RhoH Plays Critical Roles in FcεRI-Dependent Signal Transduction in Mast Cells[1]

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RhoH is an atypical small G protein with defective GTPase activity that is specifically expressed in hematopoietic lineage cells. RhoH has been implicated in regulation of several physiological processes including hematopoiesis, integrin activation, and T cell differentiation and activation. In the present study, we investigated the role of RhoH in mast cells by generating RhoH knockout mice. Despite observing normal development of mast cells in vivo, passive systemic anaphylaxis and histamine release were impaired in these mice. We also observed defective degranulation and cytokine production upon FcεRI ligation in RhoH-deficient bone marrow-derived mast cells. Furthermore, FcεRI-dependent activation of Syk and phosphorylation of its downstream targets, including LAT, SLP76, PLCγ1, and PLCγ2 were impaired, however phosphorylation of the γ-subunit of FcεRI remained intact. We also found RhoH-Syk association that was greatly enhanced by active Fyn. Our results indicate that RhoH regulates FcεRI signaling in mast cells by facilitating Syk activation, possibly as an adaptor molecule for Syk. ***The Journal of Immunology,*** **2009, 182: 957–962.**

RhoH is a newly identified hematopoietic small G protein, originally cloned as one of the genes frequently disrupted in diffuse large B cell lymphoma (1, 2). Because RhoH is defective in GTPase activity and thus constitutively active, the function of this protein was thought to be regulated by its expression level. Overexpression of RhoH inhibited RhoA/Rac/cdc42-dependent NF-κB activation in HEK293 cells (3), and it also inhibited SCF-mediated Rac1 activation in bone marrow progenitor cells (4). Knockdown of RhoH increased spontaneous LFA-1-mediated adhesion in Jurkat cells (5), and in vitro colony formation in bone marrow progenitor cells (4). Recent studies, however, have demonstrated that RhoH plays critical roles in T cell development (6, 7) by functioning as an adaptor for ZAP-70 in TCR signaling (7) via its tyrosine-phosphorylated ITAM-like motif (8). In the absence of RhoH, development of T cells in the thymus is impaired in both β-selection and positive selection, resulting in a severe reduction of mature peripheral T cells (6, 7). RhoH-deficient T cells showed defective phosphorylation of LAT and ERK upon TCR stimulation, indicating that RhoH is critical in TCR-dependent proximal signal transduction events. The precise function of RhoH in TCR signaling, however, remains controversial because there is a discrepancy in the phosphorylation status of ZAP-70 between two reports (6, 7). Furthermore, the physiological function of RhoH in other hematopoietic lineage cells is largely unknown.

Mast cells are widely distributed in the body and function as the primary effectors in immediate-type hypersensitivity reactions (9, 10). Mast cells recognize Ags via IgE and specific, high-affinity Fc receptors, termed FcεRI (11–13). FcεRI cross-linking triggers activation of Src family kinases Lyn and Fyn, and phosphorylation of ITAM motifs on the γ subunit of FcεRI complexes (14, 15). Subsequently, ZAP-70-related Syk kinase binds to phosphorylated ITAM motifs of the γ subunit and is thus activated by Src kinases (16–18). Activated Syk, in turn, phosphorylates LAT, LAT-2, and SLP-76 to form the signalosome, which transduces signals downstream, initiating Ca^{2+} mobilization, degranulation, and the expression of specific genes (19–21). The FcεRI-initiated signal cascade in mast cells is analogous to the TCR-initiated signal cascade in T cells, sharing many common molecules and features (14, 15, 22). This prompted us to investigate the function of RhoH in FcεRI signaling in mast cells.

In this study, we report the critical role of RhoH in mast cell activation. We established RhoH-deficient mice to unveil the physiological roles of RhoH in mast cells. RhoH-deficient mice showed impaired passive systemic anaphylaxis (PSA)[3] and histamine release upon challenge with the specific Ag. Our in vitro data showing impaired Syk activation with defective degranulation and cytokine production in $RhoH^{-/-}$ mast cells supports the observed in vivo phenotypes. Furthermore, we demonstrated that RhoH associates with Syk, and this interaction was greatly enhanced in the presence of constitutively active Fyn. These results suggest that RhoH acts as a positive regulator for FcεRI-mediated signal transduction by facilitating Syk activation.

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[3] Abbreviations used in this paper: PSA, passive systemic anaphylaxis; BMMC, bone marrow-derived mast cell; HSA, human serum albumin; HA, hemagglutinin.

Materials and Methods

Mice

RhoH knockout mice (Acc.No.CDB0483K) were generated using the previously described methods (23, 24). In brief, the entire ORF of the *RhoH* gene located in the third exon was replaced with a cassette consisting of a *lacZ* and *neomycin* resistance gene (*LacZ/neo*) by homologous recombination. The lengths of the homologous regions in the targeting vector were 8.2 kb and 4.8 kb at the 5′ and 3′ sides of the DT-A/LacZ/neo cassette (http://www.cdb.riken.jp/arg/cassette.html), respectively. Two mutant mouse lines were established from two independent homologous recombinant ES cell lines; no difference was found in their phenotype. All mice were maintained after five generations of backcrossing to C57BL/6J (B6) and housed under specific pathogen-free conditions in accordance with institutional guidelines.

Mast cell culture

Bone marrow-derived mast cells (BMMCs) were prepared as described (25). In brief, femurs were isolated from 8- to 20-wk-old $RhoH^{+/+}$ and $RhoH^{-/-}$ mice, and BM cells were cultured in 2% conditioned RPMI 1640 medium from X63-IL-3 cells (gift from Dr. H. Karasuyama, Tokyo Medical and Dental University, Tokyo, Japan), containing 10% heat-inactivated FBS. After at least 4 wk of culture, cells were stained with PE-conjugated anti-FcεRI Ab and allophycocyanin-conjugated anti-c-*kit* Ab (BD Biosciences) and their expression was confirmed before use in all experiments.

Plasmids

Hemagglutinin (HA)-tagged RhoH cDNA was cloned into pcDNA3 vector using a PCR-based strategy from a mouse thymus cDNA library made in our laboratory. Myc-tagged Syk and active type Fyn cloned in pcDNA3 vector were kind from Dr. T. Kurosaki (RIKEN Research Center for Allergy and Immunology, Yokohama, Japan) and Dr. T. Yamamoto (Institute of Medical Science, University of Tokyo, Tokyo, Japan).

Passive systemic anaphylaxis

Mouse IgE anti-DNP (100 μg, clone SPE-7, Sigma-Aldrich) was administered i.v. through the tail vein in volumes of 300 μl/mouse. After 24 h, 100 μg of DNP-human serum albumin (HSA) in 300 μl of PBS was injected i.v. Immediately after Ag challenge, body temperature was measured every 5 min by rectal thermometer. At 30 min following Ag challenge, mice were sacrificed and peripheral blood was taken by cardiac puncture and plasma was used for histamine enzyme immunoassay (SPI-BIO).

Degranulation assay

BMMCs (5×10^4 per well) on 96-well plates were sensitized with 1 μg/ml IgE anti-DNP for 4 h at 37°C. Next, cells were washed three times with Tyrode's Buffer (130 mM NaCl, 5 mM KCl, 1.4 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, 0.1% BSA, 10 mM HEPES (pH 7.4)) and stimulated for 30 min with the indicated concentration of DNP-HSA or A23187 (200 ng/ml, Sigma-Aldrich) in 100 μl of Tyrode's buffer. Samples were centrifuged and supernatant was collected to measure the released β-hexosaminidase. To determine the total cell content of this enzyme, the cell pellet was lysed with 0.5% Triton X-100 in Tyrode's buffer and the lysate was collected. For each sample, 50 μl of substrate solution (1.3 mg/ml 4-nitrophenyl N-acetyl-β-D-glucosaminide in 0.1 M sodium citrate (pH 4.5); Sigma-Aldrich) was added and incubated for 1 h at 37°C. The reaction was stopped by the addition of 150 μl of 0.2 M glycine solution (pH 10.7). The absorbance at 405 nm was measured in a microplate reader (Bio-Rad).

Calcium flux assay

BMMCs were sensitized with 1 μg/ml IgE anti-DNP at 10^6/ml without IL-3 and labeled with 3 μM of Fura2-AM (Invitrogen) for 30 min at 37°C. Then, cells were washed three times and resuspended in Tyrode's buffer at 5×10^5/ml. Fluorescence intensities were measured at an excitation wavelength of 340 or 380 nm and an emission wavelength of 510 nm, with a fluorescence spectrometer (Hitachi F-2500) during stimulation as indicated in Fig. 5.

Real-time RT-PCR analysis

Total RNA was isolated from 10^6 stimulated or nonstimulated BMMCs (as described above) using RNeasy kit (Qiagen), and cDNA was generated using the SuperScript III kit (Invitrogen) according to the manufacturer's instructions. Real-time PCR was conducted using the Platinum SYBR Green qPCR Supermix (Invitrogen) with specific primers for RhoH (5′gatcaggagcaacctacc ct3′/5′atgcaggaagccctgtga3′), IL-6 (5′gctaccaaactggatataatcagga 3′/5′ccaggtag ctatggtactccagaa3′), TNF-α (5′tcttctcattcctgcttgtgg3′/5′ggtctgggccatagaactga 3′), and β-actin (5′aaggccaaccgtgaaaagat3′/5′gtggtacgaccagaggcatac3′).

Immunoblotting and immunoprecipitation

For immunoblotting, BMMCs were sensitized with 1 μg/ml anti-DNP IgE at 10^6 cells/ml for 4 h without IL-3, washed three times, and then stimulated with DNP-HSA for the indicated periods. Stimulated BMMCs were lysed in lysis/wash buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris-Cl (pH 8.0), 1 mM sodium orthovanadate, 1 mM DTT, proteinase inhibitors), subjected to 7.5–12.5% SDS-PAGE and Western blotting (10^5 cells per lane). For FcRγ and Lyn, 10^6 BMMCs were lysed with lysis/wash buffer and immunoprecipitated with 1 μg of the indicated Abs, then subjected to 12.5% SDS-PAGE in tricine-SDS running buffer (0.1 M Tris, 0.1 M Tricine, 0.1% SDS for the upper chamber, 0.2 M Tris-Cl (pH 8.9) for the lower chamber), and Western blotting. For RhoH and Syk, 4.5×10^5 293T cells were transiently transfected with HA-tagged RhoH, myc-tagged Syk with or without constitutively active Fyn using FuGENE 6 (Roche). After 24 h, transfected cells were incubated with or without 30 μM PP2 (src kinase inhibitor) for 2 h before lysis. After 2 h, cells were lysed with magnesium containing lysis/wash buffer (1% NP40, 10 mM $MgCl_2$, 25 mM HEPES (pH 7.3), 150 mM NaCl, 8% glycerol, 1 mM EDTA, 1 mM sodium orthovanadate, and protease inhibitors), immunoprecipitated with 1 μg of the indicated Abs and then subjected to 12.5% SDS-PAGE in tricine-SDS running buffer. We confirmed that 30 μM of PP2 did not affect the overall tyrosine phosphorylation status of the cells, indicating that this concentration of PP2 specifically inhibits src kinases. Abs used for Western blotting and immunoprecipitation were anti-phospho Syk, -phospho PLCγ1, -phospho PLCγ2, -ERK, -phospho p38, -p38, -Lyn (Cell Signaling Technology), anti-phospho SLP76 and -phospho LAT (BD Biosciences), anti-phospho tyrosine (clone pY20, Neomarkers), anti-phospho tyrosine (clone 4G10), -myc and -HA (Roche) and -FcRγ (Millipore). We could not detect endogenous RhoH using commercially available or in-house generated RhoH Abs from rabbit or rat.

Histochemistry

To observe tissue mast cells, 6-μm paraffin sections of fixed tissues were stained with toluidine blue (Sigma-Aldrich) and the total number of mast cells in each tissue unit was counted. A skin unit was defined as the entire area of an ear section along the cartilage, and a stomach unit was defined as the entire area of a sagittal section.

Results

Generation of $RhoH^{-/-}$ mice

Mice carrying a mutant *RhoH* allele were generated from TT2 embryonic stem cells in which homologous recombination was used to replace the entire coding region located in exon 3 of the *RhoH* gene. As a result, there is no possibility of expression of any truncated forms of the protein in the mice (Fig. 1*A*). Offspring carrying the mutant allele were identified by Southern blotting (Fig. 1*B*). Homozygous *RhoH*-null mice were born at the expected Mendelian ratio.

Impaired FcεRI-mediated anaphylaxis in $RhoH^{-/-}$ mice

We found that RhoH is expressed in mast cells as well as T and B cells (Fig. 1*C*), as previously reported (4). Because the role of RhoH in immune cells other than T cells has not been characterized, we focused on mast cells in the present study. One of the major functions of mast cells is inducing allergy and anaphylaxis, therefore, we examined PSA, in which exogenously administered IgE is passively taken up by mast cells and degranulation is evoked by subsequent administration of Ag. As shown in Fig. 2*A*, the IgE-dependent PSA response in $RhoH^{-/-}$ mice, evaluated by the reduction of core body temperature after DNP-HSA injection, was smaller compared with those in $RhoH^{+/+}$ mice. As shown in Fig. 2*B*, measurement of plasma histamine concentration 30 min after DNP-HSA challenge revealed reduced secretion of histamine in $RhoH^{-/-}$ mice compared with $RhoH^{+/+}$ mice. Collectively, we demonstrated that RhoH is important in the systemic anaphylaxis reaction in vivo.

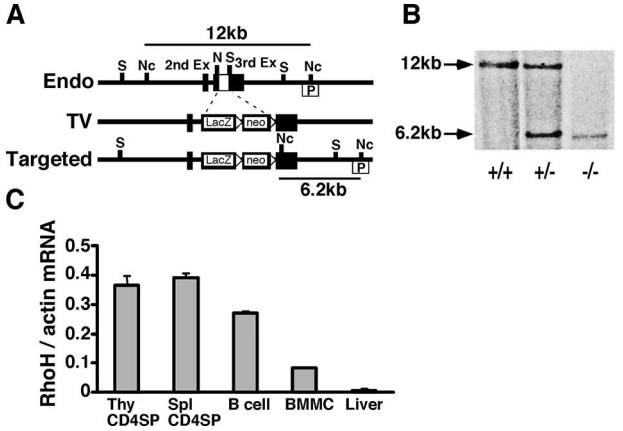

FIGURE 1. Generation of RhoH$^{-/-}$ mice. *A*, Targeting vector was designed to disrupt the *RhoH* gene by homologous recombination. The endogenous locus of *RhoH* (Endo), targeting vector (TV), and targeted locus (Targeted) are shown. The open box in the third exon represents the entire ORF of *RhoH* that was replaced by a *LacZ/neomycin* resistance gene. Restriction sites are abbreviated as follows: S, *Sal*I; Nc, *Nco*I; N, *Not*I. P in the open box indicates the location of probe for Southern blotting. *B*, Southern blot analysis of *Nco*I digested mouse genomic DNA. A 12- and 6.2-kb band represent the wild-type and targeted alleles, respectively. *C*, Expression of RhoH mRNA was detected by real time RT-PCR in the indicated cells and tissues. Results are the mean and SE from three independent experiments, $n = 9$ for each group.

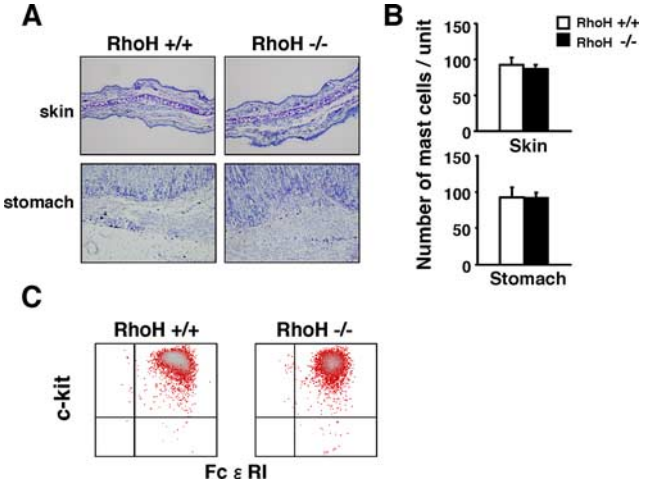

FIGURE 3. Normal development of mast cells in RhoH$^{-/-}$ mice. *A*, Mast cells in the ear skin (*upper*) and stomach (*lower*) in RhoH$^{+/+}$ and RhoH$^{-/-}$ mice were detected with toluidine blue staining. The highly metachromatic cells are mast cells. *B*, Number of mast cells in indicated tissues. Results are the mean and SE of mast cells per unit from indicated number of mice. For ear skin, $n = 4$ mice per group; for stomach, $n = 3$ per group. *C*, Expression of FcεRI and c-*kit* on the surface of BMMCs induced from RhoH$^{+/+}$ and RhoH$^{-/-}$ mice was analyzed by flow cytometry. Shown are representative data from more than five independent experiments.

Normal development of mast cells in RhoH$^{-/-}$ mice

Because we found impaired anaphylaxis in RhoH-deficient mice, we assessed the effect of RhoH disruption on mast cell development in vivo. Histological analysis by toluidine blue staining demonstrated that the anatomical distribution and morphology of connective tissue mast cells in the skin and mucosal mast cells in the stomach of RhoH$^{-/-}$ mice were comparable to those of RhoH$^{+/+}$ mice (Fig. 3*A*). The number of mast cells detected per area in these tissues was not changed in these mice (Fig. 3*B*). Growth rate and total number of in vitro induced BMMCs from RhoH-deficient mice were comparable to those from RhoH$^{+/+}$ mice (data not shown). After 4 wk culture, the proportion of FcεRI and c-*kit* double positive cells was >95% in both RhoH$^{-/-}$ and RhoH$^{+/+}$ BMMCs (Fig. 3*C*). From these results, we conclude that RhoH is dispensable for mast cell development both in vivo and in vitro.

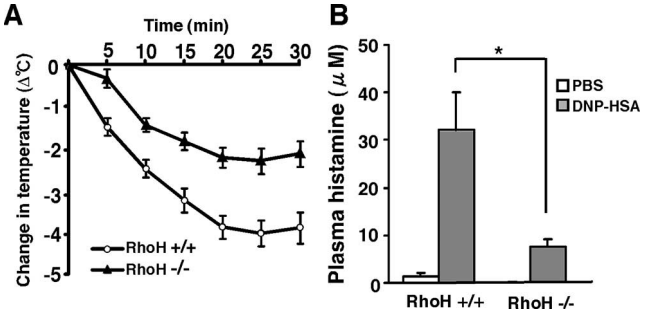

FIGURE 2. Impaired systemic anaphylaxis in RhoH$^{-/-}$ mice. *A*, RhoH$^{+/+}$ (○) and RhoH$^{-/-}$ (▲) mice were sensitized with anti-DNP IgE overnight, then challenged with DNP-HSA (100 μg), and the core body temperature was measured up to 30 min. Representative data of three independent experiments is shown. *B*, At 30 min after induction of PSA, peripheral blood was taken from RhoH$^{+/+}$ and RhoH$^{-/-}$ mice by cardiac puncture and the plasma histamine concentration was measured by EIA. Results are the mean and SE from three independent experiments. Statistical significance was determined by Welch's *t* test; *, $p < 0.05$. $n = 5$ control mice, $n = 6$ knockout mice.

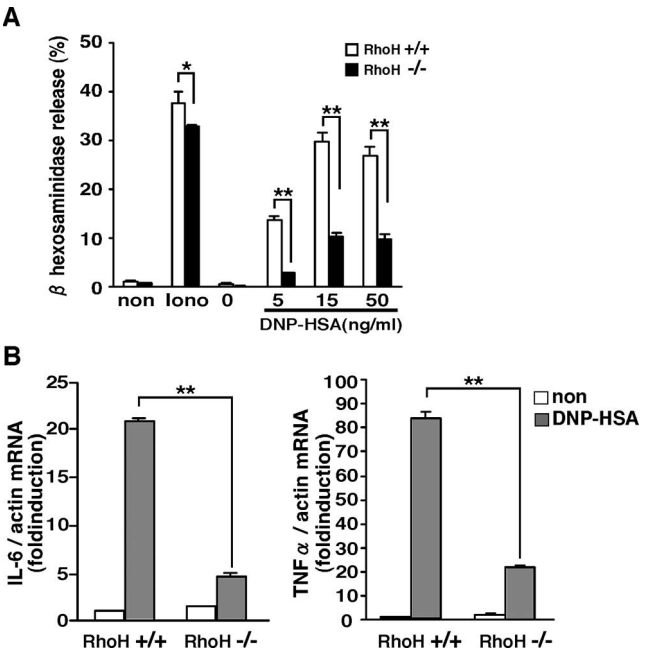

FIGURE 4. FcεRI-stimulated degranulation and cytokine production were impaired in RhoH$^{-/-}$ mast cells. *A*, BMMCs from RhoH$^{+/+}$ (□) and RhoH$^{-/-}$ (■) were sensitized with anti-DNP IgE, then 30 min after DNP-HSA (at the indicated concentrations) or A23187 (Iono, 200 ng/ml) stimulation, degranulation was assayed by β-hexosaminidase release. *B*, Anti-DNP IgE-sensitized BMMCs were stimulated with DNP-HSA (10 ng/ml) for 1 h or unstimulated. Then, IL-6 (*left*) and TNF-α (*right*) mRNA were quantified by real time RT-PCR. Results are the mean and SE from three independent experiments; $n = 9$ for each group. Statistical significance was determined by Student's *t* test; *, $p < 0.05$; **, $p < 0.01$.

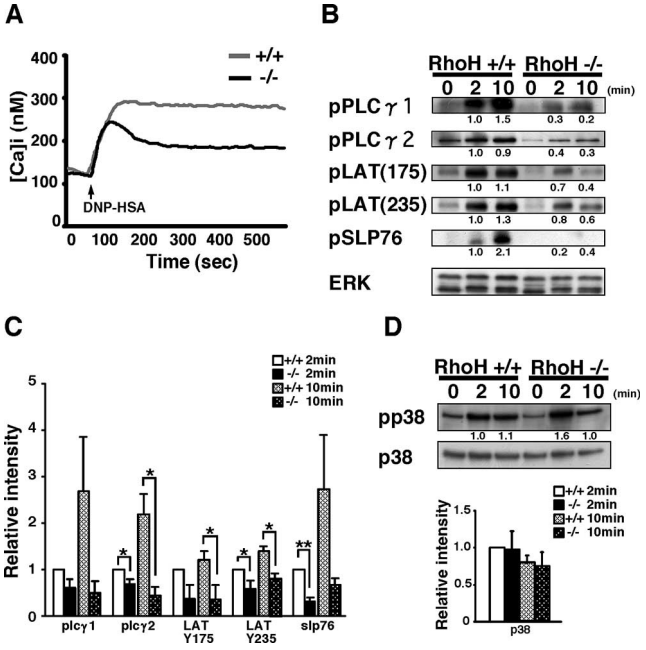

FIGURE 5. Defective FcεRI-mediated Ca^{2+} flux and adaptor phosphorylation in $RhoH^{-/-}$ mast cells. *A*, Ca^{2+} flux in $RhoH^{+/+}$ (gray line) and $RhoH^{-/-}$ (black line) BMMCs was monitored. Anti-DNP IgE sensitized BMMCs were stimulated with 100 ng/ml DNP-HSA at 70 s. Shown is representative data from two independent experiments, $n = 6$ for each group. *B*, Anti-DNP IgE sensitized BMMCs were stimulated with 10 ng/ml DNP-HSA for the indicated periods. Cell lysates were analyzed by Western blot using the indicated Abs. Numbers below the bands indicate the relative intensity of each band. Blotting for ERK was used for confirmation of equal loading to wells. Data shown is representation of three independent experiments. *C*, The bar graph represents band intensities from (*B*) relative to 2 min stimulated $RhoH^{+/+}$ in each lane. *D*, Activation of p38 in $RhoH^{-/-}$ BMMCs (*upper*). Shown is representative data from at least three independent experiments. Relative intensity of each sample is shown as in *C* (*lower*) with the mean and SE for the bar graph. Statistical significance was determined by paired *t* test; ∗, $p < 0.05$; ∗∗, $p < 0.01$.

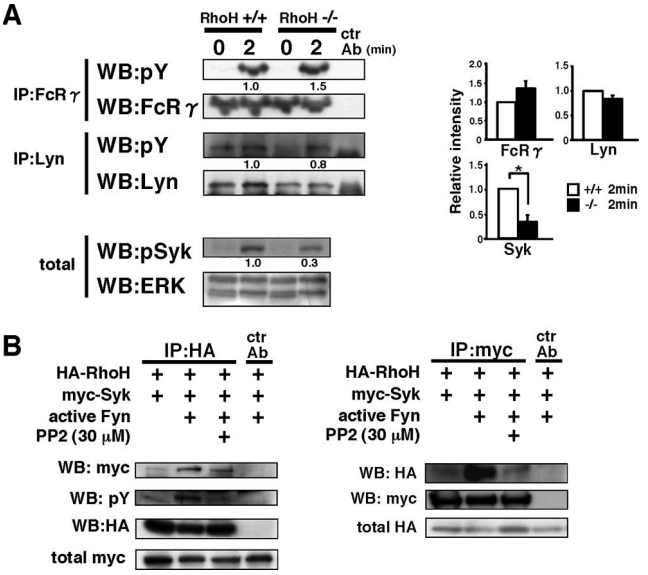

FIGURE 6. RhoH associates with Syk and regulates its activation. *A*, Anti-DNP IgE sensitized BMMCs were stimulated with 5 μg/ml anti-FcεRI for the indicated periods. Cell lysates were immunoprecipitated with anti-FcRγ Ab (*left upper*) or -Lyn Ab (*left middle*) or control Abs (ctr Ab) and analyzed by Western blotting using indicated Abs. The total lysates were analyzed by Western blotting using anti-phosphoSyk and -ERK Abs (*left lower*). The relative intensity to 2 min stimulated $RhoH^{+/+}$ BMMCs in each lane is shown in the right panel. Shown is representative data from three independent experiments and the mean and SE in the bar graph. Statistical significance was determined by paired *t* test; ∗, $p < 0.05$. *B*, 293T cells were transiently transfected with the indicated expression vectors. After 24 h, cells were incubated with or without PP2, lysed, and immunoprecipitated with anti-HA (*left*) or anti-myc (*right*) or control Abs (ctr Ab) followed by Western blotting with the indicated Abs. Total lysates were analyzed by Western blotting using anti-myc (*left*) or anti-HA Ab (*right*) to confirm the expression of myc-Syk or HA-RhoH, respectively.

FcεRI-mediated degranulation and cytokine production by $RhoH^{-/-}$ mast cells

The reduction in PSA without obvious developmental defects of mast cells in $RhoH^{-/-}$ mice indicated a functional deficiency in $RhoH^{-/-}$ mast cells. Therefore, we analyzed FcεRI-mediated degranulation of BMMCs from $RhoH^{-/-}$ mice in vitro. BMMCs were stimulated with varying doses of Ag, and degranulation was measured by the release of β-hexosaminidase, an enzyme found in mast cell granules. As shown in Fig. 4*A*, degranulation of RhoH-deficient BMMCs in response to Ag/IgE Ab-mediated cross-linking was significantly reduced.

Total β-hexosaminidase content from $RhoH^{+/+}$ and $RhoH^{-/-}$ BMMCs were comparable (data not shown), suggesting that RhoH is dispensable for granule biogenesis in BMMCs. We next measured gene expression of IL-6 and TNF-α (26), well-established targets of FcεRI signaling in mast cells. We stimulated $RhoH^{+/+}$ and $RhoH^{-/-}$ BMMCs for 1 h and then measured mRNA expression levels of IL-6 and TNF-α by real time RT-PCR. Expression of IL-6 and TNF-α in BMMCs was severely inhibited in the absence of RhoH (Fig. 4*B*).

Impaired Ca^{2+} influx and adapter phosphorylation in $RhoH^{-/-}$ mast cells

Engagement of FcεRI results in tyrosine phosphorylation of kinases and adaptors, and then an increase in intracellular Ca^{2+} concentration (27, 28). To explore the role of RhoH in FcεRI-dependent signal transduction, we first analyzed Ca^{2+} mobilization in RhoH-deficient BMMCs. As shown in Fig. 5*A*, Ca^{2+} concentration reached the maximum level within 150 s in cells from $RhoH^{+/+}$ and $RhoH^{-/-}$ mice after Ag challenge. The maximal Ca^{2+} concentration after the Ag challenge was, however, far less in RhoH-deficient BMMCs, indicating that the signaling defect in $RhoH^{-/-}$ cells lies upstream of Ca^{2+} mobilization. Indeed, phosphorylation of PLCγ1 and PLCγ2, which is essential for FcεRI-dependent Ca^{2+} mobilization, was significantly reduced in $RhoH^{-/-}$ BMMCs (Fig. 5, *B* and *C*). Because phosphorylation of the LAT adaptor is required for the activation of PLCγ1 and 2, we next investigated the phosphorylation status of LAT and SLP76 upon stimulation. As shown in Fig. 5, *B* and *C*, FcεRI-induced phosphorylation of both LAT (Y175 and Y235) and SLP76 were significantly inhibited. Conversely, the activation of p38 upon FcεRI engagement in RhoH BMMCs was comparable to that of $RhoH^{+/+}$ BMMCs (Fig. 5*D*).

RhoH associates with Syk and regulates its activation

These results imply the signal transduction upstream of these adaptors is defective in RhoH-deficient mast cells. The adaptors LAT and SLP-76 are phosphorylated by the kinase Syk upon antigenic stimulation (29), therefore we next examined the activation of Syk. We found that stimulation-dependent phosphorylation of Tyr 519/520 residues of Syk, a known determinant of its kinase activity, was severely reduced in RhoH-deficient BMMCs (Fig. 6*A*).

Therefore, we conclude that FcεRI-induced activation of Syk is dependent on RhoH, which is essential for the phosphorylation of LAT and SLP-76 adaptors as well as PLC phosphorylation and Ca^{2+} mobilization in mast cells.

Syk binds to phosphorylated tyrosines in the ITAM motif of the γ-subunit of FcεRI, and is phosphorylated by Lyn or by itself. We therefore examined phosphorylation of FcRγ and Lyn, which are the earliest events after FcεRI engagement and required for mast cells activation (30). As shown in Fig. 6*A*, phosphorylation of FcRγ and Lyn in $RhoH^{-/-}$ BMMCs was comparable to that in $RhoH^{+/+}$. These results indicate that cross-linking and activation of immediate downstream molecules of FcεRI occur normally in the absence of RhoH, and we concluded that the impaired phosphorylation of Syk is responsible for the defective FcεRI-dependent signal transduction in $RhoH^{-/-}$ BMMCs.

RhoH has been reported to associate with ZAP-70 in T cells (7). Because Syk and ZAP-70 belong to the same family and are functionally analogous, we hypothesized that RhoH might associate with Syk and act as an adaptor for Syk. To test this hypothesis, we coexpressed HA-tagged RhoH, myc-tagged Syk, and constitutively active Fyn in 293T cells and performed coimmunoprecipitation of RhoH and Syk. We found that RhoH associated with Syk and this association was enhanced by constitutively active Fyn (Fig. 6*B*). Concomitantly, RhoH was phosphorylated in the presence of active Fyn. Treatment of cells with PP2, an inhibitor of src family kinases, inhibited the phosphorylation of RhoH and the interaction between RhoH and Syk. Because FcεRI ligation activates Fyn in mast cells, RhoH would be able to interact with Syk more strongly, and therefore RhoH may function as an adaptor molecule for Syk in mast cells in a way analogous to that in T cells. This possibility may provide an explanation for the defective FcεRI signaling in $RhoH^{-/-}$ mast cells.

Discussion

Mast cells play important roles in initial immunological responses as well as in the clearance of parasitic infections (9, 10, 31, 32). One of the major functions of mast cells is the immediate release of histamine and other inflammatory mediators from their intracellular granules to increase vascular permeability upon antigenic cross-linking of surface FcεRI (33). In the present study, we demonstrated that the hematopoietic lineage specific atypical small GTPase RhoH, plays critical roles in mast cell function by facilitating phosphorylation of Syk in FcεRI-dependent signal transduction.

We observed impaired degranulation and cytokine production in RhoH deficient mast cells (Fig. 4) without obvious developmental defects of mast cells (Fig. 3). This result is consistent with the fact that FcεRI-dependent signaling is not required for mast cell development. As a matter of fact, the mast cell-related phenotype in $RhoH^{-/-}$ mice is similar to the one in mice deficient for Syk (16), LAT (34), SLP76 (35, 36), Fyn (18), or Btk (37, 38), which are all involved in FcεRI-related signaling. We observed both impaired Ca^{2+} influx and phosphorylation of PLCγ1 and 2, LAT (Y175, Y235), and SLP-76 (Y128) upon FcεRI stimulation in mast cells (Fig. 5). We saw a slight but reproducible decrease in ionomycin -induced degranulation in $RhoH^{-/-}$ BMMCs (Fig. 4*A*). Interestingly, a similar phenotype was seen in SLP76 mutant BMMCs, in which the calcium influx upon ionomycin treatment was lower than that of wild-type BMMCs (36). This might be because adaptor molecules like RhoH and SLP76 can affect the signaling events downstream of calcium release from the ER, or might indicate the existence for a possible positive feedback pathway from calcium signaling to the upstream of adaptor molecules.

RhoH was initially reported as an antagonist for the Rac1/RhoA/cdc42-dependent activation of NF-κB and p38 (3, 4), and has also been proposed to repress LFA-1 activation (5). More recently, RhoH has been reported to act as an adaptor for ZAP-70 by associating with ZAP-70 via its ITAM-like motif, and its association is enhanced upon TCR stimulation (7). ZAP-70 is not expressed in mast cells, but instead, Syk, which belongs to the same kinase family as ZAP-70, is expressed and plays essential roles in FcεRI signaling (16). In this study, we demonstrated that RhoH associates with Syk, and phosphorylated RhoH can interact with Syk more efficiently (Fig. 6*B*). Indeed, the reduced number of DP thymocytes in $RhoH^{-/-}$ mice cannot be explained by the functional defect of ZAP-70, because $ZAP\text{-}70^{-/-}$ mice showed normal β-selection (39). However, it is known that Syk and ZAP-70 have redundant roles in β-selection during T cell development (40). Therefore, our results showing that RhoH associates with ZAP-70 and Syk explain the impairment of β-selection in $RhoH^{-/-}$ mice. Considering the fact that Syk is critical in B cell development and BCR-dependent activation of B cells (41, 42), it is surprising that $RhoH^{-/-}$ mice showed normal B cell development and activation (data not shown). It is possible that B cells have another molecule having similar functions to RhoH, or the requirement for recruiting Syk to the membrane proximal could be different between BCR- and FcεRI-dependent signal transduction.

RhoH was reported to inhibit SDF-dependent activation of Rac1 in hematopoietic progenitor cells, and this was probably due to the inhibition of membrane targeting of Rac1 by RhoH (43). Therefore, the activity of Rac1 could be increased in the absence of RhoH. Indeed, Dorn et al. (6) showed that the PAK-binding activity of Rac1 in $RhoH^{-/-}$ T cells was increased without TCR stimulation. We did not observe enhanced phosphorylation of p38, a major downstream molecule of Rac1 in the absence or presence of FcεRI stimulation in $RhoH^{-/-}$ BMMCs (Fig. 5*D*), indicating that Rac1 is not over-activated in $RhoH^{-/-}$ mast cells. Precise function of RhoH on Rac1 regulation in mast cells should be elucidated in further studies.

Two independent groups have reported phenotypes of RhoH knockout mice (6, 7), and both of them showed defective T cell development and activation. Although both groups reported impaired phosphorylation of LAT (Y195) upon TCR stimulation, there is a discrepancy in the phosphorylation status of ZAP-70 (Y319) between the two groups. Dorn et al. (6) reported unaltered phosphorylation of ZAP-70, whereas Gu et al. (7) showed severely impaired ZAP-70 phosphorylation. Consequently, the former group hypothesized that RhoH is important for interaction between activated ZAP-70 and its substrate LAT, whereas the latter group hypothesized that RhoH is important for recruiting ZAP-70 to membrane proximal before activation. We observed impaired Syk activation upon FcεRI stimulation, evaluated by phosphorylation of Y519/520 in $RhoH^{-/-}$ mast cells (Fig. 6*A*), therefore, our results in mast cells are more consistent with the latter model in T cells. We also showed that RhoH and Syk can associate with each other without stimulation albeit very weakly, and the association was greatly enhanced by activation (Fig. 6*B*). From these results, we hypothesize that phosphorylated RhoH can associate strongly with Syk to keep Syk molecules at membrane proximal, thus facilitating effective activation of Syk. We tried to prove RhoH-dependent Syk recruitment by immunoprecipitaion and membrane subfractionation experiments. However, we were unable to detect membrane recruited Syk, possibly due to the fact that it is such a small proportion of total Syk (44). Future studies should clarify the function of RhoH in the recruitment of endogenous Syk in mast cells.

In the present study, we demonstrated that RhoH plays an important role in FcεRI-mediated activation of mast cells. RhoH$^{-/-}$ mice exhibited reduced systemic anaphylaxis in vivo, and RhoH$^{-/-}$ mast cells failed to degranulate and produce cytokines upon FcεRI stimulation in vitro. Because FcεRI-induced activation of Syk is dependent on RhoH, downstream events including phosphorylation of LAT, PLCγ1 and 2, and SLP-76 as well as Ca^{2+} mobilization in mast cells were all dependent on RhoH. RhoH associated with Syk when exogenously introduced, therefore it is possible that RhoH functions as an adaptor for Syk in mast cells, in a way analogous to its interaction with ZAP-70 in T cells (7). Collectively, these results indicate that RhoH positively regulates FcεRI signal transduction in mast cells. Because Syk is expressed in many kinds of hematopoietic lineage cells and involved in various ITAM-mediated signal transduction pathways (45), our current finding that RhoH facilitates Syk activation will shed new light on ITAM-based immune responses.

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Disclosures

The authors have no financial conflict of interest.

References

1. Dallery, E., S. Galiegue-Zouitina, M. Collyn-d'Hooghe, S. Quief, C. Denis, M. P. Hildebrand, D. Lantoine, C. Deweindt, H. Tilly, C. Bastard, et al. 1995. TTF, a gene encoding a novel small G protein, fuses to the lymphoma-associated LAZ3 gene by t(3;4) chromosomal translocation. *Oncogene* 10: 2171–2178.
2. Aspenstrom, P., A. Ruusala, and D. Pacholsky. 2007. Taking Rho GTPases to the next level: the cellular functions of atypical Rho GTPases. *Exp. Cell Res.* 313: 3673–3679.
3. Li, X., X. Bu, B. Lu, H. Avraham, R. A. Flavell, and B. Lim. 2002. The hematopoiesis-specific GTP-binding protein RhoH is GTPase deficient and modulates activities of other Rho GTPases by an inhibitory function. *Mol. Cell Biol.* 22: 1158–1171.
4. Gu, Y., A. C. Jasti, M. Jansen, and J. E. Siefring. 2005. RhoH, a hematopoietic-specific Rho GTPase, regulates proliferation, survival, migration, and engraftment of hematopoietic progenitor cells. *Blood* 105: 1467–1475.
5. Cherry, L. K., X. Li, P. Schwab, B. Lim, and L. B. Klickstein. 2004. RhoH is required to maintain the integrin LFA-1 in a nonadhesive state on lymphocytes. *Nat. Immunol.* 5: 961–967.
6. Dorn, T., U. Kuhn, G. Bungartz, S. Stiller, M. Bauer, J. Ellwart, T. Peters, K. Scharffetter-Kochanek, M. Semmrich, M. Laschinger, et al. 2007. RhoH is important for positive thymocyte selection and T-cell receptor signaling. *Blood* 109: 2346–2355.
7. Gu, Y., H. D. Chae, J. E. Siefring, A. C. Jasti, D. A. Hildeman, and D. A. Williams. 2006. RhoH GTPase recruits and activates Zap70 required for T cell receptor signaling and thymocyte development. *Nat. Immunol.* 7: 1182–1190.
8. Underhill, D. M., and H. S. Goodridge. 2007. The many faces of ITAMs. *Trends Immunol.* 28: 66–73.
9. Bischoff, S. C. 2007. Role of mast cells in allergic and non-allergic immune responses: comparison of human and murine data. *Nat. Rev. Immunol.* 7: 93–104.
10. Wedemeyer, J., M. Tsai, and S. J. Galli. 2000. Roles of mast cells and basophils in innate and acquired immunity. *Curr. Opin. Immunol.* 12: 624–631.
11. Garman, S. C., J. P. Kinet, and T. S. Jardetzky. 1999. The crystal structure of the human high-affinity IgE receptor (FcεRIα). *Annu. Rev. Immunol.* 17: 973–976.
12. Kinet, J. P. 1999. The high-affinity IgE receptor (FcεRI): from physiology to pathology. *Annu. Rev. Immunol.* 17: 931–972.
13. Siraganian, R. P. 2003. Mast cell signal transduction from the high-affinity IgE receptor. *Curr. Opin. Immunol.* 15: 639–646.
14. Kraft, S., and J. P. Kinet. 2007. New developments in FcεRI regulation, function and inhibition. *Nat. Rev. Immunol.* 7: 365–378.
15. Gilfillan, A. M., and C. Tkaczyk. 2006. Integrated signalling pathways for mast-cell activation. *Nat. Rev. Immunol.* 6: 218–230.
16. Costello, P. S., M. Turner, A. E. Walters, C. N. Cunningham, P. H. Bauer, J. Downward, and V. L. Tybulewicz. 1996. Critical role for the tyrosine kinase Syk in signalling through the high affinity IgE receptor of mast cells. *Oncogene* 13: 2595–2605.
17. Nishizumi, H., and T. Yamamoto. 1997. Impaired tyrosine phosphorylation and Ca^{2+} mobilization, but not degranulation, in lyn-deficient bone marrow-derived mast cells. *J. Immunol.* 158: 2350–2355.
18. Parravicini, V., M. Gadina, M. Kovarova, S. Odom, C. Gonzalez-Espinosa, Y. Furumoto, S. Saitoh, L. E. Samelson, J. J. O'Shea, and J. Rivera. 2002. Fyn kinase initiates complementary signals required for IgE-dependent mast cell degranulation. *Nat. Immunol.* 3: 741–748.
19. Siraganian, R. P., J. Zhang, K. Suzuki, and K. Sada. 2002. Protein tyrosine kinase Syk in mast cell signaling. *Mol. Immunol.* 38: 1229–1233.
20. Iwaki, S., J. Spicka, C. Tkaczyk, B. M. Jensen, Y. Furumoto, N. Charles, M. Kovarova, J. Rivera, V. Horejsi, D. D. Metcalfe, and A. M. Gilfillan. 2008. Kit- and FcεRI-induced differential phosphorylation of the transmembrane adaptor molecule NTAL/LAB/LAT2 allows flexibility in its scaffolding function in mast cells. *Cell Signal* 20: 195–205.
21. Rivera, J., R. Arudchandran, C. Gonzalez-Espinosa, T. S. Manetz, and S. Xirasagar. 2001. A perspective: regulation of IgE receptor-mediated mast cell responses by a LAT-organized plasma membrane-localized signaling complex. *Int. Arch. Allergy Immunol.* 124: 137–141.
22. Singer, A. L., and G. A. Koretzky. 2002. Control of T cell function by positive and negative regulators. *Science* 296: 1639–1640.
23. Murata, T., K. Furushima, M. Hirano, H. Kiyonari, M. Nakamura, Y. Suda, and S. Aizawa. 2004. ang is a novel gene expressed in early neuroectoderm, but its null mutant exhibits no obvious phenotype. *Gene Expr. Patterns* 5: 171–178.
24. Yagi, T., T. Tokunaga, Y. Furuta, S. Nada, M. Yoshida, T. Tsukada, Y. Saga, N. Takeda, Y. Ikawa, and S. Aizawa. 1993. A novel ES cell line, TT2, with high germline-differentiating potency. *Anal. Biochem.* 214: 70–76.
25. Yamasaki, S., E. Ishikawa, M. Kohno, and T. Saito. 2004. The quantity and duration of FcRγ signals determine mast cell degranulation and survival. *Blood* 103: 3093–3101.
26. Plaut, M., J. H. Pierce, C. J. Watson, J. Hanley-Hyde, R. P. Nordan, and W. E. Paul. 1989. Mast cell lines produce lymphokines in response to cross-linkage of FcεRI or to calcium ionophores. *Nature* 339: 64–67.
27. Eiseman, E., and J. B. Bolen. 1992. Engagement of the high-affinity IgE receptor activates src protein-related tyrosine kinases. *Nature* 355: 78–80.
28. Millard, P. J., T. A. Ryan, W. W. Webb, and C. Fewtrell. 1989. Immunoglobulin E receptor cross-linking induces oscillations in intracellular free ionized calcium in individual tumor mast cells. *J. Biol. Chem.* 264: 19730–19739.
29. Koretzky, G. A., F. Abtahian, and M. A. Silverman. 2006. SLP76 and SLP65: complex regulation of signalling in lymphocytes and beyond. *Nat. Rev. Immunol.* 6: 67–78.
30. Nadler, M. J., S. A. Matthews, H. Turner, and J. P. Kinet. 2000. Signal transduction by the high-affinity immunoglobulin E receptor FcεRI: coupling form to function. *Adv. Immunol.* 76: 325–355.
31. Marshall, J. S. 2004. Mast-cell responses to pathogens. *Nat. Rev. Immunol.* 4: 787–799.
32. Abraham, S. N., and R. Malaviya. 1997. Mast cells in infection and immunology. *Infect. Immun.* 65: 3501–3508.
33. Hide, I., J. P. Bennett, A. Pizzey, G. Boonen, D. Bar-Sagi, B. D. Gomperts, and P. E. Tatham. 1993. Degranulation of individual mast cells in response to Ca^{2+} and guanine nucleotides: an all-or-none event. *J. Cell Biol.* 123: 585–593.
34. Saitoh, S., R. Arudchandran, T. S. Manetz, W. Zhang, C. L. Sommers, P. E. Love, J. Rivera, and L. E. Samelson. 2000. LAT is essential for FcεRI-mediated mast cell activation. *Immunity* 12: 525–535.
35. Pivniouk, V. I., T. R. Martin, J. M. Lu-Kuo, H. R. Katz, H. C. Oettgen, and R. S. Geha. 1999. SLP-76 deficiency impairs signaling via the high-affinity IgE receptor in mast cells. *J. Clin. Invest.* 103: 1737–1743.
36. Wu, J. N., M. S. Jordan, M. A. Silverman, E. J. Peterson, and G. A. Koretzky. 2004. Differential requirement for adapter proteins Src homology 2 domain-containing leukocyte phosphoprotein of 76 kDa and adhesion- and degranulation-promoting adapter protein in FcεRI signaling and mast cell function. *J. Immunol.* 172: 6768–6774.
37. Setoguchi, R., T. Kinashi, H. Sagara, K. Hirosawa, and K. Takatsu. 1998. Defective degranulation and calcium mobilization of bone-marrow derived mast cells from Xid and Btk-deficient mice. *Immunol. Lett.* 64: 109–118.
38. Kawakami, Y., J. Kitaura, A. B. Satterthwaite, R. M. Kato, K. Asai, S. E. Hartman, M. Maeda-Yamamoto, C. A. Lowell, D. J. Rawlings, O. N. Witte, and T. Kawakami. 2000. Redundant and opposing functions of two tyrosine kinases, Btk and Lyn, in mast cell activation. *J. Immunol.* 165: 1210–1219.
39. Negishi, I., N. Motoyama, K. Nakayama, K. Nakayama, S. Senju, S. Hatakeyama, Q. Zhang, A. C. Chan, and D. Y. Loh. 1995. Essential role for ZAP-70 in both positive and negative selection of thymocytes. *Nature* 376: 435–438.
40. Cheng, A. M., I. Negishi, S. J. Anderson, A. C. Chan, J. Bolen, D. Y. Loh, and T. Pawson. 1997. The Syk and ZAP-70 SH2-containing tyrosine kinases are implicated in pre-T cell receptor signaling. *Proc. Natl. Acad. Sci. USA* 94: 9797–9801.
41. Cheng, A. M., B. Rowley, W. Pao, A. Hayday, J. B. Bolen, and T. Pawson. 1995. Syk tyrosine kinase required for mouse viability and B-cell development. *Nature* 378: 303–306.
42. Turner, M., P. J. Mee, P. S. Costello, O. Williams, A. A. Price, L. P. Duddy, M. T. Furlong, R. L. Geahlen, and V. L. Tybulewicz. 1995. Perinatal lethality and blocked B-cell development in mice lacking the tyrosine kinase Syk. *Nature* 378: 298–302.
43. Chae, H. D., K. E. Lee, D. A. Williams, and Y. Gu. 2007. Cross-talk between RhoH and Rac1 in regulation of actin cytoskeleton and chemotaxis of hematopoietic progenitor cells. *Blood* 111: 2597–2605.
44. Wilson, B. S., J. R. Pfeiffer, Z. Surviladze, E. A. Gaudet, and J. M. Oliver. 2001. High resolution mapping of mast cell membranes reveals primary and secondary domains of FcεRI and LAT. *J. Cell Biol.* 154: 645–658.
45. Humphrey, M. B., L. L. Lanier, and M. C. Nakamura. 2005. Role of ITAM-containing adapter proteins and their receptors in the immune system and bone. *Immunol. Rev.* 208: 50–65.