamine for 2 minutes and immediately placed in an ice-water bath. The cells were washed once with ice-cold phosphate-buffered saline containing 0.1% BSA and lysed with SDS-PAGE sample buffer. Cell lysates were separated on SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The

TABLE 1

H₄ receptor-binding proteins identified by affinity purification and nano-liquid chromatography–tandem mass spectrometry

Bands identified by SDS-PAGE were subjected to nano-liquid chromatography–tandem mass spectrometry analysis. Protein names and the Mascot score with number of matched peptides are given.

Band	Protein Name	Score (Peptides)
p50	Moesin	271 (6)
p40	Moesin	322 (6)
p40	Fructose-bisphosphate aldolase	224 (4)
p40	Ezrin	179 (4)
p40	Casein kinase 2 alpha	58 (1)
p22	GTP binding protein Rac2	33 (2)
p11	GTP binding protein Rac1	274 (7)

membrane was incubated with anti-ERK1/2 or anti-phospho-p44/p42 MAPK antibody followed by a horseradish peroxidase-conjugated secondary antibody. Signals were detected with the Chemi-Lumi One L detection kit and Hyperfilm ECL (GE Healthcare), and band intensity was quantified by densitometry. The signal for activated ERK was normalized to that of total ERK. ERK activation was expressed as the fold increase over unstimulated cells.

Calcium Mobilization Assay. Cells were suspended in assay medium (Hanks' balanced salt buffer with calcium and magnesium containing 20 mM Hepes pH 7.4 and 0.1% BSA) and loaded with 4 μM Fluo-3 AM for 30 minutes at 37°C in the presence of 2.5 mM probenecid (Wako) and 0.08% pluronic acid (Dojindo). The cells were washed three times with assay medium and plated in a black-wall 96-well plate. The plate was centrifuged for 3 minutes at 300g, and calcium mobilization was assayed with a FlexStation 3 microplate reader (Molecular Devices, San Jose, CA). The $\Delta F/F$ value was calculated according to the following formula:

$$\Delta F/F = (F - F_{rest})/F_{rest}$$

where F is the dye fluorescence at any given time and F_{rest} is the average fluorescence signal prior to histamine addition.

Lentivirus Production. To construct the short-hairpin RNA (shRNA) expression plasmids, oligonucleotides corresponding to the sense target sequence, hairpin loop (5'-TTCAAGAGA-3'), and anti-sense target sequence were synthesized, annealed together, and inserted into the pLKO1.puro vector (Addgene, Cambridge, MA). The shRNA target sequences (sense) were as follows: mouse Rac1 sh1, 5'-GACGGAGCTGTTGGTAAA-3'; mouse Rac1 sh11, 5'-CTGGAAC-CTTTGTACGCT T-3'; mouse Rac2 sh14, 5'-ATGTGATGGTGGACAGTAA-3'; and mouse Rac2 sh16, 5'-GGCCAAGGATATTGA TTC A-3'. Each shRNA expression vector was transfected into HEK293T cells along with a lentiviral packaging plasmid psPAX2 (Addgene) and envelope expression vector pVSV-G (Clontech, Fremont, CA). The virus supernatants were harvested 48 hours after transfection and concentrated by ultracentrifugation (70,000g for 2 hours at 4°C). Virus particles from a 10-cm dish were resuspended in 0.1 ml of RPMI1640 medium supplemented with 10% fetal bovine serum and stored at -80°C until use.

shRNA-Mediated Knockdown of Rac1 and Rac2 in Mast Cells. RetroNectin, a recombinant human fibronectin fragment (Takara, Kusatsu, Japan), was used for the lentiviral transduction. Briefly, a polystyrene tube (BD Falcon 2058; BD Biosciences, Franklin Lakes, NJ) was coated with 30 μg/ml RetroNectin for 2 hours at 20°C. Lentivirus (0.1 ml) was bound to the RetroNectin-coated tube by centrifugation at 1350g for 4 hours at 32°C. After removing unbound lentivirus, lineage-negative bone marrow cells (1×10^5 cells), isolated using the mouse lineage cell depletion kit (Miltenyi Biotec, Bergisch Gladbach, Germany) from a female 4- to 6-week-old Balb/c mouse, were added into the lentivirus-coated tube and centrifuged at 400g for 30 minutes to facilitate transduction. The cells were then cultured in complete RPMI1640 medium containing 50 ng/ml mouse stem cell factor (Biolegend) and 10 ng/ml mouse IL-3. Two days after transduction, puromycin was added

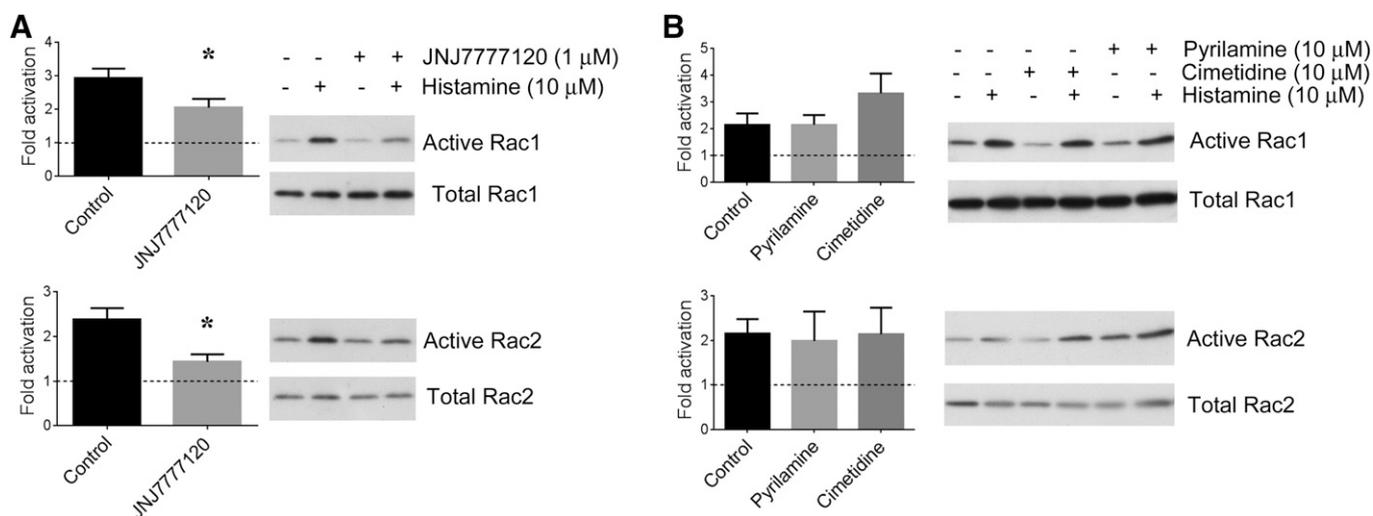


Fig. 2. H_4 receptor-mediated activation of Rac1 and Rac2. (A) BMMCs were stimulated with 10 μ M histamine alone or with an H_4 receptor antagonist, JNJ777120 (1 μ M). Active and total Rac1 and Rac2 were detected by immunoblotting. Data are expressed as fold activation over unstimulated, and are presented as the mean with S.E.M. from five independent experiments. * $P < 0.05$ by unpaired t test. A representative blot is shown in the right panel. (B) BMMCs were stimulated with 10 μ M histamine alone or with an H_1 receptor antagonist pyrilamine (10 μ M) or H_2 receptor antagonist cimetidine (10 μ M). Active and total Rac1 and Rac2 were detected by immunoblotting. Data are expressed as fold activation over unstimulated, and are presented as the mean with S.E.M. from three independent experiments. The activation of Rac1 and Rac2 in the presence of H_1 or H_2 receptor antagonist was not significantly different from that of the control by one-way analysis of variance with post-hoc Dunnett's test. A representative blot is shown in the right panel.

to a final concentration of 2 μ g/ml. Differentiation into mast cells was confirmed by the expression of Fc ϵ RI and CD117 (c-kit).

Statistical Analysis. Data are presented as the mean with S.E.M. Comparisons between two groups were assessed with unpaired t test, and among three or more groups comparisons were assessed with one-way analysis of variance followed by Dunnett's multiple comparisons test. A P value of <0.05 was considered statistically significant.

Results

Association of Rac1 and Rac2 with Histamine H_4 Receptor. To fully elucidate the signaling pathway downstream of the H_4 receptor, we sought to identify binding partners interacting with the intracellular domain of the mouse histamine H_4 receptor. For the pull-down assay, BMMC lysate was affinity-purified with bacterially expressed GST-mH4RCT as the bait. Figure 1A shows silver staining of the affinity-purified proteins separated on SDS-PAGE. Major bands that were specific for binding to GST-mH4RCT were analyzed by nano-liquid chromatography-tandem mass spectrometry (Kanno et al., 2007). Identified proteins included ERM proteins (ezrin and moesin), small G proteins (Rac1 and Rac2), and casein kinase 2 (Table 1). Furthermore, immunoblotting using specific antibodies confirmed Rac1 and Rac2 as binding partners of mouse H_4 receptor (Fig. 1B).

Direct interaction of the H_4 receptor carboxy-terminal tail with Rac1 or Rac2 was examined using bacterially expressed recombinant fusion proteins. The full H_4 receptor carboxy-terminal tail (amino acids 363-391), proximal half of the H_4 receptor carboxy-terminal tail (amino acids 363-376), and H_4 receptor third intracellular loop (amino acids 195-306) of mouse H_4 receptor expressed as GST-fusion proteins (Fig. 1C) were tested for binding with MBP-fused full-length mouse Rac1 or Rac2 in vitro. The result showed that the proximal half of the carboxy-terminus or the third intracellular loop was sufficient for H_4 receptor binding to Rac1 and Rac2 (Fig. 1D). These results indicated that Rac1 and Rac2 physically interacted with the H_4 receptor in mouse BMMCs.

Rac Activation via H_4 Receptor. We next explored whether Rac1 and Rac2 were activated by histamine in BMMC. Rac activation was assessed by a pull-down assay using PAK1-PBD to which the active GTP-bound form of Rac specifically binds. In BMMCs, 10 μ M of histamine increased the activation of Rac1 and Rac2 by 2- and 3-fold, respectively (Fig. 2). Because BMMCs express histamine H_1 , H_2 , and H_4 receptors (Ito et al., 2012), we next examined the effect of specific receptor antagonists on histamine-induced Rac activation. In the presence of 1 μ M JNJ777120, a specific H_4 receptor antagonist, histamine-induced activation of Rac1 and Rac2 was significantly attenuated (Fig. 2A). Pyrilamine and cimetidine, H_1 and H_2 receptor antagonists, respectively, failed to block the effect of histamine (Fig. 2B). These results suggested that both Rac1 and Rac2 were activated by histamine via the H_4 receptor in BMMCs.

Rac Involvement in BMMC Chemotaxis toward Histamine. Because the H_4 receptor mediates mast cell migration toward histamine (Hofstra et al., 2003), we subsequently investigated the role of Rac activation in this process. NSC23766 is a specific Rac inhibitor that effectively blocks Rac1 activation by Rac-specific guanine nucleotide exchange factor (GEF) Trio or Tiam1 (Gao et al., 2004). We found that NSC23766 inhibited BMMC chemotaxis toward histamine in a concentration-dependent manner with a 50% inhibitory concentration of 51 μ M (Fig. 3), which was comparable to its inhibitory effect on Rac-TrioN interaction in vitro or platelet-derived growth factor-induced Rac1 activation in NIH-3T3 cells (Gao et al., 2004). This result suggested that Rac activation was involved in H_4 receptor-mediated chemotaxis.

Involvement of Rac Activation in H_4 Receptor-Mediated Signaling. Because previous reports showed that histamine induces calcium mobilization from intracellular storage (Hofstra et al., 2003) and ERK activation (Desai and Thurmond, 2011) through the H_4 receptor in BMMCs, we evaluated the involvement of Rac activation in these signaling pathways. NSC23766 at 50 μ M had negligible effect on calcium

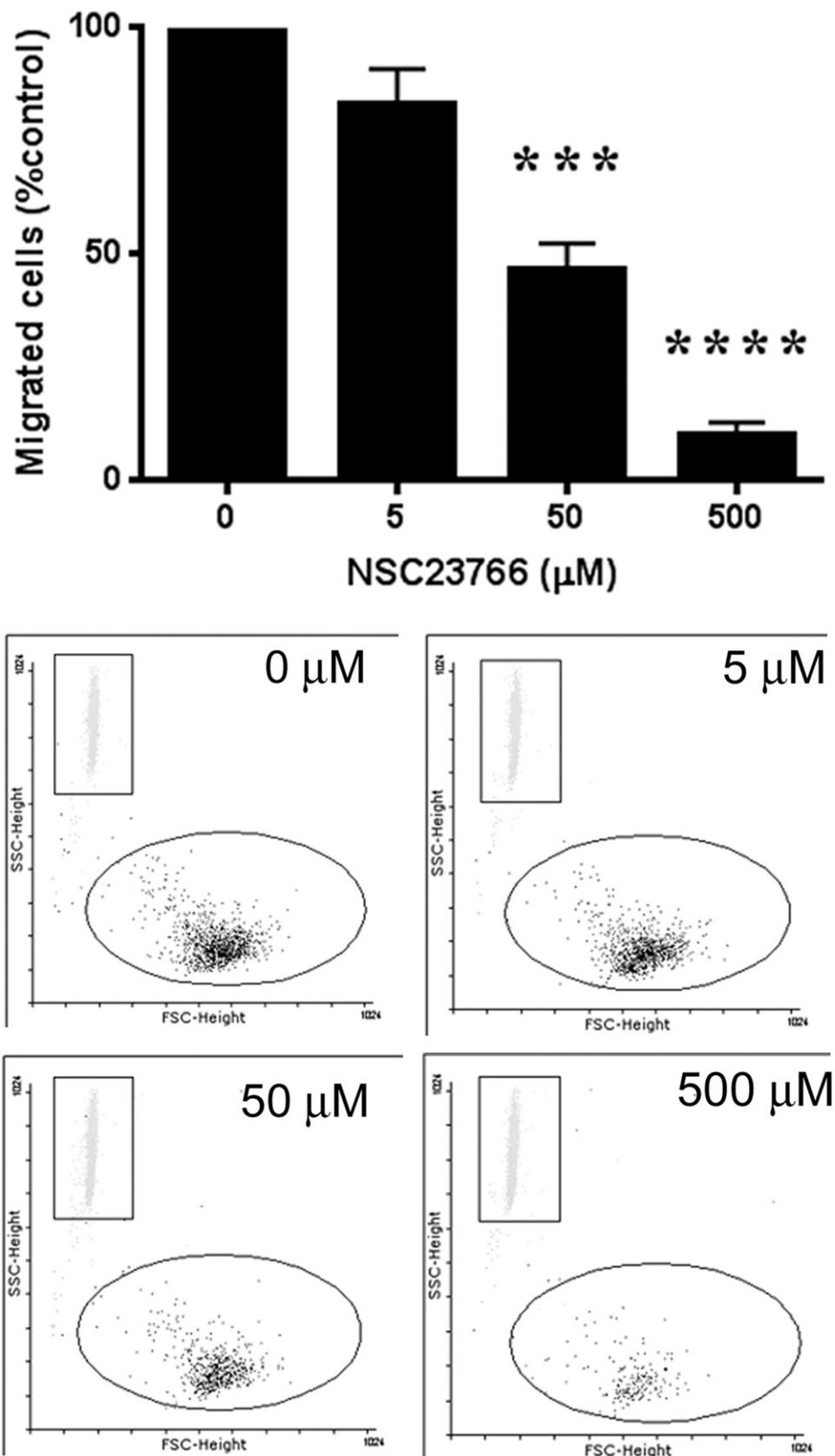


Fig. 3. Effect of Rac inhibitor NSC23766 on chemotaxis of BMMCs toward histamine. BMMCs were preincubated in the absence or presence of NSC23766 at the indicated concentrations and subjected to chemotaxis assay. Cells migrating into the lower chamber were mixed with fluorospheres and analyzed on a flow cytometer. Representative dot plots are shown at the bottom. The fluorospheres and BMMCs appear in the upper-left rectangle gate and lower oval gate, respectively. Data are expressed as the mean percentage of control (without inhibitor) with S.E.M. from three independent experiments. *** $P < 0.001$; **** $P < 0.0001$ by one-way analysis of variance with post-hoc Dunnett's test compared with the control without inhibitor.

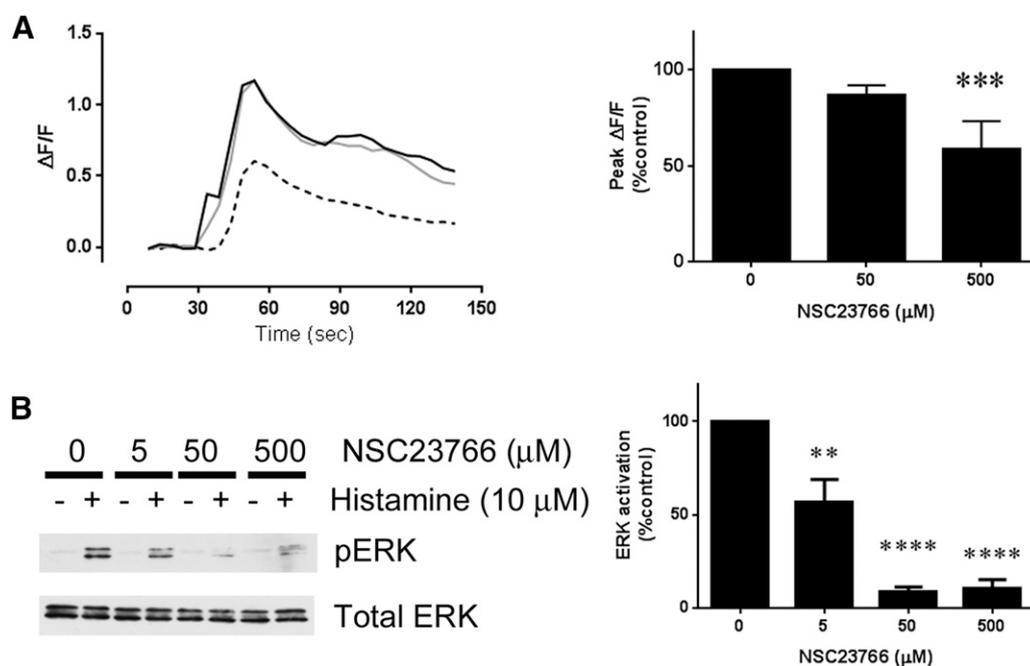


Fig. 4. Histamine-induced calcium mobilization and ERK activation are Rac dependent. (A) Effect of Rac inhibitor NSC23766 on histamine-induced calcium mobilization in BMMCs. Histamine ($10 \mu\text{M}$) was added at 30 seconds. The left panel shows a representative trace (solid black line, no inhibitor; solid gray line, $50 \mu\text{M}$ NSC23766; dashed line, $500 \mu\text{M}$ NSC23766). Data are expressed as mean percentage of the control without inhibitor with S.E.M. from at least five independent experiments. $***P < 0.001$ by one-way analysis of variance (ANOVA) with post-hoc Dunnett's test compared with the control without inhibitor. (B) Effect of Rac inhibitor NSC23766 on histamine-induced ERK activation in BMMCs. Phosphorylated and total ERKs were detected by immunoblotting, and a representative blot is shown in the left panel. Data are expressed as mean percentage of the control without inhibitor with S.E.M. from three independent experiments. $**P < 0.01$; $****P < 0.0001$ by one-way ANOVA with post-hoc Dunnett's test compared with the control without inhibitor.

mobilization. At $500 \mu\text{M}$, the inhibitor partially but significantly decreased the peak $\Delta F/F$ value (Fig. 4A), indicating that calcium mobilization through the H_4 receptor was partially mediated by Rac activation. We next evaluated whether Rac activation was involved in ERK activation. As shown in Fig. 4B, histamine-induced ERK phosphorylation was inhibited by NSC23766 in a concentration-dependent fashion. At a concentration of $50 \mu\text{M}$ or higher, ERK activation was nearly abolished (Fig. 4B), indicating that this process was fully dependent on Rac activation.

Histamine-Induced Chemotaxis was Dependent on Mitogen-Activated Protein Kinase Kinase. Next, we determined whether histamine-induced ERK activation mediated BMMC chemotaxis toward histamine. PD98059 is an inhibitor of mitogen-activated protein kinase kinase (MEK), a MAPK that phosphorylates ERK. PD98059 significantly attenuated histamine-induced BMMC migration in a concentration-dependent manner (Fig. 5A), suggesting that ERK activation was involved in H_4 receptor-mediated BMMC chemotaxis downstream of Rac activation. SP600125, an inhibitor of c-Jun N-terminal kinase (JNK), another MAPK downstream of Rac, had no inhibitory activity on histamine-induced chemotaxis of BMMCs, suggesting that JNK was not involved in this process (Fig. 5B).

PI3K-Dependent Rac Activation. Numerous Rac-GEFs are known to be activated by PI3K (Welch et al., 2003). Furthermore, H_4 receptor activation in BMMCs leads to cytokine production via the PI3K pathway (Desai and Thurmond, 2011). These findings led us to hypothesize that PI3K may be involved in H_4 receptor-mediated Rac activation and cell migration. Thus, we examined whether histamine-induced BMMC migration and Rac activation were mediated by the PI3K pathway. LY294002, a PI3K inhibitor, suppressed BMMC chemotaxis toward histamine in a concentration-dependent manner (Fig. 6A). Furthermore,

this inhibitor attenuated histamine-induced activation of both Rac1 and Rac2 (Fig. 6B). These results suggested that PI3K regulated H_4 receptor-mediated chemotaxis of BMMCs upstream of Rac activation.

Both Rac1 and Rac2 are Required for BMMC Migration. To define the specific role of Rac1 and Rac2 in histamine-induced chemotaxis of BMMCs, shRNA was used to individually knockdown Rac1 or Rac2 expression. Lineage-negative bone marrow cells were transduced with lentiviral vector expressing the individual shRNA and induced to differentiate into mast cells. After successful differentiation as assessed by the expression of both c-kit and Fc ϵ RI α (Fig. 7A), specific downregulation of Rac1 or Rac2 was confirmed by immunoblot (Fig. 7B). When compared with vector-only transduction, knockdown of Rac1 and Rac2 significantly reduced the percentage of cells that migrated toward histamine (Fig. 7C). These results suggested that both Rac1 and Rac2 were required for mast cells to migrate toward histamine.

Distinct Roles of Rac1 and Rac2 in H_4 Receptor-Mediated Signaling. We subsequently explored whether each Rac GTPase played a specific role in downstream signaling. While Rac1 downregulation had minimal effect on histamine-induced ERK phosphorylation, Rac2 knockdown had a significant inhibitory effect on ERK activation (Fig. 8A), indicating that Rac2 was mainly involved in histamine-induced phosphorylation of ERK. In a calcium mobilization assay, both Rac1 knockdowns (sh1 and sh11) resulted in significantly reduced peak calcium concentration by approximately 40% (Fig. 8B). In contrast, Rac2 knockdowns (sh14 and sh16) had minimal effect on calcium signaling; while sh16 resulted in a slight decrease in peak calcium concentration by 20%, sh14 had a negligible effect on calcium mobilization (Fig. 8B). These results suggested that Rac1 was primarily involved in calcium signaling.

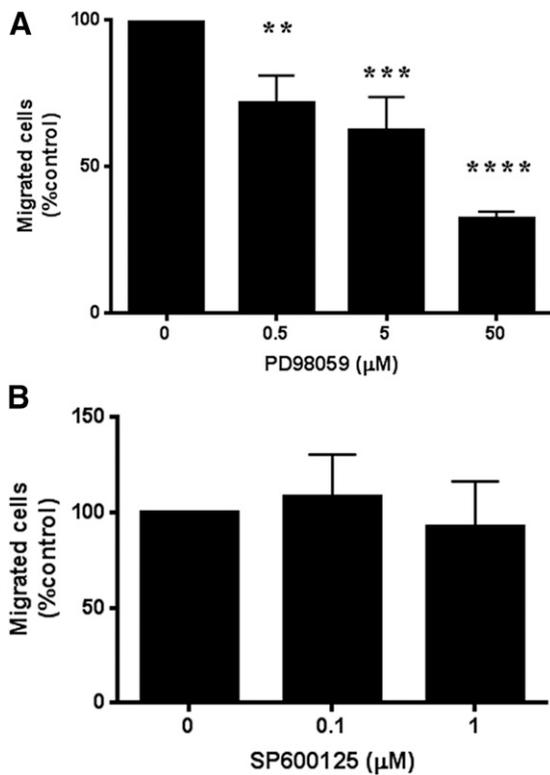


Fig. 5. Histamine-induced migration of BMMCs is MEK dependent. Effect of MEK inhibitor PD98059 and JNK inhibitor SP600125 on chemotaxis of BMMCs toward histamine. BMMCs were preincubated in the absence or presence of PD98059 (A) or SP600125 (B) at the indicated concentrations and subjected to chemotaxis assay. Data are expressed as mean percentage of the control without inhibitor with S.E.M. from three independent experiments. ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$ by one-way analysis of variance with post-hoc Dunnett's test compared with the control without inhibitor.

Discussion

In this study, we identified several intracellular binding partners of the histamine H₄ receptor by proteomics. Among

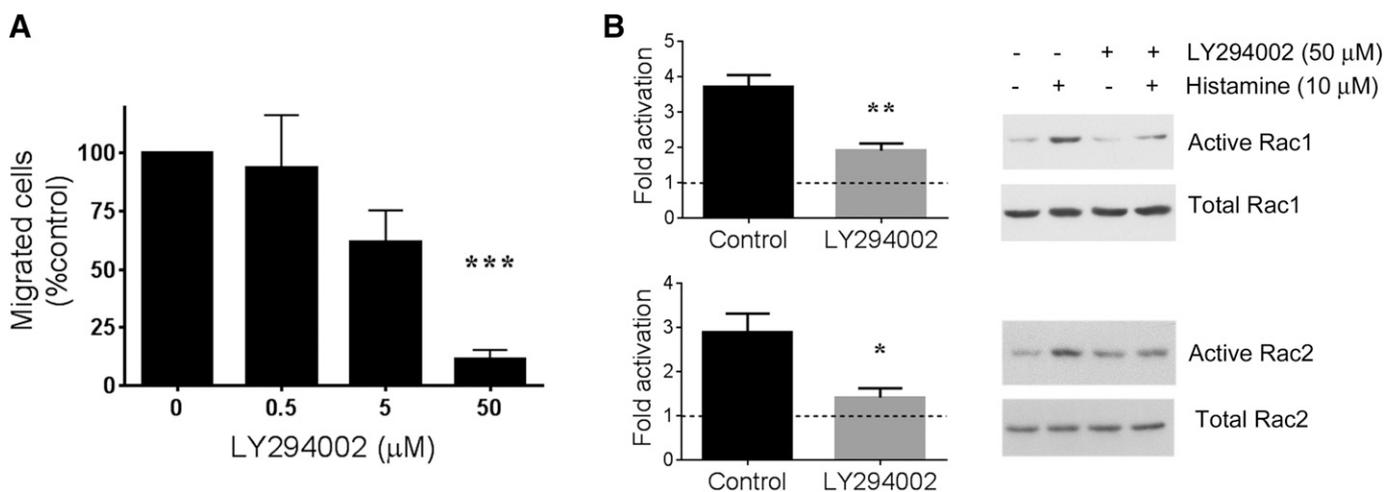


Fig. 6. Histamine-induced chemotaxis and Rac activation of BMMCs are PI3K dependent. (A) Effect of PI3K inhibitor LY294002 on chemotaxis of BMMCs toward histamine. BMMCs were preincubated in the absence or presence of inhibitor at the indicated concentrations and subjected to chemotaxis assay. Data are expressed as mean percentage of the control without inhibitor with S.E.M. from three independent experiments. *** $P < 0.001$ by one-way analysis of variance with post-hoc Dunnett's test compared with the control without inhibitor. (B) Effect of PI3K inhibitor LY294002 on histamine-induced activation of Rac1 and Rac2 in BMMCs. BMMCs were stimulated with 10 μM histamine alone or with PI3K inhibitor LY294002 (50 μM). Active and total Rac1 and Rac2 were detected by immunoblotting. Data are expressed as fold activation over unstimulated cells, and are presented as the mean with S.E.M. from four independent experiments. * $P < 0.05$; ** $P < 0.01$ by unpaired t test. A representative blot is shown in the right panel.

these proteins, the ERM proteins and casein kinase 2 were previously implicated in GPCR signaling or trafficking (Stanasila et al., 2006; Torrecilla et al., 2007). However, we were not able to confirm their binding to the histamine H₄ receptor. In contrast, we demonstrated that Rac GTPases bound to the H₄ receptor, and further evaluated these small GTPases in our study.

We showed that histamine activated small GTPases Rac1 and Rac2 through the histamine H₄ receptor in mouse BMMCs. This is the first demonstration of small GTPase activation downstream of the H₄ receptor. In addition to the functional coupling, we also demonstrated the physical association of the H₄ receptor with both Rac1 and Rac2, suggesting that the H₄ receptor forms a multiprotein complex to transduce signals efficiently and precisely. Attenuation of histamine-induced chemotaxis by a Rac inhibitor, which is known to suppress the activation of both Rac1 and Rac2 (Cancelas et al., 2005), suggested the importance of Rac1 and/or Rac2 activation in H₄ receptor-mediated BMMC migration. While Rac1 is ubiquitously expressed, Rac2 expression is specific to hematopoietic cells. Rac2-deficient mice, which have normal Rac1 expression, show functional abnormalities in multiple blood lineages, suggesting that Rac2 has specific roles that cannot be substituted by Rac1 (Roberts et al., 1999; Yang et al., 2000; Croker et al., 2002; Pradip et al., 2003). In agreement with this, specific knock-down of Rac1 or Rac2 in BMMCs revealed that both proteins were necessary for histamine-induced chemotaxis, suggesting that the two Rac GTPases have nonredundant roles.

Our results also demonstrated distinct roles of Rac1 and Rac2 in downstream signaling. Rac1 knockdown reduced histamine-induced calcium mobilization, whereas Rac2 down-regulation suppressed ERK phosphorylation. These results suggested that H₄ receptor signaling diverges to at least two distinct pathways: the Rac1-calcium pathway and Rac2-ERK pathway. Because chemotaxis involves multiple events that include sensing of attractant, polarity formation, leading edge protrusion, cell body translocation, and posterior retraction, all of which need to be tightly regulated to cooperate,

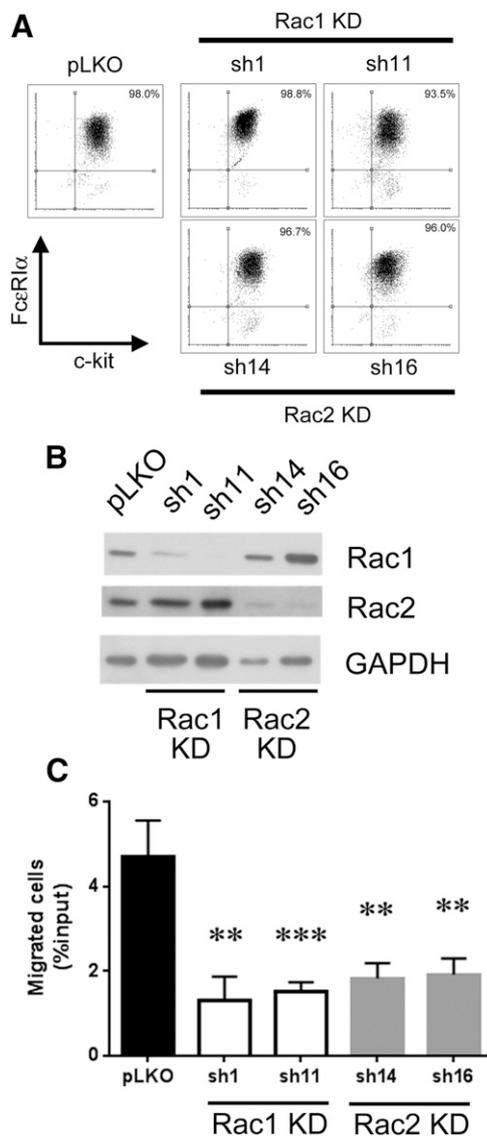


Fig. 7. Rac1 and Rac2 are not redundant in histamine-induced chemotaxis. (A) Lineage-negative [Lin (-)] bone marrow cells were transduced with a lentiviral vector to express shRNA. Transduced cells were selected by puromycin and differentiated into mast cells with 50 ng/ml stem cell factor and 10 ng/ml IL-3 for 4 weeks. The cells were stained with PE-Cy7-conjugated anti-mouse c-kit antibody and PE-conjugated anti-mouse FcεRIα and analyzed by flow cytometry. Numbers in the upper-right quadrant indicate the percentage of double positive cells. KD, knockdown. (B) Lysate of transduced cells was subjected to immunoblotting to confirm KD. (C) Effect of Rac1- or Rac2-specific shRNA expression on histamine-induced chemotaxis. Lin (-) mast cells expressing shRNA were subjected to chemotaxis assay. Data are expressed as mean percentage of migrated cells over input cells with S.E.M. from four to six independent experiments. ** $P < 0.01$; *** $P < 0.001$ by one-way analysis of variance with post-hoc Dunnett's test compared with control cells.

divergence in H_4 receptor signaling is necessary. In a previous study, inhibition of PLC completely suppressed histamine-mediated calcium mobilization and chemotaxis, suggesting that inositol 1,4,5-triphosphate (IP_3)-mediated calcium mobilization is necessary for H_4 receptor-mediated chemotaxis (Hofstra et al., 2003). Our results showed that a MEK inhibitor can significantly suppress histamine-induced chemotaxis of BMMCs, suggesting that the MEK-ERK pathway is also involved. In addition to the regulation of cell migration by various calcium-dependent proteins (Wei et al., 2012), ERK

phosphorylation is also known to regulate several proteins in chemotaxis (Huang et al., 2004); both mechanisms may work cooperatively to regulate a complex series of events to establish chemotaxis in a spatiotemporal manner.

We demonstrated that a Rac inhibitor partially but significantly suppressed histamine-induced calcium mobilization. In addition, specific knockdown of Rac1, but not Rac2, significantly attenuated calcium mobilization induced by histamine, suggesting that Rac1 was upstream of PLC to mobilize calcium from intracellular storage. Several PLC isozymes are expressed in BMMCs, including PLCβ, PLCγ, PLCδ, and PLCε (Ito et al., 2012). Among these, PLCβ and PLCγ are known to be activated by Rac GTPases (Kadamur and Ross, 2013). Rho family GTPases Rac1, Rac2, and Cdc42 can activate PLCβ2 by directly binding to the pleckstrin homology (PH) domain of the enzyme (Illenberger et al., 1997, 1998, 2003; Snyder et al., 2003). PLCβ is also activated directly by the $G\beta\gamma$ subunit, which does not involve Rac activation (Kadamur and Ross, 2013); this may explain the finding that histamine-induced calcium mobilization was only partially sensitive to the Rac inhibitor. PLCγ is generally considered to be downstream of tyrosine protein kinases; however, Piechle et al. (2005) showed that PLCγ2, but not PLCγ1, is directly stimulated by Rac1 and Rac2 independently of tyrosine phosphorylation. Rac regulates PLCγ2 by interacting with the PH domain (Walliser et al., 2008). In B cells, Rac-mediated stimulation of PLCγ2 by direct protein-protein interaction amplifies B cell receptor-induced calcium signaling (Walliser et al., 2015). In platelets, Rac1 mediates immunoreceptor tyrosine-based activation motif-dependent PLCγ2 activation (Pleines et al., 2009). A previous study revealed that the PLC- IP_3 pathway downstream of the H_4 receptor and pertussis toxin-sensitive G protein is involved in the chemotaxis of BMMCs toward histamine (Hofstra et al., 2003). Our study adds Rac1 as an important signaling molecule in this pathway.

Histamine-induced calcium signaling comprises the acute and chronic sustained phases. The rapid increase of calcium in the acute phase occurs in the absence of extracellular calcium, while the sustained phase requires extracellular calcium (Hofstra et al., 2003), suggesting that the latter phase is initiated by store-operated calcium entry. In various cell types including mast cells, store-operated calcium channels are involved in cell migration (Prakriya and Lewis, 2015; Lin et al., 2018). In addition to the IP_3 -mediated calcium increase, the sustained phase might also participate in the calcium-mediated regulation of histamine-induced chemotaxis of mast cells. In Rac1-knockdown cells or BMMCs treated with Rac inhibitor, both the acute and sustained phases appeared to be suppressed, suggesting possible involvement of Rac1 in both the acute and sustained phases of calcium signaling induced by histamine.

Our results demonstrated that Rac inhibitor completely suppressed histamine-induced ERK phosphorylation in BMMCs. Furthermore, specific knockdown of Rac2, but not Rac1, resulted in significant attenuation of histamine-induced ERK activation, suggesting that H_4 receptor-mediated ERK activation was entirely dependent on Rac2. These findings are in agreement with those of previous reports showing that Rac2 deficiency led to reduced ERK1/2 activation in N-formylmethionine-leucyl-phenylalanine-activated neutrophils or T-cell receptor-stimulated T cells (Kim and Dinauer, 2001; Yu et al., 2001). PAK1, a main downstream effector of Rac and Cdc42 GTPases, is known to phosphorylate

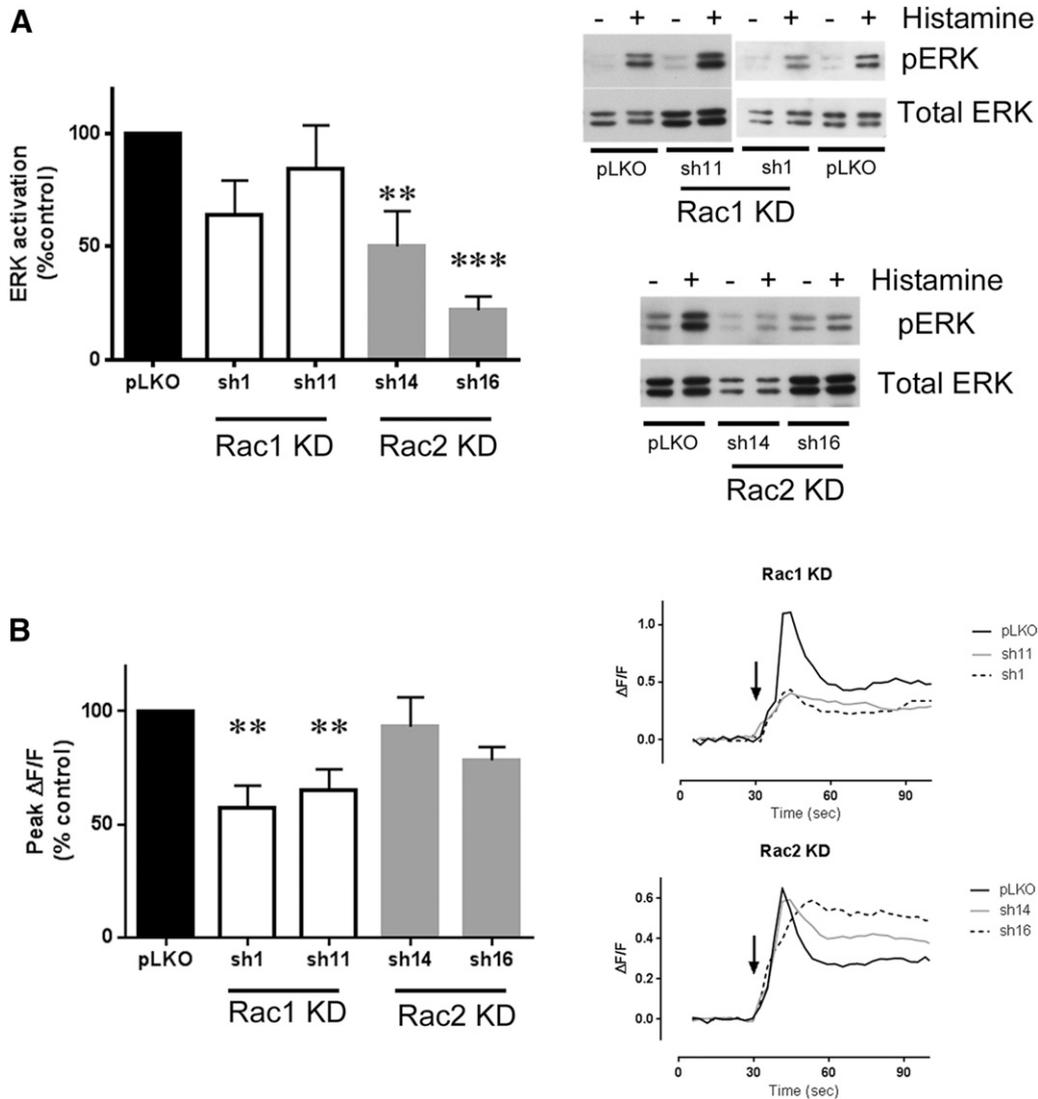


Fig. 8. Differential downstream signaling by Rac1 and Rac2. (A) Effect of Rac1- or Rac2-specific knockdown (KD) on histamine-induced ERK activation in lineage-negative [Lin (-)] mast cells. Serum-starved cells were stimulated with 10 μ M histamine for 2 minutes and subjected to immunoblotting. Phosphorylated and total ERK bands were quantified, and data are expressed as mean percentage of the control with S.E.M. from four independent experiments. ** $P < 0.01$; *** $P < 0.001$ by one-way analysis of variance (ANOVA) with post-hoc Dunnett's test compared with the control. Representative blots are shown in the right panel. (B) Effect of Rac1- or Rac2-specific KD on histamine-induced calcium mobilization in Lin (-) mast cells. Data are expressed as mean percentage of the control with S.E.M. from five to seven independent experiments. ** $P < 0.01$ by one-way ANOVA with post-hoc Dunnett's test compared with the control. Representative traces are shown in the right panel; arrows indicate the addition of 10 μ M histamine.

Raf and MEK, which are necessary for ERK signaling (Frost et al., 1997; Zang et al., 2002). This may be the mechanism by which Rac2 activation promotes the MEK-ERK pathway. In contrast to MEK inhibition, which significantly suppressed mast cell chemotaxis toward histamine, inhibition of JNK, another MAPK downstream of Rac GTPase (Timokhina et al., 1998; Gu et al., 2002; Yu et al., 2006), had negligible effect on histamine-induced migration, suggesting that the Rac-JNK pathway was not involved in H₄ receptor-mediated mast cell migration. ERK has many roles in cell migration by phosphorylating various proteins such as the myosin light chain kinase, calpain, focal adhesion kinase, and paxillin (Huang et al., 2004). Because histamine-induced ERK activation in mast cells is transient, peaking at 5 minutes and declining by 60 minutes after stimulation (Desai and Thurmond, 2011), we hypothesized that ERK activation functions in the early phase of chemotaxis. Supporting this notion, Mendoza et al. (2011)

suggested that ERK drives the initial lamellipodia protrusion by activating the WAVE2 regulatory complex.

The results of this study raised a further question on the mechanism by which the two divergent pathways are specifically regulated, because Rac1 and Rac2 are highly homologous with 92% amino acid identity. One possible mechanism is through different subcellular localization. Rac2 is known to be predominantly localized in intracellular compartments, while Rac1 is localized to the plasma membrane especially upon activation (Michaelson et al., 2001; Tao et al., 2002). Several PLCs are also known to be associated with plasma membrane where they catalyze the formation of diacylglycerol and IP₃. This different subcellular localization may be the mechanism by which calcium signaling is specifically regulated by Rac1 in histamine-stimulated BMMCs. Another possible mechanism for different Rac GTPases to mediate distinct signaling is through the action of scaffolding proteins that hold various

signaling molecules to prevent promiscuous activation. There are several scaffold proteins that are known to be involved in the MEK-ERK signaling pathway, such as the kinase suppressor of Ras, β -arrestin, and paxillin (Shaul and Seger, 2007). Further study is required to clarify the mechanism of the specific regulation of Rac1 and Rac2.

Our results also demonstrated that histamine-induced migration could be blocked by a PI3K inhibitor, suggesting that PI3K is involved in the signaling pathway downstream of the H₄ receptor. A previous report showed that histamine-induced IL-6 production in BMDCs is blocked by a PI3K inhibitor, which is consistent with our result (Desai and Thurmond, 2011). Furthermore, histamine-induced activation of both Rac1 and Rac2 was attenuated by PI3K inhibition, suggesting that PI3K was an upstream regulator of Rac GTPases. PI3K downstream of GPCRs is known to be activated by G $\beta\gamma$ subunits. A lipid messenger phosphatidylinositol (3,4,5)-triphosphate produced by PI3K is known to activate many GEFs for Rac GTPases by binding to the PH domain and exerting autoinhibition (Welch et al., 2003; Weiss-Haljiti et al., 2004). The GEF responsible for activating Rac1 and Rac2 in histamine-stimulated mast cell is yet to be determined and requires further investigation.

In conclusion, we demonstrated that small GTPases Rac1 and Rac2 physically and functionally coupled with the histamine H₄ receptor. The Rac GTPases have an essential role to diverge the signal from the H₄ receptor to at least two distinct arms, the Rac1-PLC-calcium and Rac2-MEK-ERK pathways. Thus, various events of chemotaxis can be coordinately regulated spatially and temporally in mast cells migrating toward histamine.

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Authorship Contributions

Participated in research design: Kuramasu, Yanai.

Conducted experiments: Kuramasu, Wakabayashi.

Contributed new reagents or analytic tools: Kuramasu, Wakabayashi.

Performed data analysis: Kuramasu, Wakabayashi, Inui.

Wrote or contributed to the writing of the manuscript: Kuramasu, Inui, Yanai.

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