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Gab2, via PI-3K, Regulates ARF1 in FcεRI-Mediated Granule Translocation and Mast Cell Degranulation

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Mast cells are major players in allergic responses. IgE-dependent activation through FcεR leads to degranulation and cytokine production, both of which require Gab2. To clarify how the signals diverge at Gab2, we established Gab2 knock-in mice that express Gab2 mutated at either the PI3K or SH2 domain-containing protein tyrosine phosphatase-2 (SHP2) binding sites. Examination of these mutants showed that both binding sites were required for the degranulation and anaphylaxis response but not for cytokine production or contact hypersensitivity. Furthermore, the PI3K, but not the SHP2, binding site was important for granule translocation during degranulation. We also identified a small GTPase, ADP-ribosylation factor (ARF)1, as the downstream target of PI3K that regulates granule translocation. FcεRI stimulation induced ARF1 activation, and this response was dependent on Fyn and the PI3K binding site of Gab2. ARF1 activity was required for FcεRI-mediated granule translocation. These data indicated that Fyn/Gab2/PI3K/ARF1-mediated signaling is specifically involved in granule translocation and the anaphylaxis response. The Journal of Immunology, 2011, 187: 932–941.

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Abbreviations used in this article: ARF, ADP-ribosylation factor; BMMC, bone marrow-derived mast cell; ES, embryonic stem; GAP, GTPase-activating protein; HSA, human serum albumin; KO, knockout; MS, mass spectrometry; MTM, membrane-translocating motif; NP-40, Nonidet P-40; PLCγ1, phospholipase C, γ 1; PVDF, polyvinylidene fluoride; SHP2, SH2 domain-containing protein tyrosine phosphatase-2; siRNA, small interfering RNA; WT, wild-type.

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(21). Their GTPase cycle is regulated by the guanine nucleotide exchange factors that activate ARFs and by the GTPase-activating proteins (GAPs) that deactivate them (22, 23). ARFs also regulate endoplasmic reticulum–Golgi trafficking pathways (24). Thus, ARF activity is essential for normal vesicle transport.

In this study, we found that both the PI3K and SHP2 binding sites of Gab2 were required for mast cell degranulation and the anaphylaxis response but not for cytokine production or contact hypersensitivity. We also showed that the PI3K binding site, but not the SHP2 binding site, was involved in FceRI-dependent ARF1 activation and granule translocation to the plasma membrane in the degranulation process, further dissecting the signals required for degranulation. Finally, we revealed the molecular framework of Fyn/Gab2/PI3K-dependent ARF1-mediated granule translocation and mast cell degranulation, as well as the anaphylaxis response.

Materials and Methods

Mice

We generated Gab2 knock-in mice by the insertion of cDNAs encoding aa 89–665 of either wild-type (WT) Gab2 (WT cDNA) or Gab2 mutants, by homologous recombination in embryonic stem (ES) cells, into exon 2 of the Gab2 locus. To construct the targeting vector, genomic fragments were isolated from a FvIII 129sv mouse strain genomic library. The knock-in targeting vector contained the Ncol and EcoRV genomic fragment of Gab2, which includes part of exon 2. The cDNA (codons 89–665) of the mutant or WT Gab2 was inserted into the EcoRV site in exon 2. The cDNAs also contained a poly (A) tail from a bovine growth hormone cDNA. The targeting vector was introduced into ES (B6 × 129+Ter/SvJcl) ES cell clones. Positive cell clones were identified by PCR and Southern hybridization using 5′ external probes (Supplemental Fig. 1A). ES cell clones with each mutation were microinjected into C57BL/6 blastocysts to generate chimeras and backcrossed with C57BL/6 animals. Germ-line transmission was verified by PCR. Animals were genotyped by PCR using the primers G2KI-commonS (S; Supplemental Fig. 1A) (5′-CATTTTCTC- TTCTTCCCAATG-3′ and 3′-9-AAGAAAGGTTCCTCAGGGAGTC-3′). The neomycin-resistant gene cassette was excised by crossing with the Cre deleter strain. Knock-in mice lacking the neomycin-resistance gene cassette were identified by routine PCR, using a combination of the primers.

Measurement of cytokines

IL-6 and TNF-α in cell-culture supernatants were measured with an ELISA kit (R&D Systems).

Passive systemic anaphylaxis

To induce passive anaphylaxis, mice were sensitized with an i.v. injection of 2 μg anti-DNP IgE mAb (SPE-7, Sigma); 12 h after the IgE injection, the mice were challenged i.v. with 100 μg DNP-BSA. The rectal temperature was measured with a digital thermometer (Shibaura Electronics).

RNA interference for ARF1

To knock down ARF1, BMMCs were transfected with ARF1 small interfering RNA (siRNA) or nontargeting siRNA (SMARTpool; Thermos Scientific) using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions.

Confocal microscopy and granule translocation

BMMCs were sensitized with 1 μg/ml IgE for 12 h. Before Ag stimulation, BMMCs were treated with Wortmannin (500 nM) or LY294002 (50 μM) for 2 h. The cells were then stimulated with 50 ng/ml DNP-human serum albumin (HSA) for 10 min at 37°C and then fixed and permeabilized with the Cytofix/Cytoperm Fixation/Permeabilization Solution Kit (BD Biosciences). The cells were attached to glass slides by cytofix (Thramo Shandon). Primary and secondary staining were performed on the slides using anti-ARF1 (Molecular Probes) and Alexa Fluor-conjugated anti-mouse IgG (Molecular Probes). Confocal microscopy was carried out using an Olympus Fluorescence Microscope System (Leica). The frequency of Ag-induced mast cell granule translocation was determined as described previously (11, 19). At least 90 independent GFP+ cells were counted for each experiment.

Retroviral transfection

Retrovirus transfection was performed as previously described (26). The CD63-GFP plasmid (a gift from M. Nakanishi, Aichi Gakuin University, Aichi, Japan) was cloned into the BamHI and NotI sites of the retroviral vector PMX (a gift from T. Kitamura, University of Tokyo, Tokyo, Japan). This construct was then used to transfect the 293T-based packaging cell line phoenix (a gift from G. Nolan, Stanford University, Stanford, CA) with Lipofectamine 2000 (Invitrogen) to generate recombinant retroviruses. Bone marrow cells were infected with the retrovirus in the presence of 10 μg/ml polybrene (Sigma-Aldrich) and IL-3.

Immunoblotting

BMMCs were sensitized with 1 μg/ml IgE for 12 h. The cells were then stimulated with 50 ng/ml DNP-HSA for 10 min at 37°C. Cell pellets were lysed on ice for 30 min in lysis buffer (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Nonidet P-40, 0.1% deoxycholate, 1 mM NaVO4, 3 mM EDTA, and protease inhibitor cocktail). The lysates were fractionated by electrophoresis on 5–20% acrylamide SDS gels and transferred onto a polyvinylidene difluoride (PVDF) membrane, and incubated with the appropriate Abs. The PVDF membranes were exposed to Fuji RX film (Fujifilm). Densitometric analysis was performed by LAS-1000 (Fujifilm).

Identification of proteins by peptide mass spectrometry

IgE-sensitized BMMCs were stimulated with DNP-HSA (50 ng/ml) for 15 min. The cells were fractionated into cytosolic and particulate membrane compartments (described in experimental procedures), and the lysates from particulate membrane compartments were applied to a zinc column (IMAC-Select Affinity Gel, Sigma-Aldrich). The samples were eluted by n-propanol (95%). The eluted samples were applied onto Ready Strip IPG Strips (7 cm, pH 4–7; Bio-Rad). The second dimension was performed on 10–20% polyacrylamide gels and transferred onto a Mini Problot membrane (Applied Biosystems), which was then stained with Colloidal Gold Total Protein Stain (Bio-Rad). The PVDF-immobilized proteins were reduced, S-carboxymethylated, and digested in situ with Achromobacter protease I (27). Molecular mass analyses were performed by MALDI-TOF mass spectrometry (MS) using an ABI PerSeptive Biosystem Voyager DERP (Applied Biosystems). Identification of the proteins was conducted by comparing the molecular mass determined by MALDI-TOF/MS with theoretical peptide masses from the proteins registered in NCBInr database.

Detection of GFP-bound ARF1

IgE-sensitized BMMCs were stimulated with DNP-HSA (50 ng/ml) and lysed (20 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% NP-40, 10% glycerol,
FIGURE 1. The PI3K or SHP2 binding sites of Gab2 are required for FcεRI-mediated mast cell degranulation but not cytokine production. A, IgE-sensitized BMMCs from Gab2−/−, Gab2WT/WT, Gab2ΔSHP2/ΔSHP2, and Gab2ΔPI3K/ΔPI3K knock-in mice, as well as from Gab2+/+ mice, were treated with DNP-HSA (Ag; 0, 6.25, 12.5, 25, or 50 ng/ml); degranulation was assayed by β-hexosaminidase release after 30 min of Ag stimulation. Three independent populations of BMMCs were tested in separate wells. Data are expressed as the mean ± SD. B, Production level of IL-6 in the cell-culture supernatant upon Ag stimulation (0, 5, or 10 ng/ml) for 3 h, measured by ELISA. Three independent populations of BMMCs were tested in separate wells. Data are expressed as the mean ± SD. C, Control (heterozygous Gab2 knock-in mice or Gab2+/+) and Gab2 knock-in mice were passively sensitized with anti-DNP IgE mAbs and then challenged with DNP-BSA. Systemic anaphylaxis was detected from the change in the rectal temperature over time after the allergen challenge. Data are expressed as the mean ± SD. (n = 4–6 mice per group.) Results in C show pooled data from two experiments. *p < 0.05, **p < 0.01; two-tailed Student t test.
10 mM MgCl₂, 2 mM DTT, and protease inhibitor cocktail). GTP-bound ARF1 was precipitated from the lysate with 4 μg a GST-bound peptide consisting of aa 1–226 of the ARF binding protein GGA3 and detected by immunoblotting with an anti-ARF1 Ab.

**Membrane fractionation**

Cell pellets were resuspended in 0.25 M sucrose and homogenized with 20 strokes of a 7-ml Dounce homogenizer. The lysates were centrifuged (10 min, 3300 rpm at 4°C). To monitor the translocation of ARF1 in BMMCs,

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**FIGURE 2.** PI3K is required for FcεRI-mediated granule translocation. A, Left panels, Gab2⁺/⁺, Gab2ΔSHP2ΔSHP2, or Gab2ΔPI3KΔPI3K knock-in–derived BMMCs transfected with CD63-GFP were sensitized for 12 h with IgE and then stimulated with DNP-HSA (Ag; 50 ng/ml) for 10 min. CD63-GFP (green) was visualized by confocal microscopy. Original magnification ×630. Right panel, The frequency of cells showing granule translocation to the plasma membrane was quantified. Data are expressed as the mean + SD and are representative of three experiments. *p < 0.05, **p < 0.01; two-tailed Student t test.

B, IgE-sensitized BMMCs from Gab2⁺/⁺ mice were not treated (vehicle) or were pretreated with wortmannin (500 nM) or LY294002 (50 μM) for 2 h. The cells were then stimulated with DNP-HSA (Ag; 0, 6.25, 12.5, 25, 50 ng/ml). Data are expressed as the mean + SD and are representative of three experiments. C, Left panels, BMMCs transfected with CD63-GFP were not treated (vehicle) or were pretreated with either wortmannin (500 nM) or LY294002 (50 μM) for 2 h. The cells were then stimulated with DNP-HSA (Ag; 50 ng/ml) for 10 min. CD63-GFP (green) was visualized by confocal microscopy. Original magnification ×630. Right panel, The frequency of cells showing granule translocation to the plasma membrane was quantified. Data are expressed as the mean + SD and are representative of three experiments. *p < 0.05, **p < 0.01; two-tailed Student t test.
the supernatant fractions containing the granules were subjected to ultracentrifugation at 100,000 × g for 60 min at 4˚C. The membrane pellets were resuspended in lysis buffer (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% NP-40, 1 mM NaVO₄, 3 mM EDTA, and protease inhibitor cocktail).

Statistical analysis
All data were analyzed with Statcel. Data were considered statistically significant when the p values were <0.05, obtained with a two-tailed t test.

Results
Establishment of Gab2 knock-in mutant mice
To determine how mutations at the PI3K binding sites (Y441/465/573) or SHP2 binding sites (Y603/632) of Gab2 would affect mast cell function, we inserted cDNAs encoding amino acids 89–665 of WT Gab2 (WT cDNA) or Gab2 mutants into exon 2 of the Gab2 locus by homologous recombination in ES cells (Supplemental Fig. 1A). The cDNAs encoded well-characterized Gab2 mutants: Gab2ΔSHP2 (Y603F/Y632F), Gab2ΔPI3K (Y441F/Y465F/Y573F), or Gab2WT (WT cDNA) (Supplemental Fig. 1B). The floxed neomycin-resistance cassette was excised in vivo by crossing with a Cre deleter strain. Heterozygous Gab2 knock-in mice were intercrossed to obtain homozygous mutant knock-in mice. We confirmed that the mice expressed WT Gab2 (Gab2 WT/WT), Gab2ΔPI3K/DΔPI3K, or Gab2ΔSHP2/DΔSHP2 by sequencing (data not shown). RT-PCR analysis showed that the expression level of Gab2 mRNA was comparable in the testis of Gab2WT/WT, Gab2ΔPI3K/DΔPI3K, Gab2ΔSHP2/DΔSHP2, and control mice (Supplemental Fig. 1C). The Gab2 protein levels were examined by Western blotting of lysates prepared from testes. In all of the knock-in animals, the Gab2 protein levels were 60–80% of the WT (+/+) level (Supplemental Fig. 1D). Gab2 knock-in mice were born according to Mendelian inheritance and appeared normal.

Next, we examined the phosphorylation of residues in the activation loop of Erk1/2 and Akt in Gab2 BMMCs derived from the knock-in mice. Erk1/2 phosphorylation was reduced in Gab2ΔSHP2/DΔSHP2-derived BMMCs compared with those from the other genotypes. pAkt activation was diminished in Gab2ΔPI3K/DΔPI3K-derived BMMCs (Supplemental Fig. 2). These results indicated that the interaction of SHP2 and PI3K with Gab2 was required for the full activation of the Erk/MAPK and PI3K/Akt pathways, respectively. All of the homozygous knock-in mice were viable, and breeding colonies of mutant strains were established.

The PI3K or SHP2 binding sites in Gab2 are important for mast cell degranulation and the anaphylaxis response but not for cytokine production or contact hypersensitivity
To understand the functional significance of the Gab2 mutations, we investigated mast cell degranulation, as well as cytokine. First,
we examined FcεRI-induced mast cell degranulation using a β-hexosaminidase–release assay. As shown in Fig. 1A, BMMCs from both Gab2ΔSH2/ΔSH2 and Gab2ΔPI3K/ΔPI3K mice showed a significant reduction in mast cell degranulation. Interestingly, although the Gab2-deficient BMMCs showed impaired FcεRI-induced cytokine production, these binding site mutations did not affect IL-6 production (Fig. 1B).

Consistent with these in vitro analyses, Gab2ΔSH2/ΔSH2 and Gab2ΔPI3K/ΔPI3K mice also showed impaired IgE-mediated in vivo immediate-type allergic reactions, such as the anaphylaxis response (Fig. 1C), but no impairment in the delayed-type allergic reactions, such as contact hypersensitivity (data not shown). Furthermore, sections stained with toluidine blue revealed normal numbers of mast cells in the skin, whereas the mucosal mast cells in the stomach were significantly reduced in both the Gab2ΔSH2/ΔSH2 and Gab2ΔPI3K/ΔPI3K mice (Supplemental Fig. 3A, 3B). In addition, we investigated mast cell morphology (Supplemental Fig. 3D), as well as the expression of mast cell developmental markers c-Kit and FcεRI (Supplemental Fig. 3C) and mast cell-specific protease mMCP5, mMCP6, and MC-CPA (Supplemental Fig. 3E). All of these analyses indicated that the BMMCs from Gab2 knock-in mice developed normally. Together, these data indicated that the PI3K or SHP2 binding sites of Gab2 are involved in mast cell degranulation and the anaphylaxis response but not in cytokine production or contact hypersensitivity.

**PI3K is involved in FcεRI-mediated granule translocation**

We previously showed that Gab2 is required for FcεRI-induced granule translocation (11). Therefore, we examined whether the mutation at Y603/632 (SHP2 binding sites) or Y441/465/573 (PI3K binding sites) of Gab2 affected granule translocation. As shown in Fig. 2A, the control and Gab2ΔSH2/ΔSH2 BMMCs from Gab2 knock-in mice showed normal granule translocation, whereas this process was inhibited in the BMMCs from Gab2ΔPI3K/ΔPI3K mice, suggesting that PI3K is involved in granule translocation. We used pharmacological inhibitors of PI3K to confirm this idea. Mast cells treated with wortmannin or LY294002 showed significantly decreased degranulation and granule translocation (Fig. 2B, 2C), indicating that Gab2 and PI3K are required for FcεRI-induced granule translocation but that the SHP2 binding site is not.

**FIGURE 4.** ARF1 activation is involved in FcεRI-mediated mast cell degranulation. A, Left panels, BMMCs were not treated or were treated with ARF1 peptide inhibitor (MTM-ARF1; 100 μg/ml) for 30 min and stimulated with DNP-HSA (Ag; 50 ng/ml) for the indicated times. The presence of GTP-bound ARF1 was determined by precipitation assay using GST-GGA3. The lower blot represents the total amount of ARF1 GTPase in cell lysates. Results are representative of three experiments. Right panel, ARF1 activity was quantified densitometrically. Data are expressed as the mean ± SD. B, IgE-sensitized BMMCs were treated with MTM-ARF1 for 15 min; after 30 min of Ag stimulation (DNP-HSA; 50 ng/ml), degranulation was assayed by β-hexosaminidase release. Results are representative of three experiments. C, BMMCs were treated with MTM-ARF1 (100 μg/ml) for 15 min. The production levels of IL-6 in the cell culture supernatant upon Ag stimulation (DNP-HSA; 50 ng/ml) for 3 or 6 h were measured by ELISA. Results are representative of three experiments. D, Mice were sensitized with IgE injected into the ear and then challenged with Ag (DNP-BSA) and Evans blue i.v. for 30 min. Before Ag challenge, mice were injected i.v. with MTM-ARF1 (AAVLLPVLLAAP-GNIFANLFKGLFGKKE) or MTM-control (AAVLLPVLLAAP-LFGKKE) peptide. The amount of extravasated Evans blue dye in both ears was determined by extracting the dye and measuring the OD of the extract at 620 nm. Data are expressed as the mean ± SD (n = 4 mice per group). E, IgE-sensitized BMMCs were pretreated with RNA interference for ARF1 and then incubated with or without Ag (0, 6.25, 12.5, 25, 50 ng/ml) stimulation for 30 min, after which the degranulation was assayed by β-hexosaminidase release. Results are presented as the mean ± SD of triplicate wells and are representative of three experiments. Results in D show pooled data from two experiments. **p < 0.01, two-tailed Student t test.
Identification of molecules downstream of PI3K in mast cells

We previously showed that zinc is involved in FcεRI-mediated granule translocation (19). To further characterize the zinc-dependent granule-translocation mechanism(s), we developed a screen for zinc-associated molecules in mast cells. Our strategy is summarized in Supplemental Fig. 4A. We first analyzed the proteins binding to a zinc column by isoelectric focusing and SDS-PAGE. As shown in Supplemental Fig. 4B, we obtained numerous spots from the particulate (membrane) compartment fraction of BMMCs, and found that one spot with a molecular mass of 21,000 (Mr 21K; p21) was enhanced by FceRI stimulation.

To identify p21, we performed MALDI-TOF/MS analysis and identified the protein as ARF1 (see Fig. 3A for an alignment of ARF family members [class I]). Stimulation of the mast cell FcεRI induced ARF1 activation in a time-dependent manner (Fig. 3B). Based on these results, we hypothesized that ARF1 activation is regulated by PI3K. In support of this hypothesis, we found that ARF1 activation was significantly decreased in wortmannin-treated BMMCs (Fig. 3C). These findings strongly suggested that ARF1 activity is regulated by PI3K and that ARF1 is a novel downstream target of PI3K in the FcεRI-mediated signaling pathway.

ARF1 activity is required for mast cell degranulation but not for cytokine production

To determine whether ARF1 activity is involved in FcεRI-dependent mast cell function, we used cell-permeable peptides to disrupt the protein–protein interactions between ARF1 and its potential effectors. We generated cell-permeable peptides containing a membrane-translocating motif (MTM) sequence (28) and the N-terminal portion of ARF1. This sequence was previously shown to inhibit ARF1 activity (29). We found that this fusion peptide selectively prevented ARF1 activation in response to FcεRI stimulation.
peptide (AAVLLPVALID-GNIFANLFKGLFGKKE) was efficiently delivered into BMMCs, whereas the ARF1 terminus alone (GNIFANLFKGLFGKKE) was not (data not shown).

Next, we examined whether the MTM-ARF1 peptide would inhibit FcεRI-induced activation of ARF1. As shown in Fig. 4A, MTM-ARF1 decreased FcεRI-induced activity of endogenous ARF1. MTM-ARF1–treated mast cells also showed reduced FcεRI-induced degranulation (Fig. 4B). However, MTM-ARF1 had no effect on FcεRI-induced cytokine production (Fig. 4C). Furthermore, MTM-ARF1 decreased the in vivo anaphylactic response, whereas MTM-control had no effect (Fig. 4D).

We also examined whether the knockdown of ARF1 blocked FcεRI-mediated mast cell degranulation. For this, we used ARF1 siRNA, which blocked ARF1 protein expression (data not shown). As expected, FcεRI-induced mast cell degranulation was significantly inhibited in ARF1 siRNA-transfected BMMCs (Fig. 4E). Collectively, these data indicated that ARF1 activity is required for mast cell degranulation in vivo and in vitro.

**ARF1 activity is required for FcεRI-mediated granule translocation and is regulated by the Fyn/Gab2 pathway.**

Finally, we examined whether ARF1 activity was involved in FcεRI-induced granule translocation. As shown in Fig. 5A, BMMCs treated with MTM-ARF1 showed a significant reduction in granule translocation, and, as described above, the Fyn/Gab2 pathway is required for granule translocation. Given this finding, we hypothesized that the Fyn/Gab2 pathway might regulate ARF1 activity. To investigate this possibility, we used Fyn- and Gab2-deficient BMMCs derived from their respective knockout (KO) mice and investigated their ARF1 activity. As shown in Fig. 5B, Fyn- and Gab2-deficient BMMCs showed decreased FcεRI-induced ARF1 activity. Furthermore, ARF1 activation was significantly reduced in BMMCs derived from Gab2ΔPI3K/ΔSHP2 mice but not from Gab2ΔSHP2/ΔSHP2 mice (Fig. 5C, data not shown). These results indicated that the Fyn/Gab2/PI3K signaling pathway is involved in ARF1 activation.

Next, to elucidate the role of ARF1 in granule translocation, we examined the subcellular localization of ARF1 in mast cells. BMMCs were fractionated into cytosolic and particulate membrane compartments and then immunoblotted with an ARF1 Ab. A time-dependent translocation of ARF1 from the cytosol to the membrane compartment was observed upon FcεRI stimulation (Fig. 6A). Furthermore, we investigated the colocalization of ARF1 and a granule membrane marker, CD63, by confocal microscopy. As shown in Fig. 6B, after FcεRI stimulation, a fraction of the ARF1 translocated to the granule membrane, where it colocalized with CD63. These results suggested that ARF1 and CD63 interact in the granule membrane in mast cells. Collectively, our results showed that the Fyn/Gab2/PI3K/ARF1–mediated signaling pathway was required for granule translocation in mast cells and that ARF1 activity was regulated by the Fyn/Gab2/PI3K/ARF1 signaling pathway involved in granule translocation and mast cell degranulation. We hypothesized that another Gab2 motif, such as the binding site for CrkL or Grb2, is involved in promoting cytokine production. Furthermore, we showed that the PI3K and SHP2 binding sites of Gab2 are required for the IgE-mediated anaphylaxis response but not for contact hypersensitivity.

Recently, much evidence has accumulated showing that mast cells play crucial roles in infection and chronic inflammatory diseases, including arthritis, experimental allergic encephalomyelitis, colitis, and sepsis (30–34). In chronic inflammatory diseases, a variety of mast cell–derived mediators, including protease,
cytokines, and chemokines, are likely to play critical roles. For instance, studies using the K/BxN mouse serum-transfer arthritis model confirmed that mast cells are required in chronic inflammatory diseases (30) and that mast cell-derived chemical mediators, such as proteases, contribute to its pathology (35). In addition, when atherosclerosis-prone mice deficient in the low-density lipoprotein receptor are crossed with mast cell-deficient C57BL/6-Ki<sup>W-sh/Sh</sup> mice, their progeny have a much lower incidence of atherosclerosis, indicating that mast cells can contribute to this disease. Furthermore, the formation of aneurysms in this atherosclerosis model requires mast cell-derived cytokines, such as IL-6 and IFN-γ (36).

In this study, we used Gab2 knock-in mice to show that the PI3K and SHP2 binding sites of Gab2 are specifically required for mast cell degranulation, but not cytokine production. In addition, we and another group reported that Znt5, Bcl-10, and Malt-1 are specifically required for cytokine production by mast cells but not for their degranulation (37, 38). These mice should be valuable models for further dissecting the in vivo roles of mast cells in immune and biological responses, as well as for analyzing the pathological process of a variety of immune-related diseases.

**PI3K regulates FceRI-mediated granule translocation**

In this study, we found that PI3K is required for FceRI-mediated granule translocation and mast cell degranulation. The PI3Ks are lipid kinases that catalyze the conversion of phosphatidylinositol 4,5-bisphosphate to phosphatidylinositol-3,4,5-trisphosphate. Class I<sub>α</sub> PI3K, which consists of regulatory and catalytic subunits, is involved in multiple signal-transduction cascades that regulate cell proliferation, survival, and differentiation (39). Various receptor-signaling pathways, including those mediated by KitL and FceRI, can activate the class I<sub>α</sub> PI3K in mast cells (40–43). Many investigators, including us, showed that the treatment of mast cells with the PI3K inhibitors wortmannin or LY294002 suppresses FceRI-mediated mast cell degranulation, suggesting that PI3K is required for this process (44–46). However, the precise role of class I<sub>α</sub> PI3K in mast cell degranulation has been unclear.

In this study, we showed that the PI3K binding sites, but not the SHP2 binding sites, of Gab2 are required for granule translocation, although both binding sites are important for mast cell degranulation. We dissected the degranulation process of mast cells into its component steps (11). The first is granule translocation, and the second is a plasma-granule membrane-fusion step. We postulated that the PI3K binding site of Gab2 regulates the granule-translocation step and that the SHP2 binding site contributes to plasma-granule membrane fusion. Furthermore, we identified the small GTPase ARF1 as a downstream target molecule of PI3K. ARF1 is required for vesicular trafficking (47). In addition, involvement of ARF1 in mast cell degranulation was reported using RBL-2H3 cells (48). Cockcroft et al. (48) showed that ARF1 regulates mast cell degranulation through phospholipase D activation. However, it is still unclear how ARF1 regulates the exocytotic machinery. Using siRNA and a peptide inhibitor, we showed that ARF1 is involved in FceRI-mediated granule translocation and mast cell degranulation, suggesting that PI3K and ARF1 regulate mast cell exocytotic events.

In addition, we found that phosphorylation of PLC-γ1 and calcium mobilization occurred normally in BMMCs from both Gab2<sup>−/−</sup>/SHP2<sup>−/−</sup> and Gab2<sup>−/−</sup>/PI3K<sup>−/−</sup>/ARF1<sup>−/−</sup> mice (data not shown). However, we showed that the PI3K binding site, but not the SHP2 binding site, of Gab2 is required for ARF1 activation (Fig. 5C, data not shown). Based on this observation, we hypothesized that a Gab2-dependent PI3K-signaling pathway controls ARF1 activation. Furthermore, this PI3K-mediated cascade is important for granule translocation and mast cell degranulation. Using Fyn-KO and Gab2-KO-derived mast cells, we showed that ARF1 activity is regulated by Fyn and Gab2; it was reported previously that these molecules are required for the regulation of PI3K in mast cells (4, 10). Taken together, all of these observations suggested that the Fyn/Gab2/PI3K/ARF1 signaling pathway is required for FceRI-mediated granule translocation (Fig. 7).

**The small GTPase ARF1 is a novel player in mast cell degranulation**

In this study, we found that ARF1 is required for FceRI-mediated mast cell degranulation but not cytokine production. Because we previously showed that zinc is involved in granule translocation and mast cell degranulation, we sought to identify the zinc-associated molecule(s) that regulate granule translocation by using a zinc column. We identified ARF1 in this study, although we note that ARF1 does not contain a zinc binding domain. However, ARF1 regulators, such as ARF GAP, do have this motif. We speculated that zinc regulates ARF1 activation by indirectly binding ARF1 through ARF GAP. Further biochemical analyses will clarify the mechanisms by which ARF1 elicits zinc-dependent granule translocation in mast cells.

The ARF proteins are ubiquitously expressed and highly conserved in all eukaryotes, from yeast to humans. Using yeast genetics, it was demonstrated that ARF is essential for the endoplasmic reticulum–Golgi transport step (49). However, the role of ARF in immune cells, such as mast cells, is still not well understood. In this study, we demonstrated that ARF1 is involved in FceRI-mediated exocytotic events, such as granule translocation. Furthermore, ARF1 activity is required for mast cell degranulation and the IgE-mediated anaphylaxis response. Thus, our results identified ARF1 as a novel player in FceRI-induced mast cell degranulation through granule translocation.

How does ARF1 regulate this process? We observed that FceRI stimulation can induce ARF1’s relocation to granule membranes (Fig. 6B). ARF1 is also involved in regulating the interaction of ARF GAP with the motor protein kinesin (50), raising the possibility that ARF1 promotes granule translocation by recruiting this motor protein from the cytoplasmic region to the granule membrane.

In summary, our results demonstrated a critical role for Gab2 and PI3K in FceRI-mediated granule translocation, mast cell degranulation, and anaphylaxis responses but not in cytokine production or contact hypersensitivity, in vivo and in vitro. Furthermore, we provided a mechanistic basis for this response. We showed that Gab2 is critical for PI3K-signaling pathways to modulate ARF1 activation. Our identification of the FceRI-mediated Fyn/Gab2/PI3K/ARF1-signaling axis is likely to have more general implications, such as in Fcγ- and T cell Ag receptor-mediated signaling pathways.

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**Disclosures**

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