

Inhibitory role of Munc13-1 in antigen-induced mast cell degranulation

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ABSTRACT

Secretory granules (SGs) of mast cells are lysosome-related organelles that contain various inflammatory molecules such as histamine, which are stored in the cytoplasm. Mast cell degranulation is the regulated exocytosis of SGs in response to external stimuli, such as the antigen-mediated cross-linking of the high-affinity IgE receptor, FcεRI. Upon stimulation, SGs undergo priming to become fusion-competent prior to fusing with the plasma membrane, which is mediated by Munc13-4, one of the five members of the vesicle-priming Munc13 protein family. Although Munc13-4 is shown to be crucial for mast cell degranulation, the functional involvement of other Munc13 isoform(s) remains unknown. Herein, this was investigated using the RBL-2H3 mast cell line. We found that Munc13-1 and Munc13-4 are the only Munc13 isoforms that are expressed in the RBL-2H3 cells, and Munc13-1 is distributed in the cytoplasm, but highly concentrated on the late endosome and/or lysosome. Unexpectedly, antigen-induced degranulation was considerably increased by Munc13-1 knockdown, but decreased by its overexpression. Further, we found that the hypersecretion phenotype of the Munc13-1-knockdown cells was attenuated by simultaneous Munc13-4 knockdown. These results suggested that Munc13-1 has an inhibitory role in antigen-induced mast cell degranulation, which is performed in a Munc13-4-dependent manner.

Mast cells are specialized secretory cells that play a central role in type I allergic reactions, as well as in certain innate and adaptive immune responses (1, 13). The antigen-mediated cross-linking of the high-affinity immunoglobulin E (IgE) receptor FcεRI triggers Ca²⁺-dependent exocytosis of secretory granules (SGs) referred to as degranulation, and results in the release of preformed mediators with a diversity of biological activities (26). Similar to other Ca²⁺-dependent regulated exocytosis such as neurotransmitter release, mast cell degranulation involves the fusion of SGs with the plasma membrane (PM), which is driven by a subset of the soluble *N*-ethylmaleim-

ide-sensitive fusion protein attachment protein receptor (SNARE) family of proteins, and is regulated by various proteins belonging to the Munc13, Munc18, synaptotagmin, complexin, or Doc2 families or to the Rab family of small GTPases (6, 26).

Ca²⁺-dependent regulated exocytosis is generally subdivided into three processes, vesicle docking, priming, and fusion, in chronological order (19, 31). Docking and priming are defined as a process that involves the physical contact between vesicles and the PM, and the subsequent formation of the *trans*-SNARE complex, which is ready for membrane fusion, respectively (19); however, they are thought to be closely linked processes, because a common molecular basis for their regulation has been discovered (28, 34). One of the molecules that possibly regulates both docking and priming is a protein of the Munc13 family, which contains conserved domains for the assembly of the SNARE complex, and possesses membrane-binding activity (18).

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The Munc13 family comprises the following five proteins: Munc13-1, bMunc13-2, and Munc13-3, which were originally identified as neuronal isoforms, uMunc13-2, which was identified as a ubiquitously expressed splicing variant of bMunc13-2, and Munc13-4, which was identified as a non-neuronal isoform (20). Munc13-1, u/bMunc13-2, and Munc13-3 are involved in neurotransmitter release as expected (2, 3, 23, 32); however, Munc13-1 is also required for insulin secretion in pancreatic β cells (21). On the other hand, Munc13-4 is involved in regulated exocytosis in hematopoietic secretory cells, such as cytotoxic T lymphocytes (CTLs), platelets, neutrophils, and mast cells (7, 12, 27, 30, 33, 35). It is also known that in neurons, chromaffin cells, and CTLs, multiple Munc13 isoforms are endogenously expressed, and play some redundant roles in the promotion of the docking and priming of secretory vesicles/granules (8, 10, 24, 36). In mast cells, Munc13-4 is shown to be crucial for degranulation (11, 35, 38); however, the involvement of other Munc13 isoform(s) still remains unknown.

In the present study, we found that Munc13-1 is endogenously expressed in the rat mucosal mast cell line, RBL-2H3. Therefore, we investigated its intracellular localization, implication in degranulation, and functional relationship with Munc13-4 using RBL-2H3 cells.

MATERIALS AND METHODS

Cell culture and transfection. RBL-2H3 cells were grown and transfected as described previously (15) with the following modification. To concentrate the transfectants carrying the G418-resistance gene, *Neo^r* (G418-surviving cells), over 90% of the cell population, the cell culture was continued for 30 h after the addition of G418. The control and Munc13-4-specific small interfering RNAs (siRNAs) transfected together with the plasmid carrying *Neo^r* have been described previously (15).

Preparation of rat tissues. Adult male Wistar rats (7–12-week-old) were sacrificed, and perfused with Ringer's solution, as described previously (17), followed by the removal of the brain and small intestine. Total RNA isolation and lysate preparation from tissues of these organs are described in the subsequent sections. All animal experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Iwate Medical University.

RT-PCR and quantitative real-time RT-PCR. Total RNA isolation, reverse transcription (RT), and PCR-based analyses were performed as described previously (15). For the preparation of brain mRNA using RNeasy Mini kit (QIAGEN, Hilden, Germany), a homogenization process was included according to the manufacturer's instructions. The first-strand cDNAs from mouse bone marrow-derived mast cells (BMMCs) and mouse brain were synthesized using total RNA isolated previously (16) as the template. The expression of Munc13 isoforms in the RBL-2H3 cells and in the BMMCs was analyzed by RT-PCR using the following primer pairs: 5'-CGA GCTCTGATCTCAGGACA-3' (sense) and 5'-ACA GGTCTGCAGTCCAGGCT-3' (antisense) for rat Munc13-1, 5'-AAGGTCCGACTCCAGCTGCAGG AGA-3' (sense for the u isoform), 5'-GAAGGA AAGCGAGAAGCTCTTCAGA-3' (sense for the b isoform) and 5'-AGGTGCCATCCAGGACACTC TGCTT-3' (antisense) for rat Munc13-2, 5'-CTAT TGACGAGAGTGCCTGA-3' (sense) and 5'-GGT TAGGAACTGGTCAGCAT-3' (antisense) for rat Munc13-3, 5'-ACAGAGCCATAGCAGCGGAA-3' (sense) and 5'-GAACATTTCTAGGTGGCAGG-3' (antisense) for rat Munc13-4, 5'-ACAACCTCCC TCAAGATTGTCAGCA-3' (sense) and 5'-TGCT CTCAGTATCCTTGCTGGGCT-3' (antisense) for rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-TGAGCTCTGATCTCAGGACA-3' (sense) and 5'-AGAGGTCTGCGGTCCAGGCT-3' (antisense) for mouse Munc13-1, 5'-ATGCGCC TGCCTCTCACATA-3' (sense) and 5'-CATCTG TGCCTACCCAGCTT-3' (antisense) for mouse Munc13-4, 5'-ACAACCTCACTCAAGATTGTCAG CA-3' (sense) and 5'-CTCAGTGTCTTGCTGGG GTGGGT-3' (antisense) for mouse GAPDH. The level of Munc13-1 expression in the RBL-2H3 cells and in the rat brain was analyzed by quantitative real-time RT-PCR using the following primer pairs: 5'-CGAAAGTTTCGCCACCAAATCG-3' (sense) and 5'-GCGAAGCAGTAGTCCTTCAC-3' (antisense) for Munc13-1, and 5'-ATGATTCTACCCACGGCA AG-3' (sense) and 5'-CTGGAAGATGGTGATGGG TT-3' (antisense) for GAPDH.

Plasmid construction. The siRNA expression vector, pBasi-mU6neo (Si-control), and the mammalian expression vector, pCIneo3HA (Vector), have been described previously (15, 16). For the knockdown of rat Munc13-1, the target sequences 5'-GCGGC TGAGAAGAGTTCTA-3' (KD1) and 5'-GCAAC AAGCCTGAGATCTT-3' (KD2) were obtained using Invitrogen BLOCK-iT RNAi designer (<http://>

rnadesigner.lifetechnologies.com/rnaiexpress/). The DNA fragment containing either of the sequences in both the sense and antisense directions was subcloned into the Si-control vector to produce either of the siRNA expression plasmids, Si-13-1KD1 and Si-13-1KD2, according to the manufacturer's instructions. For the expression of the hemagglutinin (HA)-tagged version of Munc13-1, rat Munc13-1 cDNA was obtained by PCR using the first-strand cDNA from rat brain as the template, and subcloned into the pCIneo3HA vector to produce the expression plasmid, pCIneo-3HA-Munc13-1 (HA-Munc13-1). The expression plasmid for the HA-tagged version of Munc13-4 (HA-Munc13-4) has been described previously (15).

Lysate preparation and western blotting. The preparation of cell lysate from the RBL-2H3 cells was performed as described previously (15). For the preparation of lysate from the brain and small intestine, a procedure of homogenization in lysis buffer was included. After protein assay using a protein assay kit (Bio-Rad, Hercules, CA, USA), the lysates were boiled, and subjected to SDS-PAGE and western blotting as described previously (16). The following primary antibodies were used: rat monoclonal anti-HA (clone 3F10; Roche Diagnostics, Indianapolis, IN, USA), rabbit polyclonal anti-Munc13-1 (cat. #126103; Synaptic Systems, Göttingen, Germany), rabbit polyclonal anti-Munc13-4 (cat. #sc-50465; Santa Cruz Biotechnology, Dallas, TX, USA), and horseradish peroxidase (HRP)-conjugated mouse monoclonal anti-GAPDH (clone 5A12; Wako Pure Chemical, Osaka, Japan). The HRP-conjugated secondary antibodies have been described previously (15). Immunoreactive bands were visualized, and the band intensity, which was normalized to the internal control, was quantified using a luminescent image analyzer, LAS3000 (GE healthcare, Pittsburgh, PA, USA), according to the manufacturer's instructions.

Immunofluorescence microscopy. The RBL-2H3 cells were immunostained as described previously (16). The following primary antibodies were used: anti-HA, anti-Munc13-1, and anti-Munc13-4, as described above, mouse monoclonal anti-CD63 (clone AD1; BD Biosciences, Franklin Lakes, NJ, USA), anti-serotonin (clone 5HT-H209; Abcam, Cambridge, UK), and anti-lysosome-associated membrane protein 1 (LAMP1; clone H4A3, Santa Cruz Biotechnology). Alexa 488-conjugated goat anti-rat immunoglobulin G (IgG), Alexa 594-conjugated

goat anti-mouse IgG, and Alexa 594-conjugated goat anti-rabbit IgG antibodies (Thermo Fisher Scientific, Waltham, MA, USA) were used as secondary antibodies. Fluorescent images of the cells were acquired as described previously (15). The extent of co-localization was expressed as Pearson's correlation coefficient (r), determined by image analysis using ImageJ Fiji (National Institution of Health, Bethesda, MD, USA).

Degranulation assay. β -hexosaminidase (β -hex) release assay was performed as described previously (15). The release of histamine and serotonin was assessed using a histamine enzyme immunoassay kit (Bertin Pharma, Montigny le Bretonneux, France) and a serotonin enzyme-linked immunosorbent assay (ELISA) kit (Enzo Life Sciences, Farmingdale, NY, USA), respectively, according to the manufacturer's instructions.

Statistical analysis. All numerical data presented in this manuscript are mean $\pm$ standard error of the mean (SEM) ($n = 4$ or 10). Statistical significance was then determined by two-tailed Student's t test.

RESULTS

Munc13-1 expression in the RBL-2H3 cells

To investigate the expression of the Munc13 isoforms in mast cells, first, we examined the expression of their mRNAs in the RBL-2H3 cells and BMMCs by RT-PCR. As shown in Fig. 1A, the primer pair for Munc13-1, u/bMunc13-2, or Munc13-3 amplified a single DNA fragment of expected size (600–700 bp) in the rat brain, and the pair for Munc13-4 did so in the RBL-2H3 cells in an RT-dependent manner as expected; however, unexpectedly, the primer pair for Munc13-1 also amplified the same fragment in the RBL-2H3 cells. Similar amplification was observed in the mouse brain and BMMCs (Fig. 1B), suggesting that Munc13-1 was expressed in the mast cells at the mRNA level. The level of Munc13-1 mRNA expression in the RBL-2H3 cells was about 1/30 of that in the rat brain, as determined by quantitative real-time RT-PCR (Fig. 1C).

Next, we examined the expression of the Munc13-1 protein in the RBL-2H3 cells by western blotting using lysates from brain and small intestine as positive and negative controls, respectively (Fig. 1D). The anti-Munc13-1 antibody detected an immunoreactive band of ~ 250 kDa in the brain lysate as expected, and a similar but faint immunoreactive band was also observed in the RBL-2H3 cell lysate

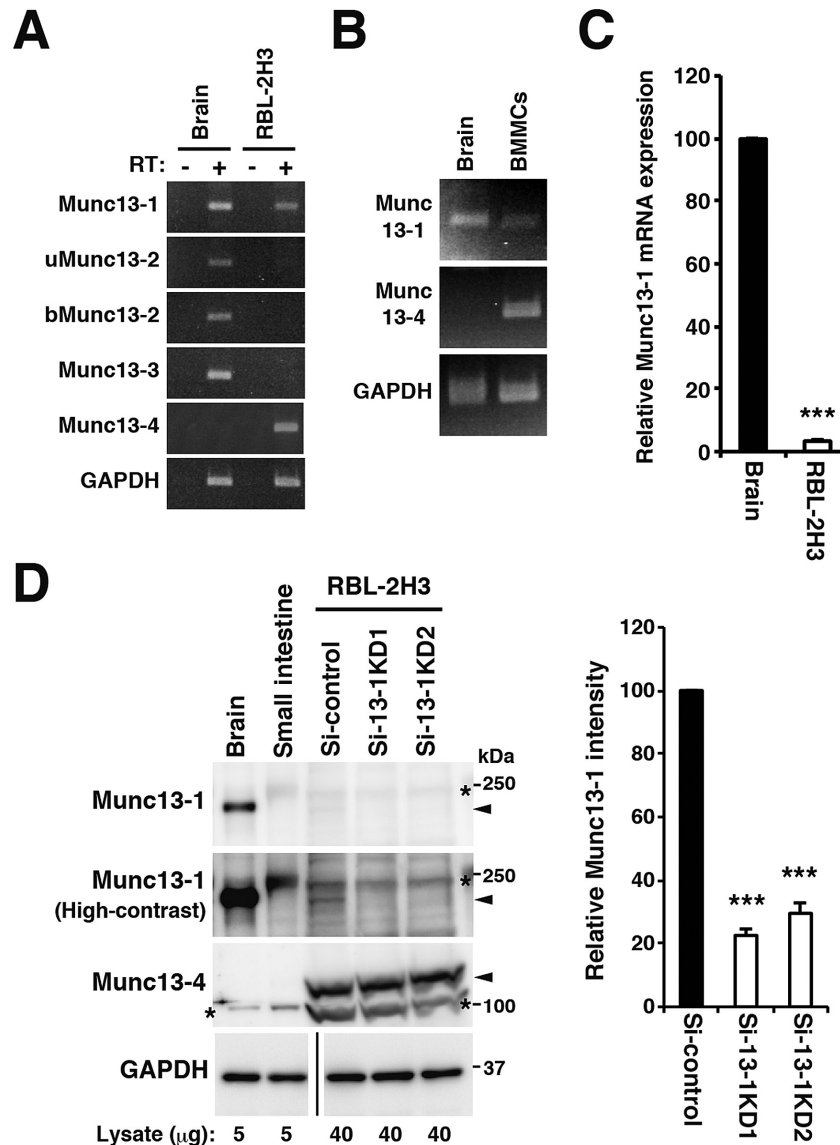


Fig. 1 Expression of Munc13-1 in the RBL-2H3 cells. **(A)** Total RNA from the RBL-2H3 cells and the rat brain was subjected to first-strand cDNA synthesis with (+) or without (–) reverse transcriptase (RT), followed by PCR amplification with primer pairs for all known Munc13 isoforms and GAPDH. **(B)** First-strand cDNAs synthesized from the mouse brain and the BMMCs were subjected to PCR amplification with primer pairs for Munc13-1, Munc13-4, and GAPDH. **(C)** Relative level of Munc13-1 expression compared with GAPDH was determined in the rat brain and the RBL-2H3 cells by quantitative real-time RT-PCR. The level of Munc13-1 mRNA expression in the rat brain was set to 100, and the relative expression level in the RBL-2H3 cells is presented as mean $\pm$ SEM ($n = 4$). *** $P < 0.001$. **(D)** Lysates prepared from the rat brain, small intestine, and G418-surviving RBL-2H3 cells transfected with Si-13-1KD1, Si-13-1KD2, or the Si-control plasmid, were subjected to western blot analyses with antibodies against Munc13-1, Munc13-4, and GAPDH. Arrowheads indicate the position of the Munc13 isoforms. Non-specific bands are marked with an asterisk. The graph represents the relative quantity of Munc13-1 in the Si-13-1KD1/2 transfectants, with the quantity in the Si-control transfectants set to 100. Bars represent mean $\pm$ SEM ($n = 4$). *** $P < 0.001$.

(Si-control). The band intensity had decreased severely (to 20–30% of that of the Si-control) in the lysate of the RBL-2H3 transfectants that expressed Munc13-1-specific siRNA (Si-13-1KD1 or Si-13-1KD2), indicating that the band represented the

Munc13-1 protein. These siRNAs did not alter the intensity of the Munc13-4 band. Taken together, these results suggested that at least two Munc13 isoforms, Munc13-1 and Munc13-4, are expressed in mast cells.

Functional involvement of Munc13-1 in the antigen-induced degranulation of the RBL-2H3 cells

To examine whether Munc13-1 was involved in the degranulation of the RBL-2H3 cells, first, we analyzed the antigen-induced release of the lysosomal enzyme β -hex, a well-known marker for mast cell degranulation (22), from the cells transfected with the expression plasmid, HA-Munc13-1. The expression of HA-Munc13-1 was confirmed by western blotting using an anti-HA antibody, which showed an immunoreactive band of ~250 kDa that disappeared because of the co-expression of Munc13-1-specific siRNA (Fig. 2A). As shown in Fig. 2B, the antigen-induced release of β -hex from the HA-Munc13-1 transfectants was less than that from the control transfectants. Next, we assessed the antigen-induced β -hex release from individual Munc13-1-knockdown RBL-2H3 cells (Si-13-1KD1 and Si-13-1KD2), and found that both the knockdown cell types showed greater β -hex release than the control cells (Fig. 2C). The time course of the antigen-induced β -hex release was unaffected by the overexpression or knockdown of Munc13-1 (data not shown).

The β -hex release assay alone cannot discriminate between degranulation and the Ca^{2+} -dependent release of lysosomes in mast cells (5), because β -hex is widely distributed in lysosome-related organelles. Therefore, we further analyzed the antigen-induced release of histamine and serotonin, the preformed mediators specifically stored in SGs (26). The Munc13-1-knockdown cells were found to exhibit greater antigen-induced release of these mediators than the control cells (Fig. 2D). Taken together, these results strongly suggested an inhibitory role of Munc13-1 in antigen-induced mast cell degranulation. However, the effect of the overexpression or knockdown of Munc13-1 almost disappeared during degranulation induced by Ca^{2+} ionophores (supporting data Fig. S1), possibly because such moderate phenotypes are neutralized by the non-physiological potent activation, as is the case of the inhibitory effect of Rab27a on the degranulation of BMMC (35).

Intracellular localization of Munc13-1 in the RBL-2H3 cells

Because Munc13-1 appeared to be involved in mast cell degranulation, next, we investigated whether Munc13-1 as well as Munc13-4 was localized on SGs in the RBL-2H3 cells by immunofluorescence microscopy. Unfortunately, both the anti-Munc13-1 and anti-Munc13-4 antibodies could detect Munc13 proteins that were expressed exogenously, but not endogenously (supporting data Fig. S2). Therefore,

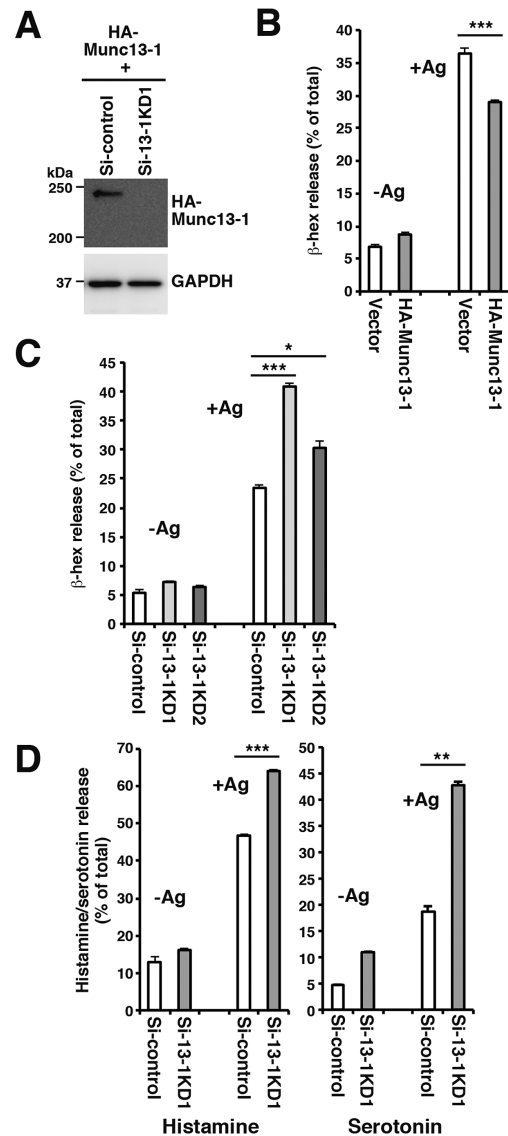


Fig. 2 Involvement of Munc13-1 in the antigen-induced degranulation of the RBL-2H3 cells. **(A)** Lysates prepared from the G418-surviving cells transfected with the HA-Munc13-1 plasmid, together with Si-13-1KD1 or the Si-control plasmid, were subjected to western blot analyses with anti-HA and anti-GAPDH antibodies. **(B)** G418-surviving cells transfected with the HA-Munc13-1 plasmid or the control vector were sensitized with IgE, followed by the stimulation with (+Ag) or without (-Ag) antigen for 20 min. After the measurement of β -hex activity in the supernatant and the cell lysate, the degree of release was expressed as the percentage of total activity. **(C)** Degree of β -hex release from the G418-surviving cells transfected with Si-13-1KD1, Si-13-1KD2, or the Si-control plasmid was examined as in (B). **(D)** G418-surviving cells transfected with Si-13-1KD1 or the Si-control plasmid were sensitized and stimulated as in (B). After the determination of the content of histamine or serotonin in the supernatant and the cell lysate using ELISA, the degree of release was expressed as the percentage of the total content. (B–D) Bars represent mean $\pm$ SEM ($n = 4$). *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$.

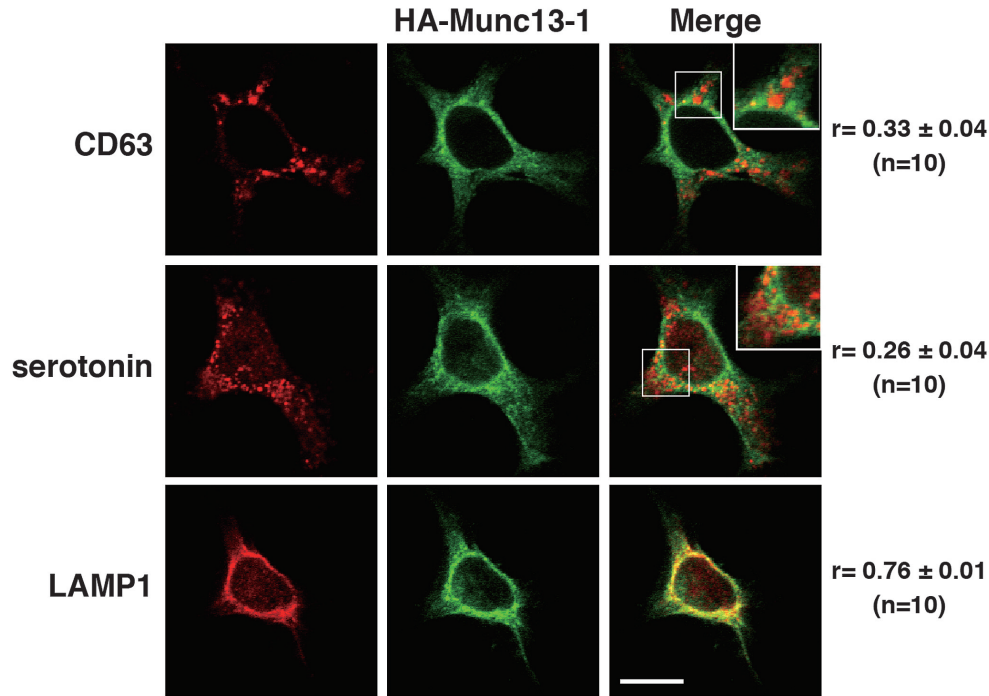


Fig. 3 Intracellular localization of Munc13-1 in the RBL-2H3 cells. G418-surviving cells transfected with the HA-Munc13-1 plasmid were fixed and immunostained with the anti-HA antibody together with one of the following antibodies against the respective organelle marker: anti-CD63 (SG), anti-serotonin (SG), and anti-LAMP1 (late endosome/lysosome). The merged image is shown on the right in each pair. In the merged image of the co-immunostaining with the anti-CD63 or anti-serotonin antibody together with the anti-HA antibody, the magnified image of the boxed region is inset. The extent of co-localization was expressed as mean $\pm$ SEM of the values of the Pearson's correlation coefficient (r) of ten stained cells. Bar, 15 μ m.

we examined the intracellular localization of HA-Munc13-1 using the anti-HA antibody, together with the antibodies against the SG markers, CD63 and serotonin, or the late endosome/lysosome marker, LAMP1. As shown in Fig. 3, HA-Munc13-1 was distributed in the cytoplasm; however, it was highly concentrated around the nucleus, whose perinuclear distribution overlapped with that of LAMP1 (Pearson's correlation coefficient, $r = 0.76 \pm 0.01$, $n = 10$), rather than that of CD63 ($r = 0.33 \pm 0.04$, $n = 10$) and serotonin ($r = 0.26 \pm 0.04$, $n = 10$). Moreover, the distribution of HA-Munc13-1 and the SG markers in the perinuclear region often appeared to be complementary (Fig. 3, insets). In contrast, the co-localization of HA-Munc13-4 with the SG markers was extensive ($r = 0.84 \pm 0.01$, $n = 10$, compared with CD63; $r = 0.79 \pm 0.02$, $n = 10$, compared with serotonin); however, the co-localization with LAMP1 was less ($r = 0.38 \pm 0.04$, $n = 10$) (supporting data Fig. S3). These results suggested that unlike Munc13-4, Munc13-1 is presented both in the cytoplasm and on the late endosome/lysosome.

Functional relationship between Munc13-1 and Munc13-4

Munc13-4 is a key regulator of mast cell degranulation (11, 35, 38). To investigate whether Munc13-1 regulates antigen-induced mast cell degranulation independent of Munc13-4, we analyzed the effect of Munc13-4 downregulation on the hypersecretion phenotype of the Munc13-1-knockdown RBL-2H3 cells (Fig. 4). The knockdown efficiency of Munc13-4-specific siRNA was confirmed previously (15). As expected, the knockdown of Munc13-1 and Munc13-4 resulted in the hyper- and hyposecretion of β -hex, respectively, when the cells were stimulated with antigen; however, little effect was observed on spontaneous β -hex release without stimulation. Notably, when the knockdown of these proteins was combined, the increase in β -hex release, which was caused by Munc13-1 knockdown, was considerably attenuated. These results suggested that in antigen-induced mast cell degranulation, Munc13-1 plays its inhibitory role in a Munc13-4-dependent manner.

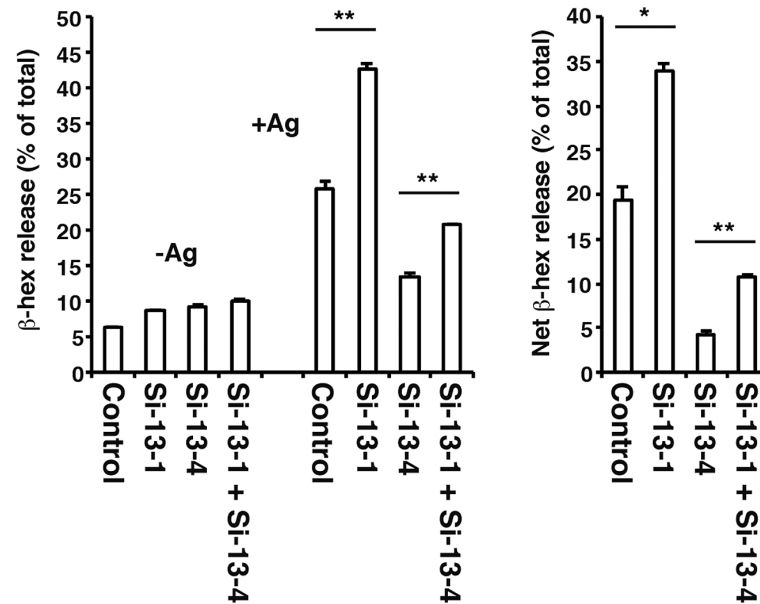


Fig. 4 Munc13-4-dependent hypersecretion phenotype of the Munc13-1-knockdown RBL-2H3 cells. G418-surviving cells transfected with the following combinations of plasmid and siRNA: Si-control plasmid with control siRNA (Control), Si-13-1KD1 plasmid with control siRNA (Si-13-1), Si-control plasmid with Munc13-4-specific siRNA (Si-13-4), or Si-13-1KD1 plasmid with Munc13-4-specific siRNA (Si-13-1 + Si-13-4), were sensitized with IgE, and stimulated with (+Ag) or without (–Ag) antigen for 20 min. The degree of β -hex release was assessed and expressed as in Fig. 2B. The net β -hex release represented the degree of antigen-induced release, from which basal release without stimulation was subtracted. Bars represent mean $\pm$ SEM ($n = 4$). ** $P < 0.01$; * $P < 0.05$.

DISCUSSION

Munc13-1 has been recently found to be expressed together with Munc13-4 in CTLs, and both the proteins have been shown to positively regulate the priming process in lytic granule exocytosis, indicating their functional overlap (10). In the present study, we showed that Munc13-1, as well as Munc13-4, is endogenously expressed in the RBL-2H3 mast cell line; however, unexpectedly, we found that in contrast to its effect on CTLs, Munc13-1 inhibited the antigen-induced degranulation of the RBL-2H3 cells. This appears to be the first report describing the inhibitory effect of the proteins of the Munc13 family on Ca^{2+} -dependent regulated exocytosis.

Similar to other secretory cells, mast cells release preformed mediators by both simple and compound exocytosis. One mode of compound exocytosis is sequential, wherein the initial SG-PM heterotypic fusion results in the formation of the fused but stabilized SGs on the PM, and deeper-lying SGs then fuse with the primary SGs. The other mode is multivesicular, wherein the SG-SG homotypic fusion results in the formation of large exocytic structures, which then fuse with the PM (29). The molecular aspects of compound exocytosis are still largely un-

known. Recently, Munc13-4 has been shown to positively regulate both the SG-PM and SG-SG fusion during mast cell degranulation by promoting the formation of their respective *trans*-SNARE complexes in a Ca^{2+} -dependent manner (38). Our double knock-down study (Fig. 4) suggested that Munc13-1 inhibited certain Munc13-4-dependent exocytic processes. What is then the mechanism of the inhibition? Based on the conserved SNARE-binding region of the Munc13 family of proteins (18, 20), Munc13-1 is likely to negatively regulate antigen-induced mast cell degranulation through its Ca^{2+} -dependent SNARE-binding activity rather than through other unknown mechanism(s), which could involve the interference of the Munc13-4-mediated formation of *trans*-SNARE complexes.

In other hematopoietic cells, Munc13-4 has been shown to function in the maturation and/or trafficking of late endosomes, in addition to its role in exocytosis (14, 25). Upon multivesicular exocytosis in mast cells, late and recycling endosomes have been shown to fuse sequentially with the growing exocytic structures formed by Munc13-4-dependent SG-SG fusion (SG-derived exocytic structures) (38). Because the SGs in the mast cells themselves possess endosomal properties, especially those of late endo-

somes (9, 37, 38), it is possible that in mast cells, the Munc13 isoforms comprehensively regulate these endosome-related processes, in addition to the fusion of the SGs/SG-derived exocytic structures with the PM. If so, the hypersecretion phenotype of the Munc13-1-knockdown RBL-2H3 cells may be an indirect consequence of the increased number, size, or secretion competence of the SG/SG-derived exocytic structures.

The opposite role of Munc13-1 in regulated exocytosis in CTLs and mast cells is possibly a result of the difference in the modes of exocytosis between these cell types. The details of mast cell degranulation have not been fully elucidated by conventional biochemical and morphological methods. Therefore, in order to gain comprehensive molecular insights into mast cell degranulation in future studies, we have to develop live-cell imaging systems suitable for the spatiotemporal capture of the images of the three modes of exocytosis, single, multivesicular, and sequential.

CONFLICT OF INTEREST

The authors have no conflict of interests.

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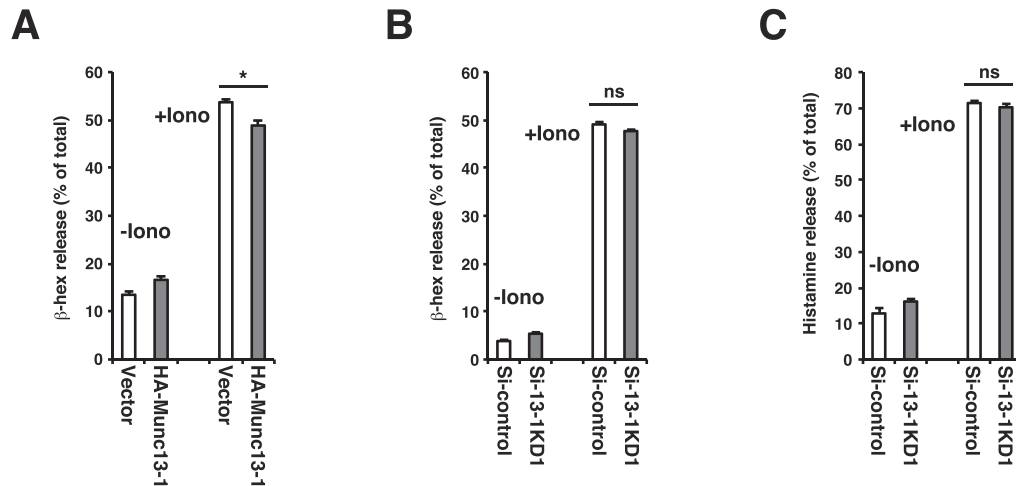


Fig. S1 Effects of the overexpression or knockdown of Munc13-1 on A23187-induced degranulation of the RBL-2H3 cells. **(A)** G418-surviving cells transfected with the HA-Munc13-1 plasmid or the control vector were stimulated with (+Iono) or without (–Iono) A23187 for 20 min in the presence of the phorbol ester, 12-*O*-tetradecanoylphorbol-13-acetate. After the measurement of β -hex activity in the supernatant and the cell lysate, the degree of release was expressed as the percentage of total activity. **(B)** Degree of β -hex release from the G418-surviving cells transfected with Si-13-1KD1 or the Si-control plasmid was examined as in (A). **(C)** Histamine content in the supernatant and the cell lysate prepared in (B) was determined by ELISA, and the degree of release was expressed as the percentage of the total content. (A–C) Bars represent mean $\pm$ SEM ($n = 4$). * $P < 0.05$; ns, not significant.

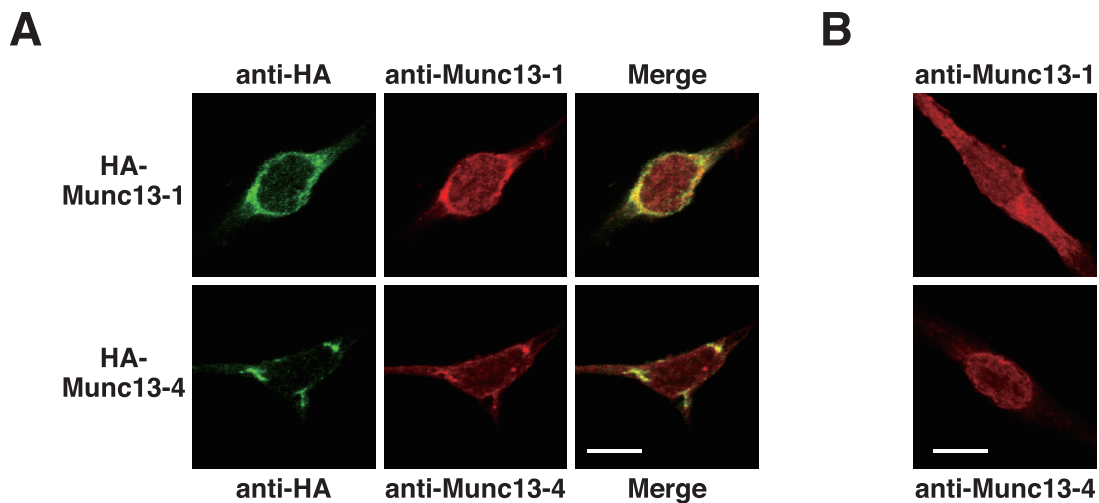


Fig. S2 Antigen recognition by the anti-Munc13-1 and anti-Munc13-4 antibodies as observed through immunofluorescent microscopy. **(A)** G418-surviving RBL-2H3 cells transfected with the HA-Munc13-1 and HA-Munc13-4 plasmids were fixed and immunostained with the anti-Munc13-1 and anti-Munc13-4 antibodies, respectively, together with the anti-HA antibody. The merged image is shown on the right in each row. **(B)** RBL-2H3 cells were fixed and immunostained with anti-Munc13-1 or anti-Munc13-4. Bars, 15 μ m.

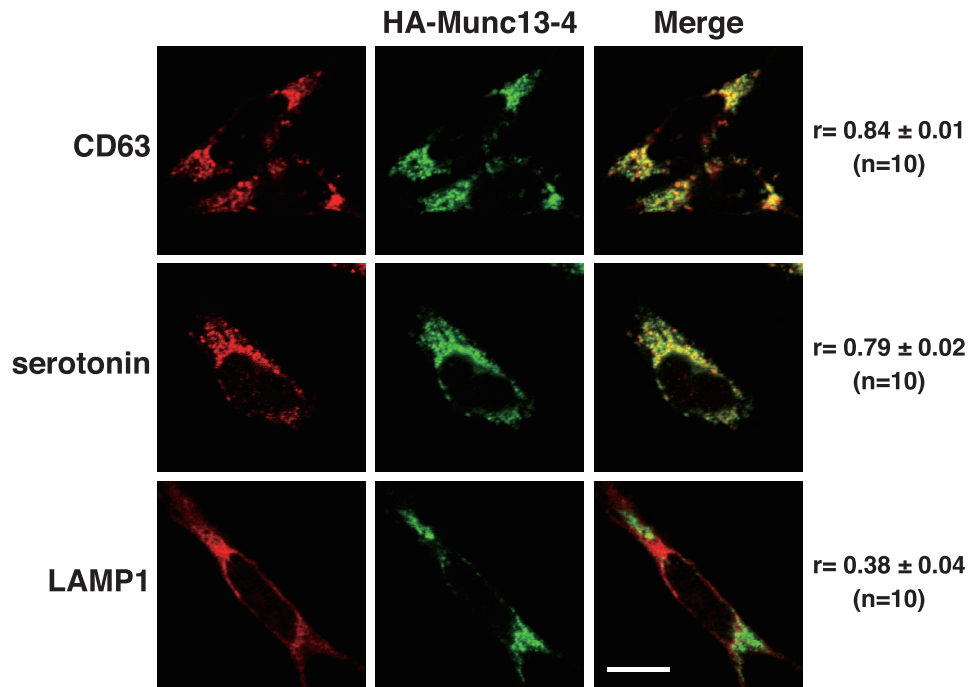


Fig. S3 Intracellular localization of Munc13-4 in the RBL-2H3 cells. G418-surviving cells transfected with the HA-Munc13-4 plasmid were fixed and immunostained with the anti-CD63, anti-serotonin, or anti-LAMP1 antibody together with the anti-HA antibody. The merged image is shown on the right in each pair. The degree of co-localization was expressed as mean $\pm$ SEM of the values of Pearson's correlation coefficient (r) of ten stained cells. Bar, 15 μ m.