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Liprin-α is involved in exocytosis and cell spreading in mast cells

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ABSTRACT

The active zone is a specialized region of the presynaptic plasma membrane where the neurotransmitter release occurs by exocytosis. Mast cells also release inflammatory mediators by exocytosis resulting in induction of allergic responses. In our previous reports, we found that active zone proteins, Munc13-1 and ELKS regulates exocytosis of mast cell positively. In this study, we investigated the involvement of liprin-α, another active zone protein, in exocytosis in mast cells.

We found that three isoforms of liprin-α, liprin-α1, -α2 and -α3 were expressed. Immunocytochemical experiments revealed that liprin-α1 resided both in the cytoplasm and on the plasma membrane. Upon stimulation with antigen, the area of a cell increased remarkably due to cell spreading and the distribution of liprin-α1 became punctuated. Interestingly, knockdown of liprin-α1 caused decrease in exocytotic release and cell spreading. These results suggest that liprin-α1 facilitates exocytosis and cell spreading, and these events might have correlated each other in mast cells.

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1. Introduction

Mast cells are specialized secretory cells that play a pivotal role in type I allergies such as pollinosis and asthma [1,2]. Type I allergic responses are evoked by inflammatory mediators secreted from mast cells by exocytosis (degranulation) upon stimulation [3]. We have studied the mechanism of exocytosis of mast cells and found that the exocytotic machinery in mast cells is similar to the processes involved in neurotransmitter release in neuronal cells. In fact, soluble N-ethyl maleimide-sensitive factor attachment protein receptor (SNARE) proteins and their binding proteins [4–12] serve as a basic machinery of exocytotic release in both cell types. However, in terms of structural features, a major difference between mast cells and neuronal cells is an active zone where exocytotic vesicle fusion occurs at nerve terminals. Synaptic vesicles are docked to a specialized region of the presynaptic plasma membrane known as the active zone [13–17], whereas secretory granules appear to be distributed uniformly throughout the mast cells.

Although various proteins involved in synaptic vesicle fusion have been previously isolated and characterized [18,19], the molecular mechanisms by which they are properly localized at the active zone remains unclear. The cytomatrix at the active zone (CAZ) is assumed to be involved in determining the site of synaptic vesicle fusion [20–22]. Thus, identifying and characterizing molecular components of CAZ is a crucial step for understanding its organization and the targeting mechanism of synaptic vesicles to the active zone. Several proteins specific to the active zone have been previously reported such as Munc13 [23], Rab3 interacting molecule1 (RIM1) [24], bassoon [25], Piccolo/Aczonin [26,27], liprin-α [28], CAZ-associated structural protein and ELKS [29–31]. Recently, we found the active zone proteins Munc13-1 [32] and ELKS [33] are expressed in mast cells, and their knockdown caused impaired exocytotic release, suggesting that these active zone proteins positively regulate exocytosis. In addition to active zone proteins, involvement of lipid rafts in exocytosis in mast cells has been suggested. The ternary SNARE complex comprised SNAP23/syntaxin-4/VAMP-2 is enriched in lipid rafts [34], and disruption of lipid rafts by methyl-β-cyclodextrin inhibits exocytotic membrane fusion [35]. Furthermore, Munc13-1 targeted to the raft domain in the plasma membrane increased exocytotic release, while Munc13-1 targeted to the non-raft region had no effect on exocytosis [32]. Therefore, probably the enriched SNARE complexes and active zone proteins are formed in a raft-like structure during exocytotic release in mast cells.

In invertebrates, liprin-α is necessary to recruit active zone proteins, synaptic vesicles, and calcium channels to the active zone [36,37]. In the present study, we examined the expression and roles of the active zone protein liprin-α, since it is essential for the formation of an exocytosis site. We detected expression of liprin-α, and found that one of its isoforms, liprin-α1, positively regulates exocytosis in mast cells. Interestingly, knockdown of liprin-α1 inhibited not only exocytotic release but also cell spreading.

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Table 1
Sequences of primer pairs used to amplify the target genes.

Target gene	Forward primer	Reverse primer
Liprin-α1	5′-GTGTCCAGTGAGGTGGAGGT	5′-CGAGGTAGCGTTTCTCAAGG
Liprin-α2	5′-AGTGGAACAAGAAGCCGAGA	5′-CCCAACTGAATACCGCAACT
Liprin-α3	5′-ATCACCACNCTGGARAAGCGCTA	5′-CMSCCATWCGYTCCTTGAGGT
Liprin-α4	5′-GTCGCTATACGGCTCTGAGG	5′-GCCAGGGTTGTTTTGGTAGA

2. Materials and methods

2.1. Cell culture

Rat basophilic leukemia (RBL-2H3) cells were cultured in Eagle's minimal essential medium purchased from Nissui (Tokyo, Japan) with 10% fetal calf serum at 37 °C in a humidified atmosphere with 5% CO_2.

2.2. Plasmid construction and transfection

Poly(A)$^+$ RNA was obtained from RBL-2H3 cells (1×10^7) with a QuickPrep Micro mRNA Purification Kit (GE Healthcare), and served as a template for cDNA synthesis with SuperScript III RT (Invitrogen). To amplify active zone proteins, degenerated primer pairs were used as summarized in Table 1.

PCR products were electrophoresed and extracted from an agarose gel to be subcloned into the TOPO/TA cloning vector pCRII (Invitrogen). Cloned PCR products were sequenced with a capillary DNA sequencer (ABI3130, Applied Biosystems) using a M13 primer.

To knockdown liprin-α1, two different target sequences were designed by BLOCK-iT™ RNAi Select. Target sequences (siRNA1: 5′-TGCGCTTACAGGCTGCACA, siRNA2: 5′-CAACACTACTGCTGTTGGA) were ligated into a siRNA expression plasmid (BLOCK-iT™ RNAi Express, Invitrogen). A negative control plasmid (pcDNA 6.2-GW/EmGFP-miR-neg control) supplied with the siRNA expression plasmid was used as the control plasmid. Plasmids expressing siRNA were electroporated in cold phosphate-buffered saline with 15 μg of plasmid DNA at 330 V and 250 μF using the Gene Pulser II (Bio-Rad). Stable transfectants were selected using blasticidin (10 μg/ml).

2.3. Antibodies

Anti-ELKS antibody was a gift from Ohtsuka [29]. Rabbit monoclonal antibody (mAb) against ELKS (anti-ELKS mAb) was raised against a peptide corresponding to amino acid (aa) 117–142, according to standard methods. Mouse anti-flotillin-2 (Becton Dickinson), goat anti-liprin-α1 (Santa Cruz Biotechnology), rabbit anti-GAPDH (Santa Cruz Biotechnology), Hilyte Fluor™555-labeled anti-rabbit IgG (Ana Spec Inc.), Hilyte Fluor™647-labeled anti-rabbit IgG (Ana Spec Inc.), and DyLight™488-labeled anti-goat IgG (ROCKLAND) antibodies were purchased from commercial sources.

2.4. Western blotting

RBL-2H3 cells (5×10^6) were lysed with lysis buffer (1% Triton X-100, 150 mM NaCl, 1 mM DTT, 0.5 mM EDTA, 1 mM PMSF, and 20 mM Tris–HCl, pH 7.5). After centrifugation at 16,000 rpm for 20 min, the supernatant was mixed with an equal volume of SDS sample buffer and boiled for 5 min. Samples were electrophoresed by SDS-PAGE and transferred to a PVDF membrane. After blocking with phosphate buffer containing 5% skim milk, blots were probed with a primary antibody for ELKS (dilution 1:1000), anti-liprin-α1 (dilution 1:200), and anti-GAPDH (dilution 1:400). After being washed with 0.1% Tween 20 in PBS, the membrane was treated with secondary antibodies conjugated with horseradish peroxidase (dilution 1:1000, Santa Cruz Biotechnology). Immunoreactivity was detected by enhanced chemiluminescence (Amersham Bioscience) using a LAS-3000 mini (FUJI FILM, Tokyo Japan). When primary and secondary antibodies were diluted, Can Get Signal™ solution 1 and 2 (TOYOBO) was used, respectively.

2.5. Assay of secreted β-hexosaminidase

Degranulation of RBL-2H3 cells was monitored by measuring the activity of a granule-stored enzyme β-hexosaminidase secreted in the cell supernatant [35]. Cells were seeded in a 24-well plate (2×10^5 cells/well). After cells were washed with HEPES-buffered saline (140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 0.6 mM $MgCl_2$, 0.1% glucose, 0.1% BSA, and 10 mM HEPES, pH 7.4), they were sensitized by anti-DNP IgE (200 ng/ml) for 15 min and incubated with an average of six DNP groups conjugated with BSA (DNP_6-BSA) in 200 μl HEPES-buffered saline for 30 min at 37 °C. Aliquots of supernatants were transferred to a 96-well plate (20 μl/well) and incubated

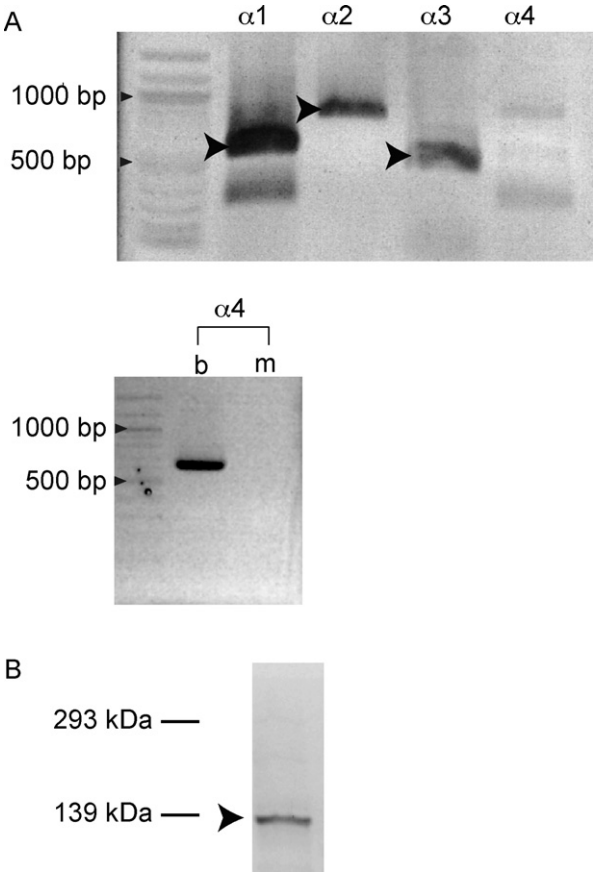

Fig. 1. Expression of liprin-α isoform in RBL-2H3 cells. (A) RT-PCR analysis of liprin-α isoforms. Sequence analysis revealed that each band indicated by an arrowhead corresponds to liprin-α1, -α2 and -α3. Liprin-α4 was not detected. Positive control of liprin-α4 is shown in the lower panel using rat brain (b: brain, m: mast cell). (B) Whole cell lysate of RBL-2H3 was electrophoresed and was subjected to Western blotting analysis using a specific antibody for liprin-α1.

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