

Rab5a Regulates Surface Expression of FcεRI and Functional Activation in Mast Cells

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Surface expression levels of high-affinity immunoglobulin E (IgE) receptors (FcεRI) on mast cells are regulated by constitutive internalization from the plasma membrane, which is thought to be an important determinant of FcεRI-mediated signaling potential. However, molecular mechanism of FcεRI trafficking has remained poorly understood. Rab proteins are small guanosine 5'-triphosphatases (GTPases) involved in the regulation of membrane traffic. In particular, Rab5 has been shown to regulate transport in the early endocytic pathway, whereas it is not known whether the FcεRI surface expression levels are regulated by Rab5. In this study, we investigated the role of individual Rab5 isoforms in mast cells by small interfering RNA knockdown method. Our results demonstrate that Rab5a knockdown enhanced FcεRI-dependent mast cell activation and upregulated FcεRI surface expression in its steady state. In contrast, Rab5c knockdown caused suppression of the activation. These findings revealed modulatory and individual roles of Rab5 isoforms in mast cell functions.

Key words mast cell; FcεRI; Rab5; endocytosis; surface expression; degranulation

Mast cells are key players in allergic responses. The aggregation of FcεRI on mast cells initiates various biochemical cascades that result in the release of inflammatory mediators. Following FcεRI ligation, the receptor is rapidly internalized by either clathrin-dependent¹⁾ or clathrin-independent, dynamin-dependent mechanisms.²⁾ Importantly, the mast cell functional response to immunoglobulin E (IgE)-dependent stimulation has been shown to be greatly influenced by surface expression levels of FcεRI in its occupied and unoccupied state.^{3,4)} The constitutive cycling of transmembrane proteins such as receptors, channels and transporters has recently been demonstrated in a wide range of cell types,⁵⁾ which is thought to be important as a determinant of surface expression levels of these proteins.

Rab proteins regulate membrane traffic in the endocytic and biosynthetic pathway.⁶⁾ Notably, Rab5 is localized to the early endosomal compartment and has been implicated in the regulation of the plasma membrane to early endosomes transport.⁷⁾ Recent studies demonstrated that RabGEF1/Rabex-5, a guanine nucleotide exchange factor (GEF) of Rab5 and Rabaptin-5, a Rab5 effector are involved in regulating of FcεRI surface expression and in mast cells activation.^{8,9)} However, the direct role of Rab5 in mast cells has not been examined yet.

Here we investigated the contribution of Rab5 to the regulation of functional activation and FcεRI surface expression in mast cells. Rab5 exists in three isoforms (Rab5a, Rab5b, and Rab5c) and are ubiquitously expressed. When overexpressed in baby hamster kidney cells, all Rab5 isoforms activate endocytosis, suggesting that the Rab5 isoforms possess redundant functions in endocytosis pathway.¹⁰⁾ To assess the importance of each Rab5 isoform for FcεRI endocytosis, we used small interfering RNA (siRNA) to efficiently suppress the expression of Rab5 proteins. The application of siRNA method revealed that the Rab5 isoforms have individual roles and that Rab5a is the main regulator of surface expression levels of FcεRI in mast cells.

MATERIALS AND METHODS

Cell Culture Bone marrow-derived mast cells (BMMCs) were prepared from BALB/c mice according to the method described previously.¹¹⁾ This study was approved by the Animal Experiment Committee in University of Toyama (Authorization No. is S-2009 INM-9) in accordance with the Guide for the Care and Use of Laboratory Animals in University of Toyama which is accredited by the Ministry of Education, Culture, Sports, Science and Technology of Japan.

siRNA-Mediated Inhibition of Rab5 Expression The siRNA sequences directed against mouse Rab5a, Rab5b, and Rab5c were selected according to the previous report.¹²⁾ These siRNAs against mouse Rab5 were obtained from B-Bridge International (Cupertino, CA, U.S.A.). Double-stranded siRNA (5 μl of 20 μM) was electroporated into 1 × 10⁶ of BMMC with Nucleofector I Device (Amaxa, Koeln, Germany) at program Y-01 using a Mouse Macrophage Nucleofector Kit (Amaxa) according to the manufacturer's instructions. A scrambled siRNA (B-Bridge International) was used as a negative control.

Quantitative Real-Time Polymerase Chain Reaction (PCR) BMMCs were cultured for 24 h after the electroporation. Total RNA was isolated from BMMCs using RNeasy Plus Micro (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Reverse transcription was performed using PrimeScript RT reagent kit (Takara Bio, Shiga, Japan) and random primers. Quantitative real-time PCR was performed using SYBR Premix Ex Taq (Takara Bio, Shiga, Japan) and Mx3000p system (Stratagene, La Jolla, CA, U.S.A.). The following primer pairs were used: Rab5a, forward 5'-GACTTAGCAAATAAAAGAGC-3' and reverse 5'-TCACTAAGGTCTACTCCTCG-3', Rab5b, forward 5'-TTCAGCTAAGACTGCTATGA-3' and reverse 5'-CTACAACACTGGCTCTTGTT-3', Rab5c, forward 5'-AAGAGAGCTGTGGAGTTTCA-3' and reverse 5'-CTCATTC-TTGGAAGCTTCT-3' and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward 5'-TGACCACAGTCCAT-

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GCCATC-3' and reverse 5'-GACGGACACATTGGGGGTA-G-3'. The PCR reaction conditions were 10 s at 95 °C, followed by 40 cycles of 5 s at 95 °C and 20 s at 60 °C. Target mRNA was normalized to GAPDH mRNA as an internal control in each sample. Results were expressed as the ratio relative to the vehicle-treated cells.

Flow Cytometry Analysis BMMCs were cultured for 48 h after the electroporation, washed with fluorescent activated cell sorter (FACS) buffer (phosphate buffered saline (PBS), 1% bovine serum albumin (BSA), 0.2% NaN₃), stained with fluorescein isothiocyanate (FITC)-labeled anti-mouse FcεRI alpha (clone MAR-1, eBioscience, San Diego, CA, U.S.A.) and PE-labeled anti-mouse CD117 (c-kit) (clone ACK45, BD Pharmingen, San Diego, CA, U.S.A.) for 30 min at 4 °C. Flow cytometric analysis was performed using FACSCalibur Cell QuestPro software version 6.0 (BD Biosciences, Franklin Lakes, NJ, U.S.A.). Percent inhibition of FcεRI expression was determined using mean fluorescence intensity (MFI), when compared with vehicle-treated cells as the baseline.

Degranulation Assay BMMCs were cultured for 48 h after the electroporation. The degree of IgE/antigen-mediated degranulation of BMMCs was assessed by measuring β-hexosaminidase release as previously described.¹¹⁾

Statistical Analyses Statistical comparisons were made using two-sample *t* test. Probability values (*p*) of <0.05 were considered as statistically significant.

RESULTS

Rab5 Expression Knockdown by siRNA To determine whether specific isoform of Rab5 affects FcεRI surface expression, siRNA duplex directed against Rab5a, -5b or -5c was introduced into BMMCs using electroporation. Quantitative real-time PCR was conducted to measure the expression levels of each Rab5-isoform mRNA. Expression levels of mRNA significantly decreased to 0.45 ± 0.17 - (Rab5a, Fig. 1A), 0.50 ± 0.16 - (Rab5b, Fig. 1B), 0.32 ± 0.06 - (Rab5c, Fig. 1C) fold in each siRNA-treated BMMCs compared with control BMMCs which were introduced with control siRNA (5–6 independent experiments, *n*=6–8). No off-target silencing was observed in each Rab5 isoform knockdown (Figs. 1A–C). These results indicate that each siRNA duplex against Rab5 isoforms successfully downregulated the Rab5 isoform expression in BMMCs.

Rab5a and Rab5c Knockdown Affects on Degranulation of Mast Cells To examine whether the knockdown of Rab5 isoform affects degranulation response to IgE-dependent stimulation, an extent of degranulation was assessed by β-hexosaminidase release. As shown in Fig. 2A, Rab5a knockdown significantly enhanced the degranulation response by 1.17 ± 0.07 -fold compared with control-siRNA treated cells. Knockdown of Rab5b did not alter the extent of degranulation (Fig. 2B). In contrast, Rab5c knockdown significantly decreased the extent of degranulation to 0.69 ± 0.13 -fold (Fig. 2C).

Rab5a Knockdown Enhances FcεRI Surface Expression Rab5 is a major regulator of early endocytic events for multiple classes of receptors.⁷⁾ However, it has been reported that there is only minimal co-localization of antigen- and IgE-bounded FcεRI with Rab5 during its internalization.¹³⁾

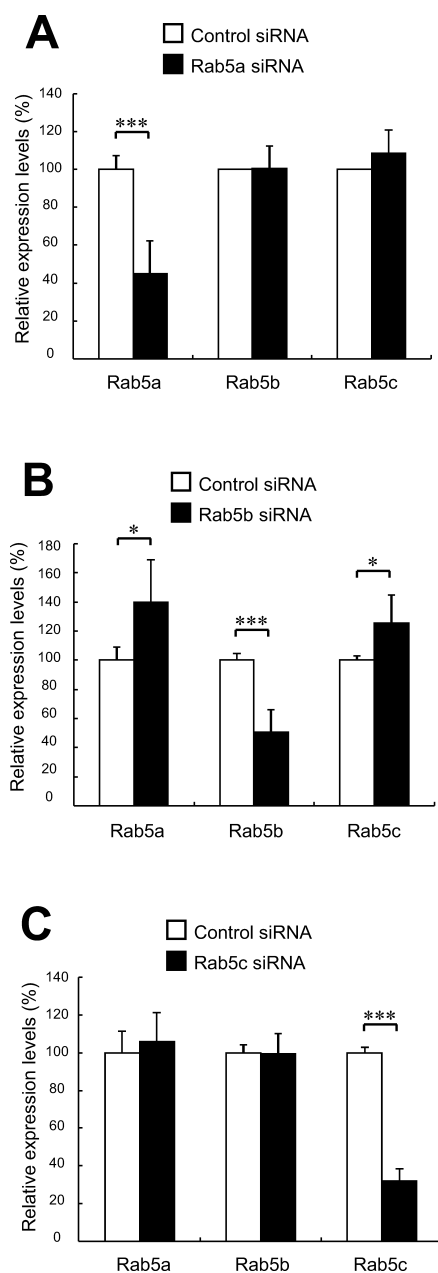


Fig. 1. Effects of siRNA on Rab5-Isoforms Expression Levels in BMMCs

1×10^6 BMMCs were transfected with $1 \mu\text{M}$ siRNAs against Rab5a (A), Rab5b (B), and Rab5c (C) (closed bars). Cells were also treated with their negative control siRNA (open bars). Twenty four hours later, cells were lysed, and the amount of Rab5-isoforms mRNA was analyzed by real-time PCR. ****p*<0.01, **p*<0.05, as determined by two-sample *t*-test (5–6 independent experiments, *n*=6–8).

Rios *et al.*, reported that Rabaptin-5, a Rab5 effector, is dispensable for internalization of antigen- and IgE-bounded FcεRI, but is required for FcεRI surface expression in the steady state.⁹⁾ These results suggest that Rab5 plays more critical roles in surface expression levels of FcεRI than in antigen- and IgE-bounded FcεRI internalization. Then to evaluate the effect of Rab5 isoform knockdown on FcεRI surface expression, siRNA-treated BMMCs were analyzed using flow cytometry. As shown in Fig. 3A, knockdown of Rab5a significantly upregulated surface expression of FcεRI by 1.14 ± 0.08 fold. In contrast, knockdown of either Rab5b or Rab5c had no significant effect on the surface expression

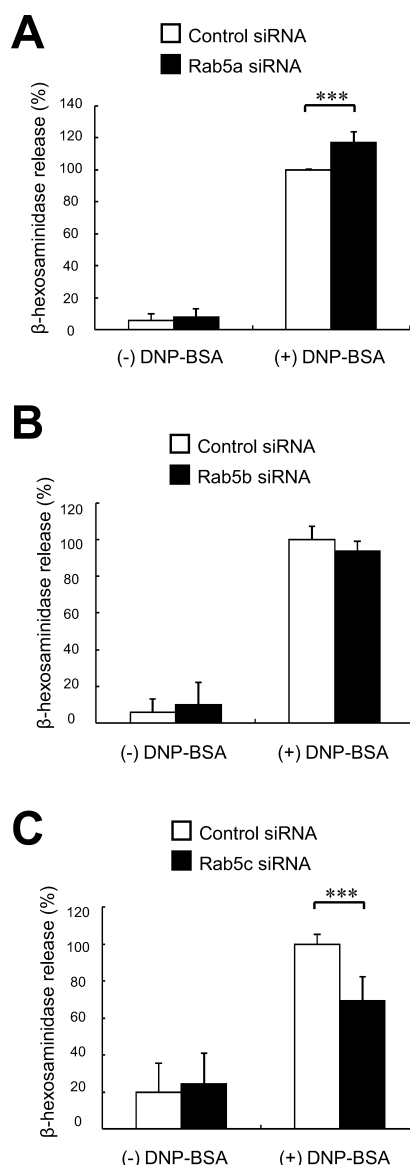


Fig. 2. Effects of Rab5-Isoforms Depletion on Degranulation in BMMCs

BMMCs were transfected with 1 μ M siRNAs against Rab5a (A), Rab5b (B), and Rab5c (C) (closed bars). Cells were also treated with their negative control siRNA (open bars). Cells were sensitized with anti-DNP IgE for 6 h, and stimulated with (+) or without (-) DNP-BSA for 1 h. The degree of degranulation was assessed by measuring β -hexosaminidase release. *** p <0.01, as determined by two-sample t -test (5 independent experiments, n =5–8).

of Fc ϵ RI (Figs. 3B,C). Surface expression levels of c-kit were not changed by knockdown of Rab5 isoforms (Fig. 3).

DISCUSSION

It is now evident that mast cell functional responses to IgE-dependent stimulation can be greatly influenced by the level of expression of Fc ϵ RI on the cell surface.¹⁴ Fc ϵ RI, like a large number of cell surface receptors, is assumed to be dynamically transported within the cell. Rab5 has been shown to be a key regulator of various receptors transport in the early endocytic pathway. However, the role of Rab5 in mast cells function has remained poorly understood. Here we show that Rab5a knockdown caused enhancement of Fc ϵ RI surface

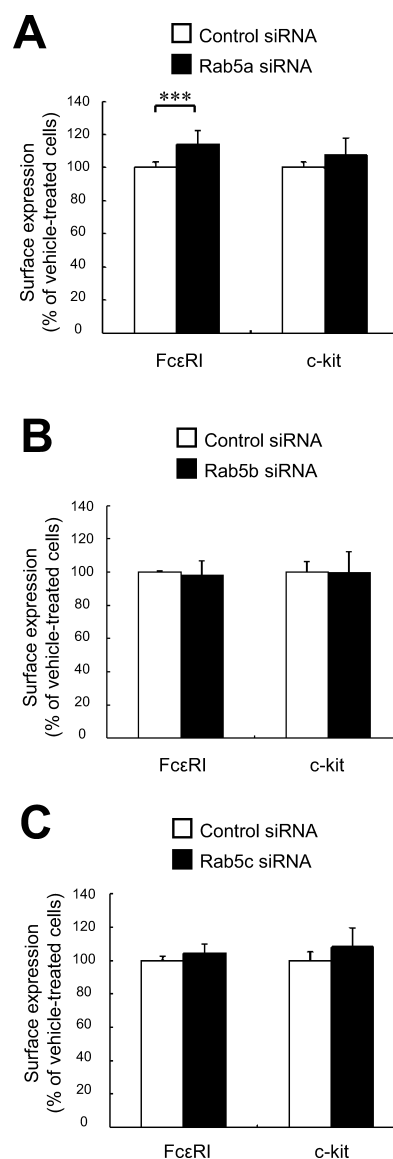


Fig. 3. Effects of Rab5-Isoforms Depletion on Surface Expressions of Fc ϵ RI and c-Kit in BMMCs

BMMCs were transfected with 1 μ M siRNAs against Rab5a (A), Rab5b (B), and Rab5c (C) (closed bars). Cells were also treated with their negative control siRNA (open bars). Their surface expression levels of Fc ϵ RI (left) and c-kit (right) were determined by flow cytometry. *** p <0.01, as determined by two-sample t -test (5 independent experiments, n =5–8).

expression in mast cells.

A recent study demonstrated that Rab5 isoforms are redundant, and significant inhibition of epidermal growth factor receptors and transferrin receptors endocytosis require the simultaneous knockdown of all three Rab5 isoforms.¹⁵ In the case of Fc ϵ RI, however, our study revealed that Rab5a alone knockdown was enough to affect the level of Fc ϵ RI surface expression among individual knockdown of three Rab5 isoforms (Figs. 3A–C). These results suggest that Rab5a is a main participant in Fc ϵ RI trafficking among Rab5 isoforms. Earlier work demonstrated that Rab5a is selectively induced in mammalian macrophages by interferon¹⁶ and that Rab5 isoforms are differentially phosphorylated *in vitro* by specific kinases.¹⁷ These findings also anticipate the distinct functions of Rab5 isoforms.

Rab5b knockdown caused upregulation of Rab5a and Rab5c expression levels (Fig. 1B), but did not alter the extent of degranulation and the surface expression levels of Fc ϵ RI (Fig. 2B). Rab5b knockdown might be counteracted by upregulation of Rab5a and Rab5c, which may explain why the Rab5b knockdown showed no obvious phenotype (Figs. 2B, 3B). Interestingly, the extent of degranulation was suppressed when the expression levels of Rab5c was downregulated (Fig. 2C). This result suggests that Rab5c might be involved in upregulation or activation of some factors besides Fc ϵ RI which contribute to the degranulation process in mast cells. Further detailed analyses are required to identify the mechanisms underlying the effect of Rab5c knockdown.

To summarize, our siRNA experiments demonstrate that Rab5a is closely involved in functional activation and Fc ϵ RI surface expression in mast cells. Furthermore, this study demonstrates that functions of three Rab5 isoforms are partially different in mast cells.

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