

Flotillin-1 Regulates IgE Receptor-Mediated Signaling in Rat Basophilic Leukemia (RBL-2H3) Cells

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Cross-linking of high-affinity IgE receptors by multivalent Ag on mast cells (rat basophilic leukemia (RBL)-2H3) induces the phosphorylation of ITAM motifs of an IgE receptor by Src family tyrosine kinase, Lyn. The phosphorylation of IgE receptors is followed by a series of intracellular signals, such as Ca^{2+} mobilization, MAPK activation, and degranulation. Therefore, Lyn is a key molecule in the activation of mast cells, but the molecular mechanisms for the activation of Lyn are still unclear. Recently, it is suggested that the localization of Lyn in lipid rafts is critical for its activation in several cell lines, although the precise mechanism is still unknown. In this study, we found that flotillin-1, which is localized in lipid rafts, is involved in the process of Lyn activation. We obtained flotillin-1 knockdown (KD)² rat basophilic leukemia (RBL)-2H3 cells, which express a low level of flotillin-1. In the flotillin-1 KD cells, we observed a significant decrease in Ca^{2+} mobilization, the phosphorylation of ERKs, tyrosine phosphorylation of the γ -subunit of IgE receptor, and IgE receptor-mediated degranulation. We also found that flotillin-1 is constitutively associated with Lyn in lipid rafts in RBL-2H3 cells, and Ag stimulation induced the augmentation of flotillin-1 binding to Lyn, resulting in enhancement of kinase activity of Lyn. These results suggest that flotillin-1 is an essential molecule in IgE receptor-mediated mast cell activation, and regulates the kinase activity of Lyn in lipid rafts. *The Journal of Immunology*, 2006, 177: 147–154.

Cross-linking of IgE receptors (FcεRI) by multivalent Ag leads to a cascade of events, which include activation of the Src family tyrosine kinase Lyn, the interaction of lipid rafts with IgE receptors, and the tyrosine phosphorylation of IgE receptors (1–7). These molecular events propagate a series of intracellular signals, which result in elevation of the intracellular Ca^{2+} concentration, translocation of MAPK from the cytoplasm to the nucleus, and, finally, exocytotic release of inflammatory mediators such as serotonin and histamine (8, 9).

Among molecules involved in the early events of mast cell activation, the tyrosine kinase Lyn plays a pivotal role in signal transduction, because the tyrosine phosphorylation of FcεRI by Lyn is the earliest detectable event before receptor cross-linking. In Lyn-deficient mast cells, subsequent intracellular signals, such as tyrosine phosphorylation of FcεRI and Ca^{2+} mobilization, are impaired (10). Therefore, the regulation of Lyn activity affects the entire cascade activated by Ag stimulation.

Recently, it has been suggested that tyrosine phosphorylation of FcεRI by Lyn is initiated by the association of Lyn with FcεRI mediated via membrane microdomains called lipid rafts (1, 6). Lipid rafts are microdomains in the plasma membrane that are rich in cholesterol, glycosphingolipid, and sphingolipids. Lipid rafts are

insoluble in nonionic detergents (Triton X-100) and include numerous signal molecules such as GPI-anchored proteins, G proteins, and Src family protein kinases (2, 3, 11, 12). Therefore, lipid rafts function as a platform from which various intracellular signals propagate. It has been reported that almost all Lyn constitutively resides in lipid rafts due to modification by unsaturated fatty acids at the N terminus (13, 14). Through the use of methyl- β -cyclodextrin (M β CD), which disrupts lipid rafts, the role of lipid rafts in FcεRI-mediated signaling has been studied intensively, particularly with regard to early events such as tyrosine phosphorylation of FcεRI and the localization of Lyn. Treatment of mast cells with M β CD causes a decrease in the level of tyrosine phosphorylation of FcεRI and changes the localization of Lyn (15, 16). According to this view, Lyn is localized in lipid rafts from which FcεRI is excluded, but FcεRI is rapidly translocated into lipid rafts after receptor cross-linking, and is phosphorylated by Lyn kinase (15, 16). In addition, Young et al. (17) provided evidence that Lyn isolated in lipid rafts has substantially higher Lyn kinase activity than that outside of lipid rafts. These results suggest that some unknown components in lipid rafts may influence the kinase activity of Lyn. However, the relationship between the activation of Lyn and lipid rafts is not yet understood.

Flotillin-1 is a novel constituent of lipid rafts (18, 19). It was initially identified as a caveolae-associated membrane protein and is a marker protein of lipid rafts, but its physiological role is not clear. Because flotillin-1 possesses recently identified, but as yet uncharacterized functional domains, such as prohibitin homology and stomatin/prohibitin/flotillin/HflK/C domains (20), it may act as a signaling molecule in numerous cells.

In the present study, we examined the possibility that flotillin-1 regulates the activity of Lyn kinase in the early process of FcεRI-mediated signaling in mast cells. We observed significant decreases in Ca^{2+} mobilization, the phosphorylation of ERKs, the tyrosine-phosphorylation level of the γ -chain of FcεRI, and degranulation in flotillin-1 KD cells. Furthermore, we found that flotillin-1 is associated with Lyn and enhances the kinase activity of Lyn.

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² Abbreviations used in this paper: KD, knockdown; RBL, rat basophilic leukemia; BMMC, bone marrow-derived mast cell; Cbp, Csk-binding protein; IP₃, inositol 1,4,5-trisphosphate; m-flotillin-1, mutated flotillin-1; m-Lyn, mutated Lyn; M β CD, methyl- β -cyclodextrin; OE, overexpressing; PVDF, polyvinylidene difluoride; SOS, son of sevenless; WT, wild type.

Materials and Methods

Materials

ATP, A23187, PMA, α -casein, and *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide were purchased from Sigma-Aldrich. Triton X-100 and PMSF were from Wako Pure Chemical. All other reagents were of the highest grade available commercially.

Cell culture

RBL-2H3 cells were cultured in Eagle's MEM from Nissui Pharmaceutical with 10% FCS (Boehringer Mannheim) at 37°C in an atmosphere of 5% CO₂.

Plasmid construction and transfection

Poly(A)⁺ RNA was obtained with a QuickPrep Micro mRNA Purification Kit (Amersham Biosciences) from 1×10^7 RBL-2H3 cells, and served as a template for cDNA synthesis with SuperScript II RT (Invitrogen Life Technologies), as reported previously (4). The primer pair for rat flotillin-1 was 5'-CTCGAGATGTTTTCACCTGTGGCCC-3' (sense; *Xho*I site is underlined)/5'-GAATTCATGCTGCTCCTTAAAGGCTTG-3' (antisense; *Eco*RI site is underlined). PCR products were extracted from agarose gel with Gene Clean (Bio 101) and subcloned into the TA cloning vector pCRII (Invitrogen Life Technologies). Cloned PCR products were sequenced with a 3100-Avant Genetic Analyzer (Applied Biosystems), and verified cDNA was ligated with pcDNA3.1⁺ for overexpression or pcDNA3.1⁻ (Invitrogen Life Technologies) for KD. RBL-2H3 cells were electroporated in cold PBS with 20 μ g of plasmid DNA at 250 V and 950 μ F using Gene Pulser II (Bio-Rad) (21).

Western blotting

RBL-2H3 cells were lysed in the lysis buffer mentioned above. Lysate was mixed with an equal volume of Laemmli sample buffer and boiled for 5 min. Samples were electrophoresed by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. After blocking with phosphate buffer containing 5% nonfat milk, blots were probed with primary Ab for flotillin-1 (clone 18; BD Transduction Laboratories), Lyn (polyclonal; Santa Cruz Biotechnology), or Cbp (H-100; Santa Cruz Biotechnology) for 1 h. After being washed with 0.1% Tween 20 in PBS, the membrane was treated with anti-mouse IgG conjugated with HRP. Immunoreactivity was detected by ECL (Amersham Biosciences) with a Lumi-Imager F1 (Roche Diagnostic Systems) and analyzed by NIH Image.

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde in HEPES buffer. After washing with HEPES buffer, cells were treated with 0.2% Triton X-100. Cells were labeled with primary Ab (2.5 μ g/ml) for Lyn for 45 min. After washing, FITC-conjugated anti-mouse IgG (Organon Teknika) was added (2.5 μ g/ml), and the mixture was incubated for 30 min. After washing, immunofluorescence images were obtained with a confocal laser microscope (LSM510; Carl Zeiss) with an argon ion laser. Samples were excited at 488 nm, and fluorescence was collected through a band filter (505–525 nm). The signal level of autofluorescence was obtained from nontransfected cells treated with the same staining procedure mentioned above. This signal level was subtracted from original fluorescent images of samples using an imaging processing software of LSM510.

Sucrose gradient centrifugation

Cells (5×10^7 cells) were harvested from culture dishes and washed twice with HEPES-buffered saline. Cells were lysed with the lysis buffer (1% Triton X-100, 5 mM EDTA, and 1 mM PMSF in TBS) for 1 h at 4°C. Aliquots (1.5 ml) of cell lysate were mixed with an equal volume of 80% sucrose in sucrose solution (1% Triton X-100, 5 mM EDTA, 120 mM NaCl, 25 mM Tris-HCl (pH 7.4), and 2 mM PMSF) and placed at the bottom of a centrifuge and overlaid with 30% sucrose solution (6 ml) and 5% sucrose solution (4 ml) in this order. The gradient was centrifuged in a SW40 Ti (Beckman Coulter) rotor at 40,000 rpm for 19 h at 4°C. Fractions (1 ml) were picked up sequentially from the top of the gradient.

Evaluation of degranulation

Degranulation of RBL-2H3 cells was monitored by measuring the activity of a granule-stored enzyme, β -hexosaminidase, secreted in cell supernatant (21). Cells were seeded in 24-well plates (2×10^5 cells/well). After washing cells with HEPES-buffered saline (140 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 0.6 mM MgCl₂, 0.1% glucose, 0.1% BSA, and 10 mM HEPES (pH 7.4)), cells were sensitized by anti-DNP IgE (200 ng/ml) for 30 min and

incubated with an average of six DNP groups conjugated with BSA (DNP₆-BSA) in 200 μ l of HEPES-buffered saline for 30 min at 37°C. For stimulation by calcium ionophore and phorbol ester, cells were stimulated by PMA (50 ng/ml) together with A23187 (1 μ M). Aliquots of supernatants were transferred to a 96-well plate and incubated with 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 Na₂CO₃-NaHCO₃ buffer, absorbance at 405 nm was measured by a microplate reader (MPR-A4; Tosoh). 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.

Intracellular Ca²⁺ measurement

RBL-2H3 cells (4.5×10^5 cells) were loaded with 2 μ M fura 2-AM (Molecular Probes) for 15 min at 37°C and sensitized with anti-DNP IgE (200 ng/ml). After the cells were incubated, they were washed twice with HEPES-buffered saline and stimulated with DNP-BSA (100 ng/ml) in the absence or presence of extracellular free Ca²⁺. The fluorescence intensities at 500 nm excited at 340 and 360 nm were measured at 37°C, and the ratio (F340/F360) was calculated using a spectrofluorometer equipped with a personal computer (RF-5300PC; Shimadzu Scientific Instruments).

Immunoprecipitation and in vitro kinase assay

RBL-2H3 cells were lysed in 0.5 ml of lysis buffer (1% Nonidet P-40, 0.1% SDS, 150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 5 mM EDTA, and 1 mM PMSF). After cells were incubated, the lysate was centrifuged at $14,300 \times g$ for 20 min to remove nuclei and insoluble remnant. The supernatant was incubated overnight with 0.5 μ g of anti-Lyn polyclonal Ab (Santa Cruz Biotechnology) or anti-Fc ϵ R γ -subunit polyclonal Ab (Upstate Biotechnology), and then rotated for 3 h with 30 μ l of ImmunoPure Immobilized Protein G (Pierce) at 4°C. Immunoprecipitates were washed five times with lysis buffer and boiled with Laemmli sample buffer. For the detection of phosphorylation of IgE receptor, immunoprecipitates were analyzed by Western blotting described above using anti-phosphotyrosine Ab (PY20; Santa Cruz Biotechnology). For in vitro kinase assay, immunoprecipitates were washed twice with lysis buffer without detergent and washed twice with kinase assay buffer (20 mM Tris-HCl (pH 7.4), 10 mM MgCl₂, and 1 mM Na₃VO₄). After washing, Lyn immunoprecipitate was subjected to an in vitro kinase assay. Lyn immunoprecipitate was mixed with α -casein (Sigma-Aldrich) as an exogenous substrate and incubated for 30 min at 37°C (17). To terminate the phosphorylation reaction, Laemmli sample buffer was added and the mixture was boiled for 5 min. Phosphorylation of α -casein was detected by Western blotting with PY20.

Assay of the association of Lyn and flotillin-1

We used the CheckMate Mammalian Two-Hybrid System (Promega) to study the association of Lyn and flotillin-1. Briefly, the day before transfection, NIH3T3 cells were seeded in 60-mm dishes at a density of $\sim 1 \times 10^5$ cells. Each plate of cells was transfected with a total of 5.4 μ g of plasmid DNA (mixture of 1:1:1, pBIND-flotillin-1 vector, pACT-Lyn vector, and pG5luc vector) by lipofection using cholesterol-3-carboxyamido ethylene-*N*-hydroxyethylamine as a cationic lipid (22). The cells were harvested and lysed with Picagene cell lysis buffer. Firefly and *Renilla* luciferase activities were quantified with Dual-Luciferase Reporter Assay System (Toyo Ink) using a Luminometer. The results are expressed as a ratio (firefly luciferase activity/*Renilla* luciferase activity).

Results

Expression level of flotillin-1 and its effects on degranulation in mast cells

To investigate the effects of flotillin-1 on Fc ϵ RI-mediated signaling in mast cells, we transfected RBL-2H3 cells with the flotillin-1 gene in an antisense direction and generated three KD clones in which the expression level of flotillin-1 was decreased. We also obtained four overexpressing (OE) clones in which the expression level of flotillin-1 was enhanced. These transfected KD or OE clones behaved similarly. Fig. 1*a* shows the Western blotting analysis of flotillin-1 using whole cell lysates. Expression of intrinsic flotillin-1 was detected in wild-type (WT) RBL-2H3 cells at 48 kDa. We detected lower and higher expressions of flotillin-1 in KD and OE clones, respectively. Because flotillin-1 resides in lipid rafts, we ascertained the expression of flotillin-1 in lipid rafts by

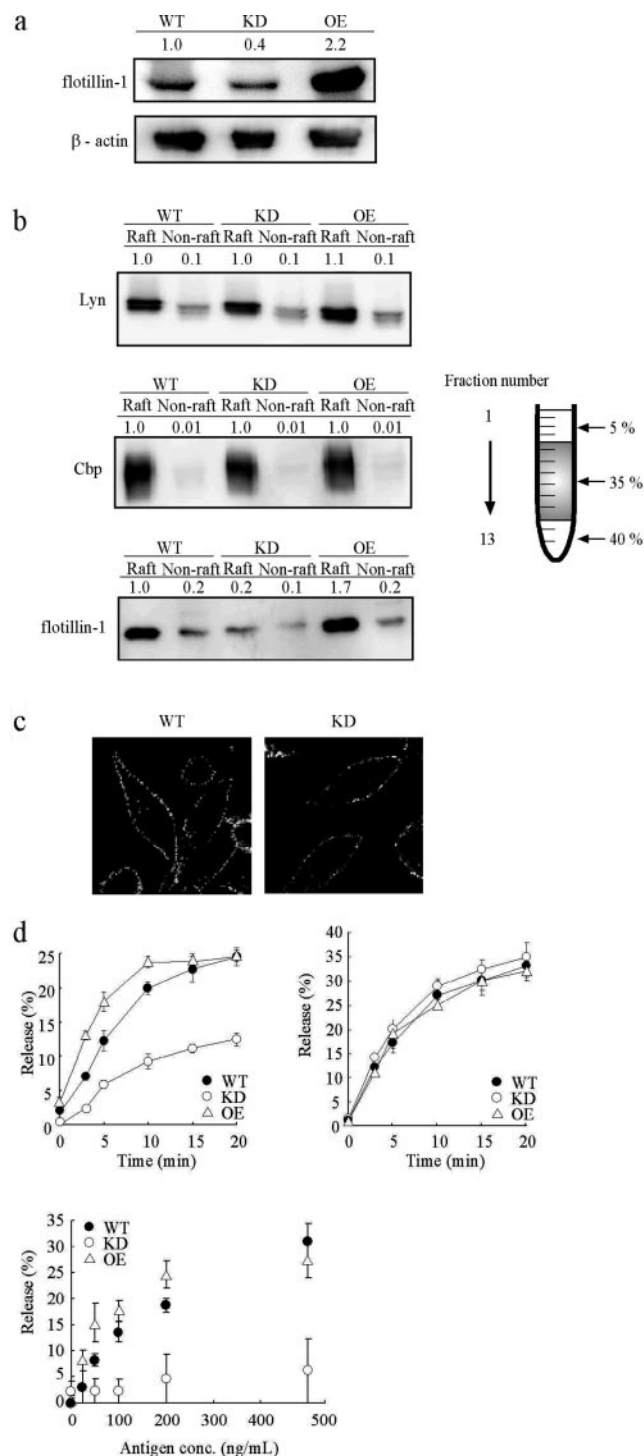


FIGURE 1. Expression level of flotillin-1 and its effects on degranulation. *a*, Western blotting analysis for the expression of flotillin-1. Samples were prepared from cells (WT RBL-2H3, WT; flotillin-1 KD cells, KD; and flotillin-1-OE cells, OE) and electrophoresed by SDS-PAGE. After the samples were transferred to a PVDF membrane, blots were probed with primary Ag for flotillin-1 and visualized with anti-mouse IgG conjugated with HRP using chemiluminescence methods. A band for flotillin-1 was detected at 48 kDa. The expression of β -actin is shown in the lower panel. Figures above each lane stand for relative expression level of flotillin-1 when the level in WT is unit. *b*, Western blotting analysis of raft proteins, Lyn, Cbp, and flotillin-1 in raft and nonraft fraction in WT, KD, and OE cells. Figures above each lane stand for relative expression level of each protein when the level in raft fraction in WT is unit. *c*, Immunostaining of Lyn in WT and KD cells. *d*, The effects of the expression level of flotillin-1 on degranulation. *Left*, Cells (●, WT; ○, KD; △, OE) were incubated with

isolating Triton X-100-insoluble fractions. The expression of flotillin-1 was detected in the lipid raft fraction, and the expression levels in rafts in WT, KD, and OE cells were proportional to those in whole cell lysates (data not shown). Because flotillin is localized in lipid rafts, expression level of flotillin-1 might cause instability of lipid rafts. To examine this possibility, we investigated the expression of Lyn and Csk-binding protein (Cbp). Lyn and Cbp, both of which play key roles in signal transduction in mast cells, were localized in lipid rafts, while Fc ϵ RI was excluded from rafts (6, 15). Therefore, we examined the expression of Lyn and Cbp in raft and nonraft fractions to check the stability of rafts. Fig. 1*b* shows the expression of Lyn and Cbp in lipid rafts (fractions 4 and 5). The expression of Lyn and Cbp in lipid rafts did not change significantly in KD and OE cells. We also conducted an immunocytochemical study of Lyn. Both in WT and KD, similar distribution of Lyn on the plasma membrane was observed (Fig. 1*c*). Together with the Western blotting analysis, it is suggested that reduction of flotillin-1 does not affect the stability of lipid rafts.

Using KD and OE clones, we examined the effects of the expression level of flotillin-1 on exocytotic release (degranulation). Degranulation was evaluated by quantifying secreted β -hexosaminidase and expressed as percentage of release normalized to the total β -hexosaminidase content (Fig. 1*d*). The total amount of β -hexosaminidase did not change by knocking down flotillin-1. When cells were stimulated with Ag (Fig. 1*d*, left), degranulation in KD cells was remarkably decreased compared with that in WT cells. In OE cells, significant enhancement of degranulation was observed especially in early period after stimulation, and this is consistent with the result in KD cells. These changes in Ag-induced degranulation depending on expression level of flotillin-1 were observed in a wide range of concentration of Ag (Fig. 1*d*, lower left). In contrast, degranulation induced by PMA and the calcium ionophore A23187 was not affected in either KD or OE cells (Fig. 1*d*, right). Stimulation with PMA and A23187 mimics the activation of mast cells by bypassing events before Ca^{2+} increase. These results suggest that flotillin-1 affects signal transduction upstream of Ca^{2+} increase rather than exocytotic fusion machinery.

Effects of the expression level of flotillin-1 on Ca^{2+} mobilization and activation of ERK

Because flotillin-1 seems to be involved in signal transduction upstream of Ca^{2+} , we observed Ca^{2+} mobilization induced by Ag stimulation. Fig. 2*a* shows the time course of the intracellular Ca^{2+} concentration in WT, KD, and OE cells. A remarkable attenuation of Ca^{2+} mobilization was observed in KD cells. Similar attenuation was observed in other KD clones, and the effects were dependent of expression level of flotillin-1, supporting the notion that flotillin-1 regulated cellular response positively. In contrast, enhancement of the Ca^{2+} increase was observed in an early phase in OE cells.

Cross-linking of Fc ϵ RI by Ag triggers a series of protein phosphorylation reactions, phosphorylation of Fc ϵ RI by Lyn, followed

IgE (200 ng/ml) and stimulated with Ag (200 ng/ml). The quantity of β -hexosaminidase in the supernatant is expressed as the percentage of total β -hexosaminidase. Each bar represents the mean $\pm$ SD. Time course of β -hexosaminidase release at the indicated times is shown. *Right*, Cells (●, WT; ○, KD; △, OE) were incubated with IgE (200 ng/ml) and stimulated with A23187 and PMA. *Lower left*, Dose-response curve of degranulation. Cells (●, WT; ○, KD; △, OE) were sensitized with IgE (200 ng/ml) and stimulated with various concentrations of Ag. Degranulation at 10 min after stimulation is plotted against concentration of Ag.

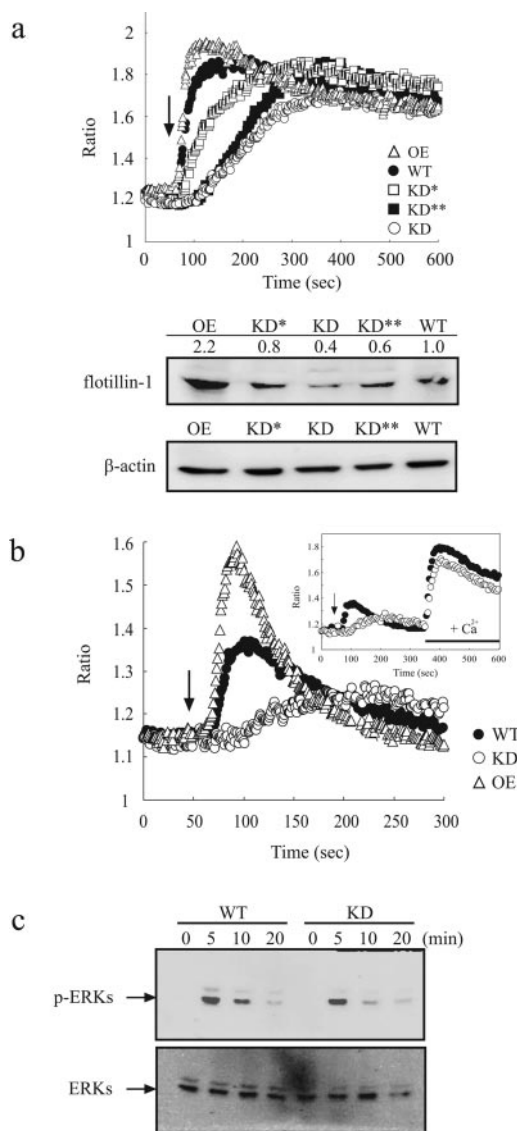


FIGURE 2. Effects of flotillin-1 on Ca^{2+} mobilization and phosphorylation of ERKs. *a* and *b*, Time course of intracellular Ca^{2+} concentration in WT (●), three different KD clones (KD, ○; KD*, □; and KD**, ■), and OE (Δ) cells. Cells were sensitized with IgE (final: 200 ng/ml), loaded with fura 2-AM (2 μM) for 15 min, and stimulated with Ag (final: 200 ng/ml) at the time indicated by an arrow in the presence (*a*) or absence (*b*) of extracellular calcium ion. Expression levels of flotillin-1 are shown in lower panel of *a*. Figures above each lane stand for relative expression level when the level in WT is unit. In the inset of *b*, Ca^{2+} was added in the extracellular medium (final free Ca^{2+} concentration was 1 mM) in the period indicated by a solid line. Ratios (F340/F360) of fluorescence intensity at 340/360 nm were plotted against time. *c*, Western blotting of phospho-ERKs and ERKs. Samples were prepared from WT and KD cells at the indicated times (0, 5, 10, and 20 min) after stimulation and electrophoresed by SDS-PAGE. After the samples were transferred to a PVDF membrane, blots were probed with anti-phospho-ERK Ab (upper panel) and anti-ERK Ab (lower panel), respectively, and visualized with anti-mouse IgG conjugated with HRP using chemiluminescence methods.

by the phosphorylation of Syk, Btk, and phospholipase C (22–24). Activation of phospholipase C generates inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol by catalyzing the hydrolysis of phosphatidylinositol 4,5-bisphosphate. IP_3 induces Ca^{2+} release from calcium stores via IP_3 receptor, resulting in an increase in the intracellular Ca^{2+} concentration. This Ca^{2+} release causes the depletion of Ca^{2+} from calcium stores and activates Ca^{2+} influx

from the extracellular medium through unidentified Ca^{2+} channels in the plasma membrane. To identify the Ca^{2+} mobilization pathway that is affected by flotillin-1, we examined Ca^{2+} mobilization in the absence of extracellular Ca^{2+} . As shown in Fig. 2*b*, Ca^{2+} increase due to Ca^{2+} release from intracellular calcium stores was severely inhibited in KD cells, while a prominent increase was observed in OE cells. These results indicate that flotillin-1 regulates processes before Ca^{2+} release from the intracellular calcium stores. In contrast to this, Ca^{2+} increase due to Ca^{2+} influx from extracellular medium was not affected severely, as shown in the inset in which Ca^{2+} was added in the extracellular medium after recovery to the basal level of Ca^{2+} concentration. In mast cells, Ca^{2+} influx is mediated by store-operated Ca^{2+} channel, which is activated by depletion of the intracellular Ca^{2+} store. Therefore, KD of flotillin-1 caused inhibition of early Ca response due to Ca^{2+} release from the store, but caused enough depletion of the store to activate store-operated Ca^{2+} channel.

In addition to Ca^{2+} mobilization, cross-linking of IgE receptor by Ag stimulates the MAPK cascade (8, 25). Phosphorylation of Fc ϵ RI by Lyn activates the ERK cascade via Shc/growth factor receptor-bound protein 2/son of sevenless (SOS). A growth factor receptor-bound protein 2 binds to a guanine nucleotide-exchange factor SOS through the SH3 domain. SOS activates membrane-bound Ras-GDP by exchange activity. Activated Ras activates Raf-1, which in turn activates MEK by phosphorylation. MEK phosphorylates ERK-1 and ERK-2 by phosphorylation. Other than this well-characterized pathway, increase in intracellular Ca^{2+} concentration or activation of protein kinase C alone stimulates ERK, and pathways involving Vav and Rac have been reported (26–28). Thus, we examined whether flotillin-1 affects phosphorylation of the MAPK cascade. Activation of ERKs in WT and KD cells was assessed by the phosphorylation level with Western blotting using a specific Ab for the phosphorylated forms of ERK-1 and ERK-2. As shown in Fig. 2*c*, the phosphorylation level of ERKs increased for up to 5 min after Ag stimulation and then gradually decreased in WT cells. In contrast, in KD cells, the phosphorylation level of ERKs at 5 min after stimulation was lower than that in WT cells, and decreased much rapidly. Together with the results regarding the inhibition of Ca^{2+} mobilization in flotillin-1 KD cells, it is suggested that flotillin-1 is involved in the activation of Lyn or in the phosphorylation of Fc ϵ RI by Lyn, because both Ca^{2+} mobilization and the MAPK cascade share the same upstream event: phosphorylation of Fc ϵ RI by Lyn.

Effects of flotillin-1 on the tyrosine phosphorylation of IgE receptor

To determine the effects of the expression level of flotillin-1 on the phosphorylation of Fc ϵ RI, we performed immunoprecipitation of IgE receptor and Western blotting analysis of the phosphorylated γ -chain with anti-phosphotyrosine Ab (PY20). As shown in Fig. 3*a*, the phosphorylation level of the γ -chain was decreased in KD cells. The phosphorylation level was quantified by the NIH Image program and plotted against time (Fig. 3*b*). In WT cells, the phosphorylation level of tyrosine on γ -chains showed an initial sharp increase after stimulation, and then gradually declined. In contrast, in KD cells, no significant increase in the phosphorylation level was observed after stimulation. These results indicate that flotillin-1 is involved in the tyrosine phosphorylation of Fc ϵ RI. Because flotillin-1 does not possess a kinase domain, it is not likely that flotillin-1 itself directly phosphorylates tyrosine residues of γ -chain of Fc ϵ RI. Therefore, it is reasonable to regard flotillin-1 as a regulatory protein that regulates the tyrosine kinase activity of Fc ϵ RI.

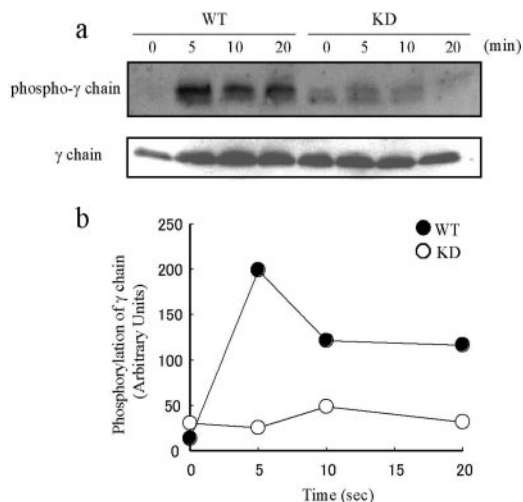


FIGURE 3. Effects of the expression level of flotillin-1 on the tyrosine phosphorylation of γ -chain. *a*, Western blotting analysis of tyrosine phosphorylation in IgE receptor. After cells were preincubated with IgE, cells were stimulated with Ag for IgE. Samples were prepared at the indicated times (0, 5, 10, and 20 min) and electrophoresed by SDS-PAGE. After samples were transferred to a PVDF membrane, blots were probed and visualized with anti-phosphotyrosine Ab (PY20) conjugated with HRP (*upper panel*). Expression of total γ -chain is shown in the *lower panel*. *b*, The level of tyrosine phosphorylation on γ -chain was quantified using the NIH Image program and plotted against time (WT, ●; KD, ○).

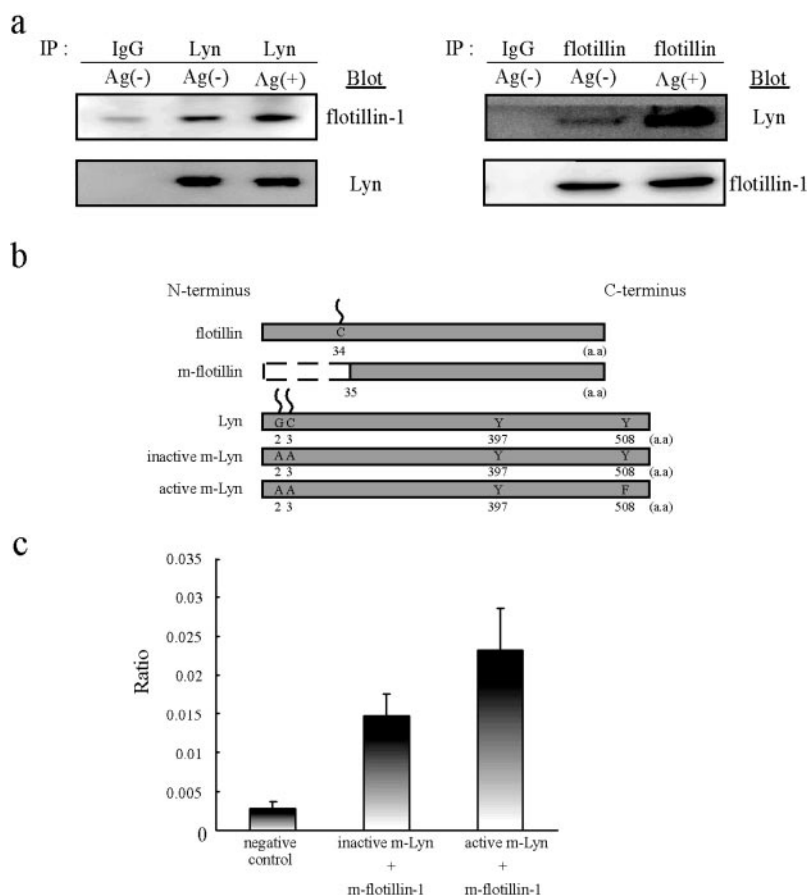
Flotillin-1 is associated with protein kinase Lyn

To elucidate the mechanism by which flotillin-1 regulates the tyrosine phosphorylation of Fc ϵ RI, we investigated the interaction of

flotillin-1 with tyrosine kinase Lyn. Aggregation of IgE receptors by Ag induces ITAM phosphorylation of the β - and γ -chains of the receptor by a mechanism that is not yet completely understood, but is dependent on Src family tyrosine kinase, Lyn. Lyn and flotillin-1 are known to reside in lipid rafts due to saturated fatty acid modification of the N terminus. Therefore, we examined whether or not flotillin-1 is associated with Lyn in lipid rafts using an immunoprecipitation assay. As shown in Fig. 4*a* (*left*), flotillin-1 was detected in Lyn immunoprecipitate prepared from WT cells before and after Ag stimulation, and the amount of flotillin-1 associated with Lyn after stimulation was about twice as much as that before stimulation, suggesting that some fraction of flotillin-1 is constitutively associated with Lyn in resting cells, and this association of flotillin-1 with Lyn is increased by Ag stimulation. Interaction between flotillin-1 and Lyn was confirmed by detection of Lyn in flotillin-1 immunoprecipitate (Fig. 4*a*, *right*).

Flotillin-1 possesses a conserved cysteine residue at the N terminus (Cys³⁴) for palmitoylation (29). Lyn also possesses two fatty acylation sites for myristoylation and palmitoylation at Gly² and Cys³, respectively, at the N terminus (13, 14). Therefore, it is difficult to exclude the possibility that flotillin-1 interacts with Lyn nonspecifically through these fatty acids in the immunoprecipitation experiments described above. To study the molecular interaction between flotillin-1 and Lyn without the involvement of lipid modification, we expressed mutated flotillin-1 (m-flotillin-1) and Lyn (m-Lyn), which cannot be modified by lipids (Fig. 4*b*). For flotillin-1, the first 34 aa containing palmitoylation sites were deleted. For Lyn, Gly² and Cys³ were substituted with Ala. Lyn possesses a negative-regulatory tyrosine residue at the C terminus (Tyr⁵⁰⁸). Before stimulation, this tyrosine residue is phosphorylated and the phosphotyrosine interacts with the Src homology 2

FIGURE 4. Interaction of flotillin-1 with Lyn. *a*, The interaction between flotillin-1 and Lyn was analyzed by immunoprecipitation and Western blotting. Cells were lysed and centrifuged to remove the nucleus and detergent-insoluble materials. Lysates were immunoprecipitated with anti-Lyn Ab (*left*) or anti-flotillin-1 Ab (*right*) or normal rabbit IgG. Immunoprecipitants were analyzed by immunoblotting with anti-flotillin-1 Ab or anti-Lyn or anti-Lyn Ab. *b*, Schematic representation of mutant molecules used in mammalian two-hybrid experiments. To express flotillin-1 and Lyn without lipid modification, mutations were introduced at acylation sites (m-flotillin and m-Lyn). In addition, a negative-regulatory tyrosine at 508 in the N terminus was replaced by phenylalanine to make Lyn unable to be phosphorylated (active m-Lyn). In inactive m-Lyn, Tyr⁵⁰⁸ is intact and subject to phosphorylation to make Lyn inactive. *c*, Protein interaction analyzed by a mammalian two-hybrid system. NIH 3T3 cells were transfected with suitable vectors (pBIND + pACT + pG5luc, pBIND-m-flotillin-1 + pACT-inactive m-Lyn + pG5luc, and pBIND-m-flotillin-1 + pACT-active m-Lyn + pG5luc). The transfectants were then lysed with lysis buffer. Firefly and *Renilla* luciferase activities were quantified with the Dual-Luciferase Reporter Assay System. The results are expressed as ratios (firefly luciferase activity/*Renilla* luciferase activity). Error bars stand for SD ($n = 3$).



domain of its own N terminus, forming an intramolecular closed configuration, which has no kinase activity. Upon stimulation, the phosphotyrosine is dephosphorylated, and, subsequently, Lyn becomes kinase active due to its open configuration. To examine the effects of the active/inactive form of Lyn on molecular interaction, we introduced another mutation in m-Lyn in addition to lipid modification sites. An active m-Lyn is a constitutively active type, in which a negative-regulatory tyrosine is replaced with Phe (Fig. 4b). In contrast, an inactive m-Lyn conserves a negative-regulatory tyrosine, Tyr⁵⁰⁸. The interaction of m-flotillin-1 with m-Lyn was investigated *in vivo* (in NIH3T3 cells) using a mammalian two-hybrid system, which is a modification of the original yeast two-hybrid system. As shown in Fig. 4c, firefly luciferase activity due to the interaction of m-flotillin-1 with m-Lyn was detected. In addition, we found that luciferase activity for the association of flotillin-1 with active m-Lyn was greater than that with inactive m-Lyn, which has a regulatory tyrosine. These results indicate that flotillin-1 interacts with Lyn directly regardless of lipid modification. Furthermore, the affinity of flotillin-1 with Lyn is dependent on the state of Lyn and a higher affinity is observed when Lyn is in active form. The results obtained by the two-hybrid system are consistent with those of the immunoprecipitation assay (Fig. 4a), because Ag stimulation increases the proportion of the active form of Lyn. Together with the results of immunoprecipitation using RBL-2H3 cells, these findings suggest that the interaction of flotillin-1 with Lyn is specific and direct, and the affinity of these proteins is increased by Ag stimulation.

Flotillin-1 regulates kinase activity of Lyn

As noted above, Fig. 4 shows that flotillin-1 interacts with Lyn. Next, we examined whether or not this association with flotillin-1 affects the kinase activity of Lyn. To monitor the kinase activity of Lyn, we performed an *in vitro* kinase assay using dephosphorylated α -casein as an exogenous substrate. The kinase activity of isolated Lyn was evaluated in terms of the amount of phosphorylated α -casein by Western blotting with phospho-Tyr Ab (PY20). Fig. 5 shows the phosphorylation of α -casein by Lyn isolated by immunoprecipitation from WT and KD cells. Regardless of Ag activation, the level of tyrosine phosphorylation of α -casein in KD cells was decreased compared with that in WT cells probably due to the lower association of flotillin-1 with Lyn in KD cells (Fig. 5). Without Ag activation, relative phosphorylation level significantly decreased to 0.5 ± 0.32 (mean $\pm$ SD; $n = 4$) in KD cells compared with WT cells ($p < 0.01$ by Student's *t* test). Ag stimulation significantly increased phosphorylation level to 1.9 ± 0.35 ($n = 4$) in WT cells compared with before stimulation ($p < 0.01$). In con-

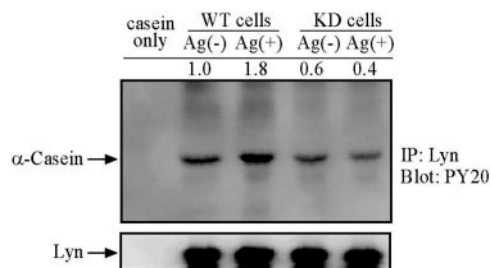


FIGURE 5. Regulation of kinase activity of Lyn by flotillin-1. Cell lysates prepared from WT and KD cells with or without Ag stimulation were immunoprecipitated with anti-Lyn Ab and subjected to an *in vitro* kinase assay. Dephosphorylated casein was incubated with immunoprecipitated Lyn and analyzed by Western blotting with anti-phosphotyrosine Ag (PY20).

trast, phosphorylation level did not change significantly between before (0.5 ± 0.32) and after (0.4 ± 0.21 ; $n = 4$) Ag stimulation ($p > 0.5$).

Discussion

The present results suggest that flotillin-1 may be involved in Ag-induced signal transduction. IgE receptor (Fc ϵ RI) consists of α -, β -, and γ -chains. ITAM in the β - and γ -chains is rapidly phosphorylated by Lyn, Src family tyrosine kinase, after cross-linking of Fc ϵ RI by Ag. Substantial evidence in the literature suggests that lipid rafts are involved in early signal transduction in immune cells after receptor stimulation. Lyn is a protein that is localized in rafts and is a key molecule in initiating intracellular signal cascades after receptor stimulation. In mast cells, cross-linked IgE receptor is translocated into rafts, in which the receptor is tyrosine phosphorylated by Lyn (5 - 7). We showed that flotillin-1, which is also localized in rafts, regulates the kinase activity of Lyn in mast cells. In flotillin-1 KD cells, both Ca²⁺ release from the calcium store and ERK activation (Fig. 2) were inhibited, suggesting that flotillin-1 works at common upstream process of these two events, probably tyrosine phosphorylation of Fc ϵ RI. The inhibition observed in flotillin-1 KD cells is similar to those obtained in bone marrow-derived mast cells (BMMC) from *lyn*^{-/-} mice. Nishizumi and Yamamoto (10) found that Ag-induced Ca²⁺ mobilization, tyrosine phosphorylation of the β - and γ -subunits of IgE receptors, and degranulation were impaired in BMMC from *lyn*^{-/-} mice, although phosphorylation of ERK was suppressed partially. The similarity of the properties of flotillin-1 KD cells in our study and BMMC from *lyn*^{-/-} mice also suggests that flotillin-1 regulates kinase activity of Lyn. Therefore, we hypothesized that the interaction of flotillin-1 with Lyn regulates the activation of Lyn kinase and enhances Lyn kinase activity, which phosphorylates the β - and γ -chains of Fc ϵ RI in RBL-2H3 cells by Ag stimulation, because flotillin-1 does not possess a kinase domain. To verify this hypothesis, we first performed an immunoprecipitation assay and a mammalian two-hybrid system assay to ascertain the direct binding of flotillin-1 with Lyn (Fig. 4). Next, we performed an *in vitro* kinase assay to evaluate the phosphorylation activity of Lyn kinases in the presence or absence of flotillin-1 (Fig. 5). Our results suggest that some fraction of flotillin-1 is constitutively and directly associated with Lyn in resting cells, and this association enhances the kinase activity of Lyn. In addition, we found that this association is enhanced by Ag stimulation. Our data provide a new insight into the early phase of IgE receptor-mediated signal transduction. In this model, the association of Lyn with flotillin-1 is necessary for the activation of Lyn. Because both flotillin-1 and Lyn are localized in lipid rafts, it is expected that treatment of cells with M β CD, which disrupts lipid rafts, would cause a change in the localization and association of Lyn and flotillin-1 in lipid rafts and reduce the activity of Lyn kinase, resulting in impaired signal transduction, as reflected in Ca²⁺ mobilization and degranulation. In fact, previous results obtained using M β CD seem to be consistent with our model (15, 16). In addition, the finding of Young et al. (17) that Lyn in lipid rafts has substantially greater kinase activity than that of Lyn outside of lipid rafts supports our model. We showed that flotillin-1 is associated with Lyn, but the molecular bases for this interaction are not yet clear. Based on the results of a motif assay, flotillin-1 has two potential tyrosine phosphorylation sites. Lyn might associate with flotillin-1 through this phosphotyrosine. The interaction between flotillin-1 and Fyn (Src family kinase) was reported in neuronal cells and T cell line (Jurkat cell) by Stuermer et al. (30); therefore, regulation of Src kinase-mediated signal transduction by flotillin-1 could occur in various types of cell. Because Fyn has been shown to be in mast cells, it is possible that

KD of flotillin-1 inhibits Ag-induced responses through an effect on Fyn. Odom et al. (31) and Parravicini et al. (32) showed that Fyn is key for mast cell degranulation even in the absence of Lyn. However, it is unlikely that flotillin-1 regulates degranulation through only Fyn-mediated pathway, because Ag-induced Ca^{2+} response was normal in mast cells derived from *fyn*^{-/-} mice, while KD of flotillin-1 inhibited not only degranulation, but also Ca^{2+} response, as shown in Fig. 2. Flotillin-1 might regulate both Lyn and Fyn, and the inhibition of degranulation caused by KD of flotillin-1 might be partly through Fyn-mediated pathway.

The present results suggest that flotillin-1 may recruit an unknown tyrosine kinase protein and phosphorylates a tyrosine in the kinase domain of Lyn. Although flotillin-1 is often used as a marker of caveolae or lipid rafts, its physiological role has not yet been established. Flotillin-1 and its associated proteins, CAP and Cbl, play a crucial role in the signal transduction of insulin-stimulated glucose transport (33–36).

The activity of Lyn is regulated positively and negatively by the phosphorylation at Tyr³⁹⁷ and Tyr⁵⁰⁸, respectively. After stimulation, dephosphorylation at Tyr⁵⁰⁸ makes Lyn assume an open form and phosphorylation at Tyr³⁹⁷ in the kinase domain enhances the kinase activity of Lyn. Therefore, flotillin-1 might affect the phosphorylation level to regulate kinase activity of Lyn. In mast cells, Ag stimulation increases the phosphorylation level of Tyr³⁹⁷. However, the phosphorylation level of Tyr⁵⁰⁸ in the basal state is very low (17), and Tolar et al. (37) reported the increase in Tyr⁵⁰⁸ phosphorylation after Ag stimulation. These results suggest that kinase activity of Lyn is regulated primarily by Tyr³⁹⁷, and Tyr⁵⁰⁸ plays a lesser role in mast cells. After stimulation, the association of Lyn with flotillin-1 increased (Fig. 4) and no clear increase in the phosphorylation level of α -casein was observed after stimulation in KD cells. Therefore, enhancement of the association of Lyn with flotillin-1 might be involved in the regulation of phosphorylation at Tyr³⁹⁷, which regulates kinase activity of Lyn positively (38). Because flotillin-1 itself does not have kinase activity, it might serve as an adaptor protein that recruits kinase to phosphorylate Lyn at Tyr³⁹⁷. Recently, lipid rafts have been reported to play important roles in signal transduction in various cells. The involvement of flotillin-1 in Lyn-mediated signal transduction in mast cells implies that flotillin-1 could regulate Src family kinase or a raft-dependent pathway in various cells other than mast cells. Because most of Lyn and flotillin-1 are localized in lipid rafts, we think the possibility that flotillin regulates Lyn outside rafts is not so high. However, flotillin and Lyn also reside outside rafts, and we do not exclude the possibility that those molecules outside rafts interact each other. It would be interesting if this is the case, but we do not have any evidence to demonstrate the activity of Lyn is regulated by flotillin outside rafts.

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Disclosures

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