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Orai-2 is localized on secretory granules and regulates antigen-evoked Ca^{2+} mobilization and exocytosis in mast cells

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ABSTRACT

The increase in intracellular Ca^{2+} through the Ca^{2+} channel is an indispensable step for the secretion of inflammatory mediators by mast cells. It was recently reported that Orai-1 is responsible for the Ca^{2+} influx that is activated by depletion of stored Ca^{2+}. There are three isoforms of Orai: Orai-1, Orai-2, and Orai-3; however, isoforms other than Orai-1 are poorly understood. We found that Orai-2 is expressed and localized on secretory granules in RBL-2H3. Ca^{2+} release from Ca^{2+} store, induced by antigen stimulation, was significantly attenuated by knockdown of Orai-2, while that induced by thapsigargin was not affected. Furthermore, exocytotic release induced by antigen stimulation was inhibited in knockdown cells. This observation suggests a new role of Orai isoforms in secretory cells.

1. Introduction

Mast cells play an essential role in allergic responses by secreting inflammatory mediators such as histamine, leukotrienes and cytokines [1–3]. The secretion of these mediators is induced by the cross-linking of high-affinity receptors for IgE ($Fc_{\varepsilon}RI$) expressed on the cell surface. The cross-linking of receptors causes activation of phospholipase C, then IP_3 and diacylglycerol are produced from PIP_2. IP_3 induces Ca^{2+} release from the intracellular Ca^{2+} store, which is followed by Ca^{2+} influx through Ca^{2+} channels on the plasma membrane. The increase in intracellular Ca^{2+} induces exocytotic release of histamine, and the synthesis and secretion of inflammatory mediators such as leukotrienes and cytokines. Therefore, it is extremely important to study the mechanism of Ca^{2+} increase. We have previously studied the mechanism of exocytotic release of histamine, including Ca^{2+} signaling, in mast cells [4–7]. The main pathway for entry of extracellular Ca^{2+} across the plasma membrane is store-operated Ca^{2+} entry (SOCE) through Ca^{2+} release-activated calcium (CRAC) channels [8–10]. It was recently revealed that Orai is a CRAC channel that is responsible for SOCE, in association with STIM, which functions as Ca^{2+} sensor in the ER [11–13]. It was demonstrated in mast cells that Orai-1 functions as a CRAC channel [10,14–16].

The three isoforms of Orai exhibit similar characteristics such as high Ca^{2+} selectivity and inward rectification; although some differences in electrophysiological and pharmacological properties among isoforms were reported, particularly for Orai-3 [17–19]. However, the role of isoforms other than Orai-1 is still poorly studied.

In the present study, we investigated the expression and roles of these isoforms of Orai in mast cells. We found that Orai-2 is localized on the secretory granules of RBL-2H3 cells, and regulates Ca^{2+} mobilization from intracellular Ca^{2+} store and exocytotic release.

2. Materials and methods

2.1. Cell culture

RBL-2H3 was cultured in Eagle's minimal essential medium (Nissui, Tokyo, Japan) with 10% fetal bovine serum (GIBCO, Grand Island, NY), at 37 °C in a humidified atmosphere with 5% CO_2.

2.2. PCR

Poly(A)+ RNA was obtained from RBL-2H3 cells using a QuickPrep micro mRNA purification kit (GE Healthcare, Little Chalfont, UK), which served as a template for cDNA synthesis using SuperScript III RT (Invitrogen, Grand Island, NY, USA). To amplify Orais, PCR was carried out with FastStart Taq DNA polymerase (Roche Diagnostics, Basel, Switzerland). The primer pairs used to amplify isoforms of Orai were 5′-ACAGCAATCCGGAGCTTCC-3′ (sense)/5′-TCCTTGACGGAGTTGAGGTTG-3′ (antisense) for Orai-1, 5′-GTGCCGAGCTCAATGTGC-3′ (sense)/5′-GACCACGAAGATGAGACCCAC-3′ (antisense) for Orai-2, and 5′-CCACCAGGCCTGCTAGTG-3′ (sense)/5′-AAATGTAGGGCAAAGGCCA-3′ (antisense) for Orai-3.

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PCR products were electrophoresed and extracted from the agarose gel, and subsequently subcloned into the pGEM/TA cloning vector (Promega, Fitchburg, WI, USA). Cloned PCR products were sequenced with an M13 primer, using a capillary DNA sequencer (ABI Prism 3130; Applied Biosystems, Foster City, CA) to confirm the expression.

The expression level of each isoform of Orai was evaluated by Real-time PCR. Total RNA was obtained from RBL-2H3 cells (1×10^5 cells) by RNeasy Mini Kit and RNase-Free DNase Set (Qiagen, Venlo, Netherlands). After reverse transcription (High Capacity cDNA Reverse Transcription Kit; Applied Biosystems), real-time PCR was performed using TaqMan probes (7300 Real Time PCR System; Applied Biosystems). As a control, β-actin was amplified with a primer pair 5′-AAGTCCCTCACCCTCCCAAAAG-3′ and 5′-AAGCAATGCTGTCACCTTCCC-3′ (POWER SYBR® Green PCR Master Mix (2×); Applied Biosystems).

2.3. Plasmid construction

For the expression of GFP-conjugated Orai-2, cDNA of Orai-2 was obtained from RBL-2H3 cells by RT-PCR using a primer pair (5′-AAGCTTATGGGTCACGTGACGGC-3′/5′-GTCGACAACACCACCTGT AGGCTTCTCTC-3′, Hind III and Sal I sites are underlined). After verification of the sequence of PCR products with a capillary DNA sequencer (ABI Prism 3130), cDNA of Orai-2 was subcloned into a pEGFP-N1 vector (Clontech, Mountain View, CA, USA) between the Hind III and Sal I sites.

To knockdown Orai-2, RNAi expression vector (BLOCK-iT Pol II miR RNAi Expression Vector Kit; Invitrogen) was used. To construct the expression vector of miRNA for Orai-2, 5′-TGCTGACCACCTG TAGGCTTCTCTCGGTTTTGGCCACTGACTGACCGAGAGAACTACAGGT GGT-3′ as a top strand and 5′-CCTGACCACCTGTAGTTCTCTCGGT CAGTCAGTGGCCAAAACCGAGAGAAGCCTACAGGTGGTC-3′ as a bottom strand were inserted into pcDNA 6.2-GW/EmGFP-miR. For the expression of YFP-conjugated VAMP-7, cDNA of each SNARE was inserted into pEYFP-C1 (Clontech), as previously reported [23].

Plasmids expressing siRNA, GFP-Orai-2, and YFP-VAMP-7 were introduced into RBL-2H3 cells by electroporation, and stable transfectants were selected with G418 (500 μg/ml).

2.4. Western blotting

To prepare whole cell lysate, RBL-2H3 cells (1×10^6) were lysed in cold lysis buffer (20 mM Tris, 150 mM NaCl, 0.5 mM EDTA, 1% TritonX-100, and 1 mM PMSF). The suspension was clarified by centrifugation, and the resulting supernatants were solubilized by treatment with 2× sample buffer (4% SDS, 12% 2-ME, 20% glycerol, 0.1 mg/ml BPB, and 100 mM Tris; pH 6.8) at 100 °C for 5 min. Proteins were separated by SDS–polyacrylamide gel electrophoresis and then transferred to PVDF membrane with an electroblotter. After blocking with 5% skim milk, the membranes were probed with one of the following primary antibodies: anti-Orai-1 antibody (1:500 dilution; Sigma–Aldrich), anti-Orai-2 antibody (1:1000 dilution; Santa Cruz Biotechnology), and anti-β-actin (dilution 1:1000; Sigma–Aldrich). After washing with 0.1% Tween 20 in PBS, the membrane was treated with secondary antibodies, anti-mouse IgG conjugated with horseradish peroxidase (dilution 1:1000; MBL) or anti-goat IgG conjugated with horseradish peroxidase (1:1000, Santa Cruz Biotechnology). Immunoreactivity was detected by enhanced chemiluminescence (ECL, Amersham Bioscience) using a LAS-3000 mini imaging system (FUJI FILM, Tokyo Japan).

2.5. Isolation of secretory granules

Secretory granules were prepared from RBL-2H3 cells following the method previously reported with a slight modification [20,21]. In brief, RBL-2H3 cells (7×10^7) were washed with PBS and suspended in homogenization buffer (0.25 M sucrose, 1 mM $MgCl_2$, 800 U/ml DNase I, 10 mM HEPES (pH 7.4), 1 mM PMSF, and a cocktail of protease inhibitors (Complete mini; Roche Diagnostics). Cells were then disrupted by 3 cycles of freezing and thawing, followed by 20 passages through a 21-gauge needle. After centrifugation for 5 min at 170*g*, the supernatant (1.5 mL) was loaded onto a sucrose gradient (2.0, 1.5, 1.0, and 0.45 M sucrose; 2.5 mL each from top to bottom) and centrifuged for 18 h at 100,000*g* (SW40Ti; BECKMAN). Twenty-two fractions (500 μL each) were collected from the top of the gradient.

2.6. Fluorescence imaging

Cells (1×10^5) were seeded in a ZOG-3 glass-bottom chamber (Elekon Science, Chiba, Japan) and incubated for 18 h. Fluorescent images were taken with a confocal laser scanning microscope (LSM-510META; Carl Zeiss, Göttingen, Germany) with a 63× objective lens (Plan-Apochromat 63×/1.4 oil). To detect EGFP, samples were excited at 488 nm with an Ar laser, and fluorescence was observed with an LP505 filter. For visualization of secretory granules, cells were stained with Lyso Tracker Red DND-99 (Molecular Probes, Eugene, OR, USA) and excited at 543 nm with a HeNe laser, and fluorescence was observed with an LP560 filter.

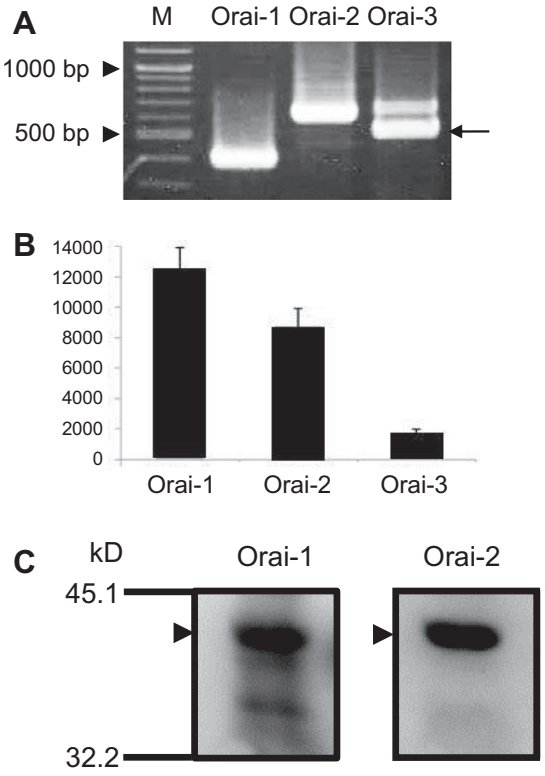

Fig. 1. Expression of Orais in RBL-2H3 cells. (A) Expression of three isoforms was detected by RT-PCR, using specific primer pairs. The leftmost lane (M) is for size marker (100 base ladder marker). Arrow heads indicate bands of 500 and 1000 bp. PCR products were sequenced by a DNA sequencer, and expression of Orai-1, Orai-2, and Orai-3 was confirmed. In the lane for Ora-3, the lower band indicated by an arrow was found to be Orai-3. (B) Expression level of each isoform of Orai was evaluated by real-time PCR using a TaqMan probe. Each bar represents copy number per total RNA (mean ± SD, $n = 3$). Orai-1 and Orai-2 were major isoforms expressed in RBL-2H3 cells. (C) Expression of Orai-1 and -2 was detected by Western blotting analysis. Total cell lysate of RBL-2H3 (20 μg protein per lane) was subjected to SDS–PAGE and transferred to PVDF membrane. Expression of Orais was detected by treating membranes with specific antibodies for Orai-1 (left) and -2 (right) at the positions indicated by arrow heads.

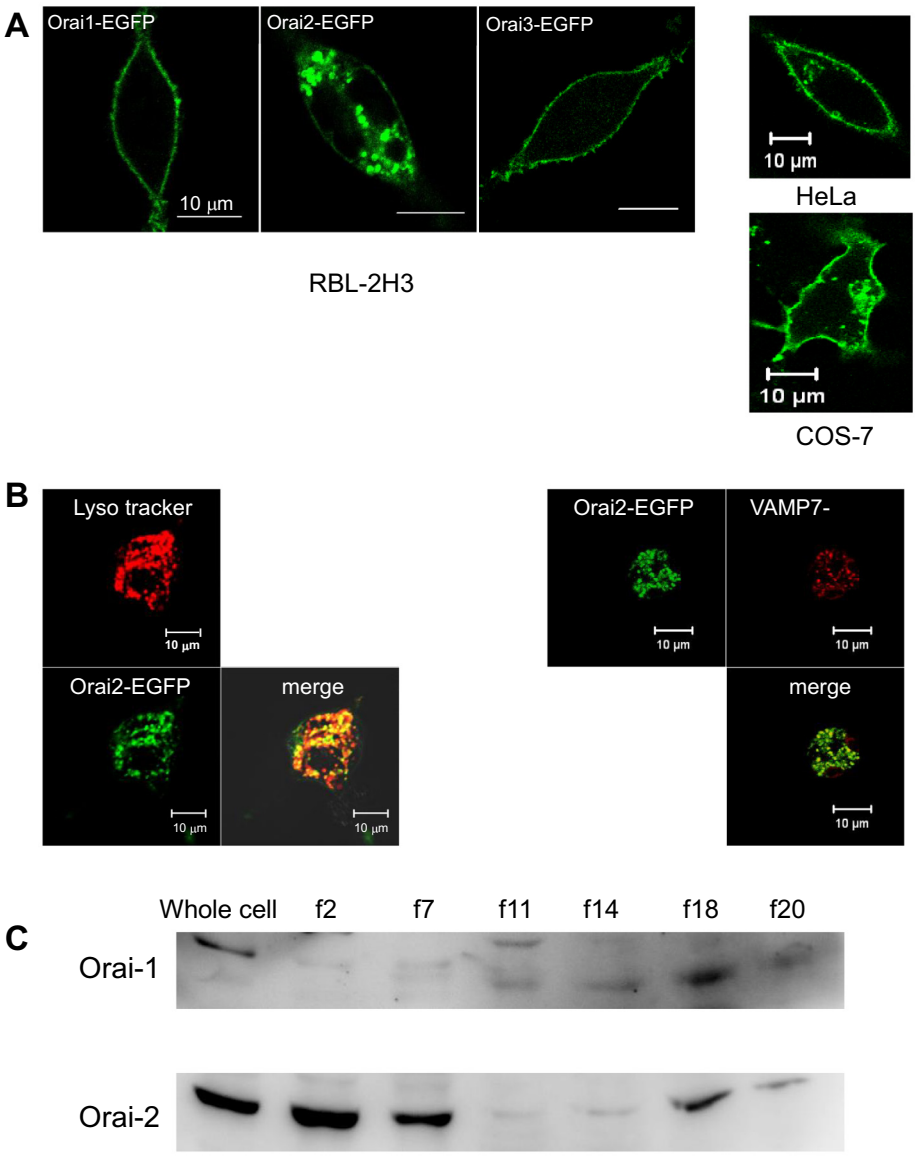

Fig. 2. Intracellular localization of Orais in RBL-2H3 cells. (A) Fluorescence image of each isoform of Orais expressed as a fusion protein with GFP [Orai-1 (left), Orai-2 (center), Orai-3 (right)]. Orai-2 was mainly localized on secretory granules, but also detected on the plasma membrane. Fluorescence images of Orai-2-EGFP expressed in HeLa (right upper panel) and COS-7 (right lower panel) are shown. In contrast to its position on RBL-2H3 cells, Orai-2 was localized on the plasma membrane in HeLa and COS-7. Bar indicates 10 μm. (B) Secretory granules were stained with Lyso Tracker (left) and VAMP-YFP (right). Most fluorescence derived from Orai-2-GFP was co-localized with secretory granules. (C) Secretory granules were fractionated by sucrose density gradient centrifugation. Expression of Orai-1 (upper panel) and Orai-2 (lower panel) in collected fractions was examined by Western blotting. Out of 22 fractions, 6 fractions (f2, f7, f11, f14, f18, and f20) and whole cell lysate (far left) were subjected to Western blotting analysis. Orai-2 was detected in fractions that corresponded to granule fractions.

2.7. Measurement of intracellular Ca^{2+}

RBL-2H3 cells were loaded with Fura2/AM (Molecular Probes) and sensitized with anti-DNP IgE for 30 min at 37 °C. After washing twice with Ca^{2+}-free HEPES-buffered saline (140 mM NaCl, 5 mM KCl, 0.6 mM $MgCl_2$, 0.1% glucose, 0.1% BSA, 0.1 mg/mL sulfinpyrazone, and 10 mM HEPES; pH 7.4), cells were stimulated with DNP_6-BSA (200 ng/ml) or thapsigargin in the absence of Ca^{2+}. After a transient increase in intracellular Ca^{2+}, concentration of Ca^{2+} extracellular buffer was increased to 0.5 mM. The fluorescence intensities with excitation at 340 and 360 nm were measured, and the ratio (F340/F360) was calculated by a spectrofluorometer linked to a personal computer (RF-5300PC; Shimadzu, Japan). During measurement of the intracellular Ca^{2+} concentration, cells were kept at 37 °C.

2.8. β-Hexosamindase assay

Degranulation of RBL-2H3 cells was monitored by measuring the activity of a granule-stored enzyme, β-hexosaminidase, secreted in cell supernatant. Cells were loaded with Fura2/AM and sensitized with anti-DNP IgE for 30 min, and then stimulated with antigen (DNP_6-BSA) or thapsigargin for 30 min at 37 °C. Aliquots of supernatant were incubated with a substrate solution (2 mM *p*-nitrophenyl-N-acetyl-β-D-glucosaminide in 100 mM citrate, pH 4.5) for 1 h at 37 °C. After the reaction was terminated with Na_2CO_3–$NaHCO_3$ buffer, absorbance at 405 nm was measured. Release activity relative to the total β-hexosaminidase content of the cells was calculated. Total β-hexosaminidase content was determined by dissolving cells with 0.1% Triton X-100.

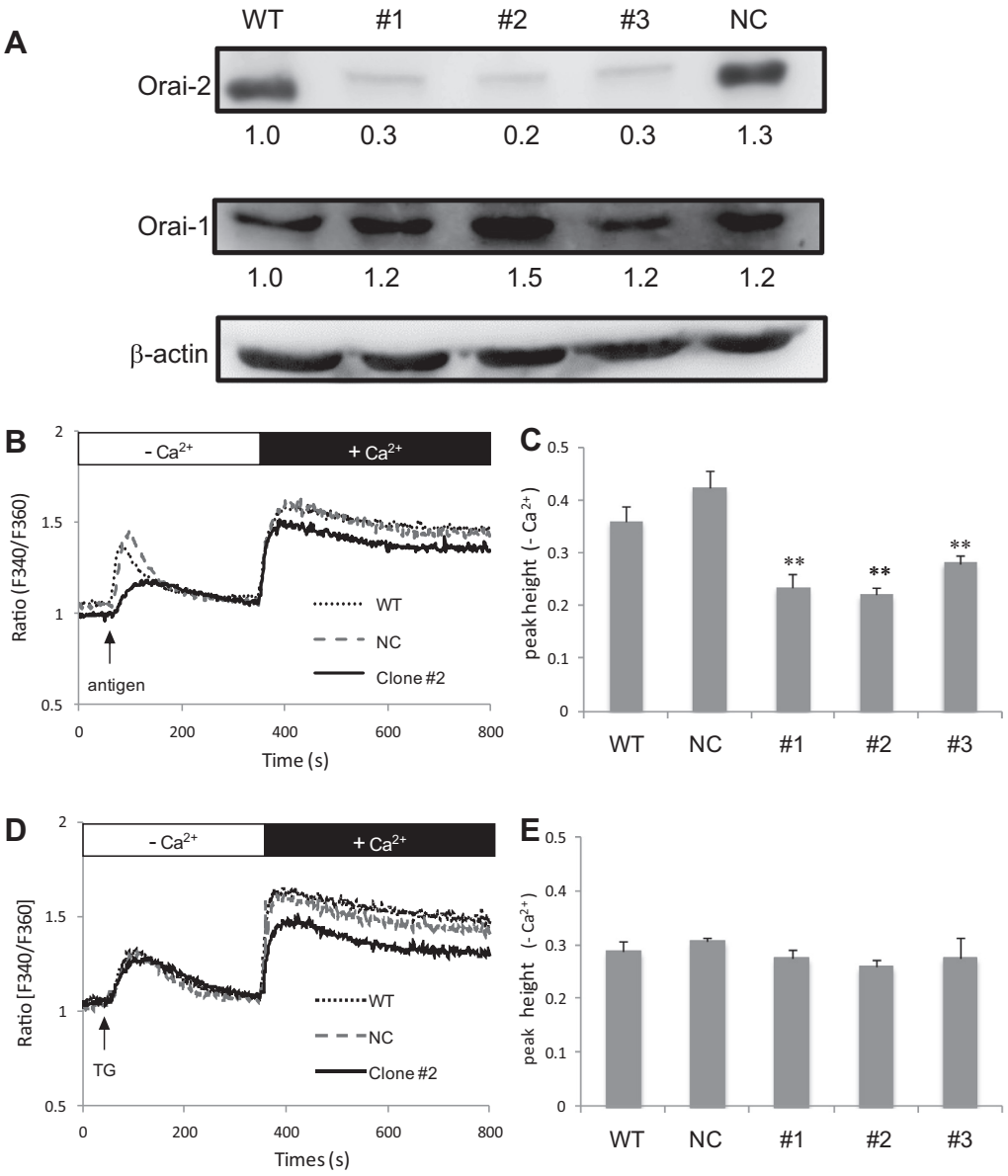

Fig. 3. Effects of knockdown of Orai-2 on intracellular Ca^{2+} mobilization. (A) Expression of Orai-2 in three knockdown clones (clones #1 to #3), wild type (WT), and negative control transfected with control plasmid (NC). Expression of β-actin is shown in the lower panel. Expression of proteins were quantified with ImageJ and corrected with the expression level of β-actin. Expression levels of Orai-1 and -2 in each clone relative to that of wild type were shown below the panel of bands. (B) Time course of intracellular Ca^{2+} concentration (ratio of fluorescence intensity excited at 340 and 360 nm) induced by antigen stimulation is shown. In the absence of extracellular Ca^{2+} (white bar), cells were stimulated at the time indicated by an arrow. After recovery to the basal level, extracellular Ca^{2+} concentration was increased to 0.5 mM (black bar). Each line represents the average value of 6 independent measurements. The solid line represents data from knockdown clone #2. Ca^{2+} increase due to Ca^{2+} release from the intracellular Ca^{2+} store was attenuated, compared to the wild type (dotted line) and negative control (dashed line). (C) Ca^{2+} increase induced by antigen stimulation in the absence of extracellular Ca^{2+} was expressed as a peak height from the basal level before stimulation to the peak after stimulation (mean ± SE, $^{**}p < 0.01$ vs NC). (D) Time course of intracellular Ca^{2+} concentration induced by thapsigargin (100 nM) stimulation is shown. In the absence of extracellular Ca^{2+} (white bar), cells were stimulated at the time indicated by an arrow (TG). After recovery to the basal level, extracellular Ca^{2+} concentration was increased to 0.5 mM (black bar). Each line shows the average value of 6 independent measurements. The solid line is data of knockdown clone #2. Ca^{2+} increase due to Ca^{2+} release from the intracellular Ca^{2+} store was attenuated compared to wild type (dotted line) and negative control (dashed line). (E) Ca^{2+} increase induced by thapsigargin stimulation in the absence of extracellular Ca^{2+} was expressed as a peak height from the basal level before stimulation, to the peak after stimulation (mean ± SE).

3. Results and discussion

3.1. Expression of Orais in mast cells

We examined the expression of the three Orai isoforms in mast cells by RT-PCR. We sequenced PCR products and found that all of these isoforms were expressed in RBL-2H3 (Fig. 1A). The lower band indicated by an arrow in the lane of Orai-3 was found to be amplified products derived from Orai-3. Thus, we determined the expression level of each isoform by real time PCR. As shown in Fig. 1B, Orai-1 is the most abundant isoform and Orai-2 is highly expressed, whereas Orai-3 expression is extremely low compared with that of Orai-1 and Orai-2.

The expression of the major isoforms of Orais, Orai-1 and Orai-2, was confirmed by Western blotting analysis of total cell lysate (Fig. 1C).

3.2. Intracellular localization of Orais in mast cells

We then investigated the intracellular localization of each isoform of Orai in RBL-2H3 cells. Each isoform was expressed as a fusion protein with green fluorescence protein (GFP) in RBL-2H3

cells. Since Orai is a Ca^{2+} channel that is responsible for Ca^{2+} influx from extracelluar medium, it is expected that any isoforms of Orai are localized on the plasma membrane regardless of isoforms. Interestingly, Orai-2 expressed as a fusion protein with GFP was mainly localized on secretory granules rather than the plasma membrane, while Orai-1 and Orai-3 were distributed on the plasma membrane (Fig. 2A, left panel). To examine whether or not this localization of Orai-2 is specific to RBL-2H3, GFP-conjugated Orai-2 was expressed in HeLa and COS-7 cells. As shown in Fig. 2A (right panel), it was distributed on the plasma membrane like Orai-1 and Orai-3 were distributed on the plasma membrane in RBL-2H3 cells. Therefore, the distribution on secretory granules is seemed to be specific to RBL-2H3 cells. To confirm the localization of Orai-2 on secretory granules, secretory granules were stained with YFP-conjugated VAMP-7 and Lyso-tracker Red. RBL-2H3 cells expressing Orai-2-EGFP were treated with Lyso-tracker Red (Fig. 2B, left panel) because it is localized in secretory granules in mast cells [22]. Most GFP-positive granules were stained by Lyso-tracker. We previously reported that VAMP-7, one of v-SNARE, is expressed on secretory granules in RBL-2H3 cells [23]. When VAMP-7 conjugated with YFP was expressed together with Orai-2-EGFP, both fluorescence proteins were co-localized on secretory granules (Fig. 2B, right panel). These double staining experiments suggest that Orai-2 is mainly localized on secretory granules.

We examined the localization of Orai-2 by biochemical analysis. Using sucrose gradient centrifugation, secretory granules were prepared from RBL-2H3 cells. Secretory granules were collected in first the 10 fractions. Although smiling effect caused downward shift of bands at center, Orai-2 was clearly detected in the fractions that corresponded to secretory granules (f2 and f7), while Orai-1 was not detected in granule fractions.

3.3. Roles of Orai-2 in intracellular Ca^{2+} mobilization

To elucidate the role of Orai-2 in mast cell activation, we examined the effects of knockdown of Orai-2 on intracellular Ca^{2+} dynamics. We knocked down the expression of Orai-2 by siRNA, and established three clones expressing lower levels of Orai-2 (Fig. 3A). We also checked the expression level of Orai-1, because Orai-1 is the most probable candidate that could be affected by siRNA for Orai-2, resulting in changes in Ca mobilization and degranulation. As shown in Fig. 3A, the expression of Orai-1 was not attenuated, although it was increased in clone #2. To distinguish Ca^{2+} release from the intracellular Ca^{2+} store and Ca^{2+} influx from the extracellular solution, we stimulated cells in the absence of extracellular Ca^{2+}. After a transient increase and recovery to the basal level of intracellular Ca^{2+} concentration, extracellular Ca^{2+} was increased to 0.5 mM. In fact, when cells were stimulated with antigen, Ca^{2+} increase because of Ca^{2+} release from the intracellular Ca^{2+} store was significantly attenuated (Fig. 3B and C). On the other hand, Ca^{2+} influx from the extracellular solution was slightly inhibited (Fig. 3B). This weak inhibition of Ca^{2+} influx may be explained by the incomplete depletion of the Ca^{2+} store or the attenuated expression of Orai-2 on the plasma membrane. The mechanism whereby Orai-2 on secretory granules affects the Ca^{2+} release from the Ca^{2+} store remains to be elucidated. When cells were treated with thapsigargin (which reduces stimulation of the Ca^{2+} store) Ca^{2+} increase because of Ca^{2+} release from the Ca^{2+} store was not affected (Fig. 3D and E). Ca^{2+} influx was weakly inhibited in knockdown cells in spite of store depletion by thapsigargin (Fig. 3D). This suggests that Orai-2 on the plasma membrane contributes to Ca^{2+} influx, although its expression on the plasma membrane is low.

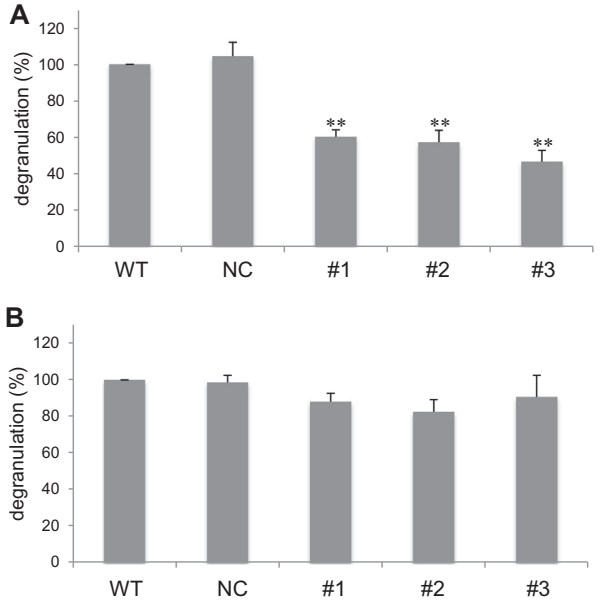

Fig. 4. Effects of knockdown of Orai-2 on degranulation. (A) Degranulation activities evoked by antigen stimulation were compared. Relative activity to wild type is shown (mean ± SE, $^{**}p < 0.01$ vs NC). Degranulation was significantly inhibited in knockdown cells. (B) Degranulation activities evoked by thapsigargin stimulation were compared. Activity relative to wild type is shown (mean ± SE).

3.4. Effects of knockdown of Orai-2 on exocytotic release

Exocytotic release of inflammatory mediators (degranulation) from mast cells is triggered by intracellular Ca^{2+} increase. Therefore, it is likely that degranulation is affected by knockdown of Orai-2. We examined the degranulation activity of mast cells evoked by antigen or thapsigargin.

When cells were stimulated with antigen, degranulation was significantly inhibited (Fig. 4A). On the other hand, degranulation was not affected when stimulated with thapsigargin (Fig. 4B). One possible reason for the inhibition of antigen-evoked exocytosis is the lower intracellular Ca^{2+} concentration in knockdown cells, as shown in Fig. 3. However, the phase of sustained increase of Ca^{2+} was not greatly inhibited. It is possible that Orai-2 on the secretory granules regulates degranulation through a mechanism other than Ca^{2+} increase.

Several observations regarding different properties among isoforms of Orai, especially Orai-3, have been recently reported [24]. For example, Na^+ currents from Orai-3 are much larger in magnitude than Orai-1 Na^+ currents [18]. Orai-3 is activated by 2-APB, while Orai-1 and Orai-2 are inhibited by the compound [19]. However, Orai-2 has been poorly studied. In the present study, we found interesting facts regarding intracellular localization of Orai-2, which may shed light on a new aspect of Orai-2 that differs from other isoforms.

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