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Functional analysis of flotillins in G protein signaling

（G タンパク質シグナルにおける flotillin の機能解析）

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Introduction

Heterotrimeric GTP-binding regulatory proteins (G proteins) transduce many extracellular signals, such as photons, odorants, hormones, and neurotransmitters, from heptahelical G protein-coupled receptors (GPCRs) to intracellular signaling molecules [1]. G proteins are composed of α, β, and γ subunits, and the latter two subunits usually form a heterodimer. Both α subunits and βγ dimers stimulate downstream effector molecules independently or cooperatively. The α subunits consist of about 20 members and are divided into 4 subgroups, Gαs, Gαi, Gαq, and Gα12/13, on the basis of their amino acid sequences and effectors [2, 3]. Gαq subfamily members activate phospholipase Cβ (PLCβ), which hydrolyses phosphatidylinositol-4,5-bisphosphate, yielding two second messengers, diacylglycerol and inositoltrisphosphate. Recently, several Gαq-binding proteins, which act as other candidate effectors and regulators of Gαq, were found [4]. Because Gαq couples with a large number of GPCRs and induces their cellular action in a cell-type and receptor-specific manner, the Gαq-binding proteins should be involved in the organization and diversity of Gq-mediated intracellular signaling pathways.

Lipid rafts are the microdomains of cell membranes enriched in a distinct set of lipids, such as cholesterol and sphingolipids [5, 6]. Signal-transducing molecules, such as non-receptor tyrosine kinases, receptor tyrosine kinases, and small GTPases, are enriched in lipid rafts, resulting in the compartmentalization of a certain process of signal transduction [7, 8]. Biochemical techniques and a fluorescence resonance energy transfer (FRET) method indicated that some GPCRs, G proteins, and their effectors localize in lipid rafts or dynamically move in and out of the domains [9, 10]. Furthermore, it was reported that a number of signaling outputs elicited by G protein activation were inhibited or potentiated by treatment with cholesterol-depleting reagents, such as methyl-β-cyclodextrin (MβCD), which

are known to disrupt lipid rafts. This growing evidence has suggested that lipid rafts play a role in G protein signaling by affecting downstream signaling activation and signaling specificity. However, the detailed mechanisms whereby lipid rafts engage in G protein signaling remain elusive.

Flotillin-1/reggie-2 and its paralogue, flotillin-2/reggie-1, were first identified as the proteins whose expression was increased during axon regeneration in gold fish [11]. Bickel *et al*. independently identified flotillins as proteins that are highly enriched in the detergent-resistant low-density membrane (DRM) [12]; thereby, flotillins are used as a lipid raft marker protein. Flotillins harbor the evolutionary conserved stomatin/prohibitin/flotillin/HflK/C (SPFH) domain [13] at their amino terminus and share about 50% amino acid identity. The expression of flotillins is regulated in the developmental processes and cell differentiation in several organisms or cultured cells [12, 14-18]. Additionally, it was reported that the expression of flotillin-1 is increased during the progression of Alzheimer's disease pathology [19]. Correlation of flotillin-2 expression and melanoma progression was also reported [20]. Although their function remains ill-defined, these lines of evidence implicate that flotillins participate in important cellular functions during developmental processes and pathogenesis.

In the present study, I identified lipid raft resident proteins, flotillin-1/reggie-2 and flotillin-2/reggie-1, as Gαq-interacting proteins. Knockdown of flotillins attenuated UTP-induced activation of p38 mitogen-activated kinase (MAPK) but not that of extracellular signal-regulated kinase (ERK) 1/2. The activation of p38 MAPK was dependent on Src family kinases and lipid raft integrity. These results suggest that flotillins mediate Gq-induced p38 MAPK activation through Src family tyrosine kinase in lipid rafts.

Materials and Methods

Materials

Antibodies against Gαq/11, GST, p38 MAPK, c-Src, and phosphotyrosine were purchased from Santa Cruz Biotechnology. Antibodies against ERK1/2, phospho-ERK1/2, and phospho-p38 MAPK were obtained from Cell Signaling Technology. Antibodies against flotillin-1 and flotillin-2 were purchased from BD Transduction Laboratories. Anti-His (Tetra-His) antibody was obtained from Qiagen. GTPγS, methyl-β-cyclodextrin, and anti FLAG (M2) antibody were purchased from Sigma-Aldrich. PP2, U-73122 and PMA were purchased from Calbiochem. YM-254890 was a gift from Jun Takasaki (Astellas Pharma).

Plasmid construction

Complementary DNA of a GTPase-deficient Q209L mutant of Gαq (GαqQL) was subcloned into a yeast two-hybrid bait vector, pGBKT7. Flotillin-1 cDNA was amplified from the total RNA of human fetal brain using reverse transcription polymerase chain reaction (RT-PCR) and then cloned into the mammalian expression vector pCMV5 and FLAG tag expression vector pCMV5-FLAG and the bacterial expression vectors pGEX4T-1 and pET28a. Flotillin-2 cDNA was amplified from the total RNA of HeLa cell and subcloned in the same way as flotillin-1. The fragments of flotillin-1-N (1-192 amino acids), -ΔN (38-192 amino acids), -C (193-427 amino acids), flotillin-2-N (1-195 amino acids), -ΔN (43-195 amino acids), and -C (196-428 amino acids) were amplified and ligated to pCMV5-FLAG.

Cell culture, transfection, and RT-PCR

Human epithelial 293 cells (HEK293) and HeLa cells were maintained in Dulbecco's modified Eagles medium containing 100 μg/ml kanamycin and 10% heat-inactivated fetal bovine serum. The cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2. Plasmid DNAs were transfected into HEK293 cells by the calcium-phosphate precipitation method. The final amount of transfected DNA for a 35-mm culture dish was adjusted to 5 μg by pCMV5. The siRNAs SMARTpool targeted for flotillin-1 and flotillin-2 were purchased from Dharmacon. The siRNAs were transfected into HeLa cells using the LipofectAMINE^{TM}2000 transfection reagent (Invitrogen) according to the manufacturer's protocol. Total RNA was extracted by TRIzol (Invitrogen) and used for RT-PCR analysis.

Immunoprecipitation and immunoblotting

Transfected cells were lysed in 300 μl of a lysis buffer (20 mM HEPES-NaOH, pH 7.5, 3 mM $MgCl_2$, 100 mM NaCl, 1 mM dithiothreitol, 1 mM phenylmethane sulfonylfluoride, 1 μg/ml leupeptin, 1 mM EGTA, 1 mM Na_3VO_4, 20 mM β-glycerophosphate, and 0.5% Nonidet P-40) per 35-mm dish. The extracts were centrifuged at 16,200×g for 10 min. The supernatants were incubated with anti-FLAG antibody (4 μg/ml) and protein G-Sepharose for 1.5 h at 4 °C. The immunoprecipitates were washed with a lysis buffer three times, denatured by adding an SDS-PAGE sample buffer, and subjected to SDS-PAGE. The proteins were transferred to PVDF membranes, blocked with 5% non-fat skim milk, and immunoblotted with antibodies.

MAP kinase assay

Cells were starved in serum-free DMEM 24 h before the addition of 10 μM UTP. Inhibitors were added 30 min before the addition of the stimuli. In the case of methyl-β-cyclodextrin, cells were pretreated for 1 h. Cells were stimulated with UTP for 5 min. Cell lysates were prepared using a modified RIPA buffer (20 mM HEPES-NaOH, pH 7.5, 3 mM $MgCl_2$, 100 mM NaCl, 1 mM dithiothreitol, 1 mM phenylmethane sulfonylfluoride, 1 μg/ml leupeptin, 1 mM EGTA, 1 mM Na_3VO_4, 20 mM β-glycerophosphate, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS). The protein concentration was determined by the Bradford method. The samples were subjected to SDS-PAGE and immunoblotted with phospho-MAPK antibodies. The signal intensities were quantified using LAS-1000 image analyzer (Fujifilm).

Measurement of intracellular calcium mobilization

The intracellular calcium mobilization was measured using the fluorescent Ca^{2+} indicator Fura-2 acetoxymethyl ester (Fura-2/AM) (Dojin Kagaku). Briefly, the cells cultured on 96-well culture plate were washed with a wash buffer (20 mM Hepes-NaOH (pH 7.5), 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, and 5.6 mM glucose) and loaded with 2 μM Fura-2/AM for 30 min at 37 °C and then washed twice with a wash buffer to remove the extracellular dye. Fluorescence was measured at 37 °C in an ARVO 1420 multilabel counter (PerkinElmer) equipped with a 340 nm and 390 nm excitation filters and a 510 nm emission filter. The cells were pretreated with or without 10 μM U-73122 for 5 min before the measurement. Fluorescence was monitored for 20 seconds prior to UTP (10 μM) addition and then every second for 40 seconds following addition of UTP.

Purification of recombinant proteins

The *E. coli* strain BL21-CodonPlus (DE3)-RIL (Stratagene) carrying the expression plasmids of GST-fused or hexahistidine-tagged proteins was cultivated in the presence of 0.1 mM of isopropyl 1-thio-β-D-galactoside at 25°C for 4 h. GST-fusion proteins were purified by using glutathione-Sepharose (GE Healthcare). Hexahistidine-tagged flotillin-2 was purified in the presence of 8 M urea using Ni-NTA agarose (Qiagen). Urea was removed by dialysis before storage at -80°C. Gαq and hexahistidine-tagged Gβ1γ2 were expressed in a baculovirus-Sf-9 cell system and purified as described previously [21].

In vitro *pull-down assay*

An *in vitro* pull-down assay was performed as described by Carman *et al*. with some modifications [22]. Recombinant Gαq (25 nM) was incubated with hexahistidine-tagged flotillin-2 (500 nM) immobilized to Ni-NTA agarose or GST-fused deletion mutants of flotillins (500 nM) immobilized to glutathione-Sepharose in a binding buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM $MgSO_4$, 6 mM 2-mercaptoethanol, 0.05% Lubrol, and 5% glycerol) for 2 h at 4 °C, followed by washing three times with a binding buffer. Gαq was pretreated with or without 100 μM GDP, 100 μM GTPγS, or 100 μM GDP-AlF_4^- for 2 h at 25 °C.

Isolation of the detergent-resistant low-density membrane

The detergent-resistant low-density membrane (DRM) was prepared from HeLa cells. Briefly, after a washing with ice-cold PBS, HeLa cells from a 100-mm dish were harvested in 600 μl of an extraction buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EGTA, 1 mM

EDTA, and 3 mM $MgCl_2$) supplemented with 1% Triton X-100. The cell lysate was homogenized by passing through a 25-G needle 20 times. Then, the lysate was adjusted to 40% of sucrose by the addition of an equal volume of an 80% sucrose solution prepared in an extraction buffer and by rocking for 30 min at 4 °C. The diluted lysate was transferred to an ultracentrifugation tube, overlaid by a discontinuous sucrose gradient of 1.8 ml of a 30% (w/v) and 0.6 ml of a 5% (w/v) sucrose solution, and centrifuged at 41,000 rpm for 16 hours in an SW55Ti rotor (Beckman Coulter). After the centrifugation, the sample was fractionated into 6 fractions (0.6 ml each) sequentially from the top of the gradient. Each fraction was subjected to SDS-PAGE and immunoblot analysis.

Immunofluorescence microscopy

HeLa cells were cultured on glass coverslips (Matsunami) for 24 h before fixation. The cells were washed with PBS and fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. After 5 min permeabilization with 0.1% Triton X-100 in PBS, the cells were incubated with a blocking buffer (10% fetal bovine serum in PBS) for 30 min, followed by incubation with antibodies for flotillin-1 (1:50 dilution), flotillin-2 (1:1000 dilution) or Gαq (1:300 dilution) in a blocking buffer for 12 h at 4 °C. After washing with PBS, the cells were incubated with Alexa Fluor 488-conjugated anti-mouse or anti-rabbit IgG antibodies (Molecular Probes) (1:1000 dilution) and mounted with Perma Fluor (Shandon). Fluorescent images were collected using an LSM510 laser-scanning confocal microscope (Zeiss) equipped with a Plan-Neofluar 40 × /0.75 NA objective.

Results

Identification of flotillin-1 as a Gαq-binding partner in yeast two-hybrid screening

To identify the novel regulators or effectors of Gαq, yeast two-hybrid screening was performed in our laboratory. Constitutively activated form of Gαq (Q209L) was used as a bait and human fetal brain cDNA library was used as a prey. As a result, clones encoding Ric-8A and flotillin-1 were identified as described in Master thesis of Hiroko Nishii. Ric-8A had already been reported to be a binding partner of Gαq [23, 24].

Gαq binds to flotillin-1 and flotillin-2 in HEK 293 cells

Next, I investigated the interaction between Gαq and flotillin-1/2 in HEK293 cells by co-expression and co-immunoprecipitation. As shown in Fig.1A, both the wild-type (WT) and GTPase-deficient forms (QL) of Gαq were co-immunoprecipitated with N terminally FLAG-tagged flotillin-1 by anti-FLAG antibody. Flotillins-2, a paralogue of flotillin-1, also interacted with Gαq WT/QL. To identify the binding region for Gαq, I constructed N- and C-terminal-deleted mutants, as shown in Fig.1C, and performed co-immunoprecipitation experiments. Since Gαq was co-immunoprecipitated with the C-terminal-deleted mutant, the N-terminal region (192 amino acids for flotillin-1 and 195 amino acids for flotillin-2) containing the SPFH domain was sufficient for the interaction with Gαq (Fig.1B). The interaction was lost when the N-terminus portion containing the first hydrophobic stretch was deleted. This result suggested that the first 38-amino acid region in flotillin-1 or 43-amino acid region in flotillin-2 is critical for the interaction. In the case of flotillin-2, Gαq weakly

interacted with its C-terminal half.

Gαq binds to flotillin-1 and flotillin-2 in vitro

I attempted to confirm these protein-protein interactions by using purified recombinant proteins. Gαq and flotillins were purified from insect cells and *E. coli*, respectively, as described in Materials and Methods. To examine whether the interaction is dependent on the activation states of Gαq, an *in vitro* pull-down assay was performed in the presence of GDP, GTPγS, or GDP plus AlF_4^-. As shown in Fig. 2A, the amounts of Gαq pulled-down by hexahistidine-tagged flotillin-2 did not vary among these conditions, and this result was consistent with that from co-immunoprecipitation experiments. The conformation change was validated by using hexahistidine-tagged RGS4, which specifically interacts with the AlF_4^--bound form of Gαq. Although I performed this experiment only by using flotillin-2 because full-length flotillin-1 was not available, the GST-fused N-terminal half of flotillin-1 also bound to Gαq independently of its nucleotide binding states (Fig. 3). These results indicated that Gαq directly binds to flotillins independently of its nucleotide binding state. I also constructed GST-fused N- and C-terminus constructs of flotillins. As in the co-immunoprecipitation experiments, Gαq bound to the N-terminal region of flotillin-1 and to N- and C-terminal region of flotillin-2 (Fig. 2B).

Gq-coupled P2Y receptor activation induces p38 MAPK activation dependently on Src kinase and lipid rafts in HeLa cells

Not only receptor tyrosine kinases but also GPCRs induce MAPK activation, having an influence on cell proliferation [25]. Gαq mediates MAPK activation in a certain cell type. I

used HeLa cells to analyze the Gq-mediated MAPK activation. In these cells, flotillin-2 localized throughout the cell, but flotillin-1 mainly resided in nucleus (Fig. 4). This observation was consistent with a previous report in which differential localization of flotillins were observed in PC-3 cells [26].

When HeLa cells were stimulated by UTP, which is an agonist for the P2Y Gq-coupled receptor, phosphorylation of two types of MAPKs, ERK1/2 and p38 MAPK, could be detected (Fig. 5A). This MAPK phosphorylation was attenuated by the Gq-specific inhibitor YM-254890 [27], which inhibits the nucleotide exchange reaction of Gαq [24]. The effect of other inhibitors commonly used for signal transduction studies was also examined. Among those tested, the pretreatment of the Src family kinase inhibitor PP2 blocked p38 MAPK phosphorylation but had little effect on ERK1/2 phosphorylation (Fig. 5A, B). The result with PP2 was consistent with a previous report, in which it was demonstrated that the Gq/11-induced activation of p38 MAPK was dependent on Src kinases in HEK293 cells [28]. Interestingly, although the phospholipase C inhibitor U-73122 could inhibit UTP-induced intracellular Ca^{2+} mobilization (Fig. 5C), the phosphorylation of ERKs and p38 MAPK was not affected by U-73122 (Fig. 5A, B).

In addition to these inhibitors, methyl-β-cyclodextrin (MβCD), which is known to disrupt the lipid raft structure, also inhibited p38 MAPK phosphorylation (Fig. 6A, B). Since the detergent-resistant low-density membrane (DRM) used to be characterized as lipid rafts, I assessed the localization of Gαq and Src kinases in DRM and examined the effects of MβCD by sucrose density-gradient centrifugation. Whereas flotillins were highly concentrated in DRM, a part of Src and a small portion of Gαq were detected in DRM (Fig. 6C). When cells were treated with MβCD, Gαq and Src were completely lost in DRM, but the localization pattern of flotillins was not changed (Fig. 6C, D). This result implied that Gαq and Src were kept apart from flotillins by MβCD treatment and indicated that Gq-coupled receptor-induced

p38 MAPK activation is dependent on Src family kinases and lipid rafts and requires the colocalization of the Gαq and Src kinase with flotillins in lipid rafts.

p38 MAPK activation, but not ERK1/2 activation, is attenuated by the knockdown of flotillin-2

To investigate the involvement of flotillins in Gq-mediated MAPK activation, I carried out RNA interference-mediated gene silencing using a synthetic small interfering RNA (siRNA) oligonucleotide targeted for flotillin-1 (siFlot1) and flotillin-2 (siFlot2). The siRNA targeted for luciferase was used as the control. As shown in Fig. 7A, the protein expression of flotillin-1 was diminished to 24% of the control level on the average when siFot1 was transfected into HeLa cells, although the expression of Gαq was not changed. When siFlot2 was transfected, the flotillin-2 protein was reduced to 30%, and, noticeably, flotillin-1 protein was reduced to 50%. Since the amount of flotillin-1 mRNA was not changed by the knockdown of flotillin-2 (Fig. 7B), the reduction of flotillin-1 expression was not due to an off-target effect of siFlot2. This phenomenon was also observed in flotillin-2 null mutants of *Drosophila* [29]. Therefore, the requirement of flotillin-2 for the stabilization of flotillin-1 appears to be conserved in mammals.

Using these cells, UTP-induced MAPK activation was examined. Fotillin-2 knockdown showed a significant reduction of p38 MAPK phosphorylation but not ERK1/2 phosphorylation, although the knockdown of flotillin-1 had no effect (Fig. 7C, D). In contrast, flotillin-2 knockdown was not affect the p38 MAPK activation induced by PKC activator phorbol 12-myristate 13-acetate (PMA), suggesting that flotillin-2 specifically regulates the p38 MAPK activation signaling pathway (Fig. 7E, F). Since Src kinases seemed to mediate the p38 MAPK activation (Fig. 5A, B), I examined the effect of flotillin knockdown on

Gq-mediated protein tyrosine phosphorylation. Upon the addition of UTP, the accumulation of tyrosine-phosphorylated proteins could be detected in the total cell lysate (Fig. 8A). This tyrosine phosphorylation was inhibited by YM-254890 and PP2 pretreatment, indicating the involvement of Gq and Src kinases (Fig. 8B). The knockdown of flotillins reduced this increase of tyrosine phosphorylation (Fig. 8A, arrowhead). This result suggested the involvement of flotillins in Gq-mediated Src kinase activation.

Since it was conceivable that flotillins could recruit Gαq to lipid rafts, thereby facilitating the activation of its downstream signaling pathway, I examined the effect of flotillins knockdown on DRM localization of Gαq. However, as shown in Fig. 9, the knockdown of flotillins did not affect the DRM localization of Gαq.

Fig. 1

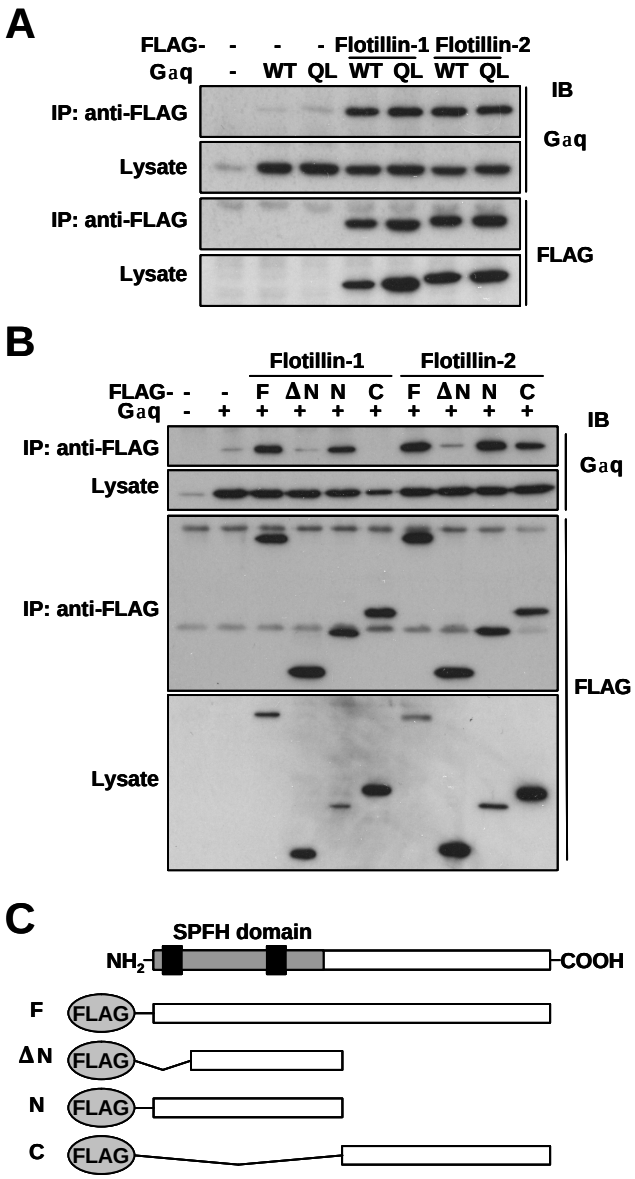

Fig. 1. Interaction of Gαq with flotillins in HEK293 cells. (A) The expression vector harboring Gαq (WT) or its constitutively activated mutant (QL) was co-transfected into HEK293 cells with the vector harboring FLAG-tagged flotillin-1 and flotillin-2. The transfected cells were lysed, and flotillins were immunoprecipitated by the anti-FLAG antibody M2. The immunoprecipitates and lysates were subjected to SDS-PAGE and immunoblot analysis with antibodies for Gαq and FLAG. (B) Gαq (WT) and FLAG-tagged flotillins and their deletion mutants were co-transfected into HEK293 cells. The transfectants were subjected as described above. (C) Schematic structure of flotillins and their deletion mutants used in B. The mutants are flotillin-1-N (1-192 amino acids), -ΔN (38-192 amino acids), -C (193-427 amino acids), flotillin-2-N (1-195 amino acids), -ΔN (43-195 amino acids), and -C (196-428 amino acids). The gray region represents the SPFH domain, and black bars indicate hydrophobic stretches.

Fig. 2

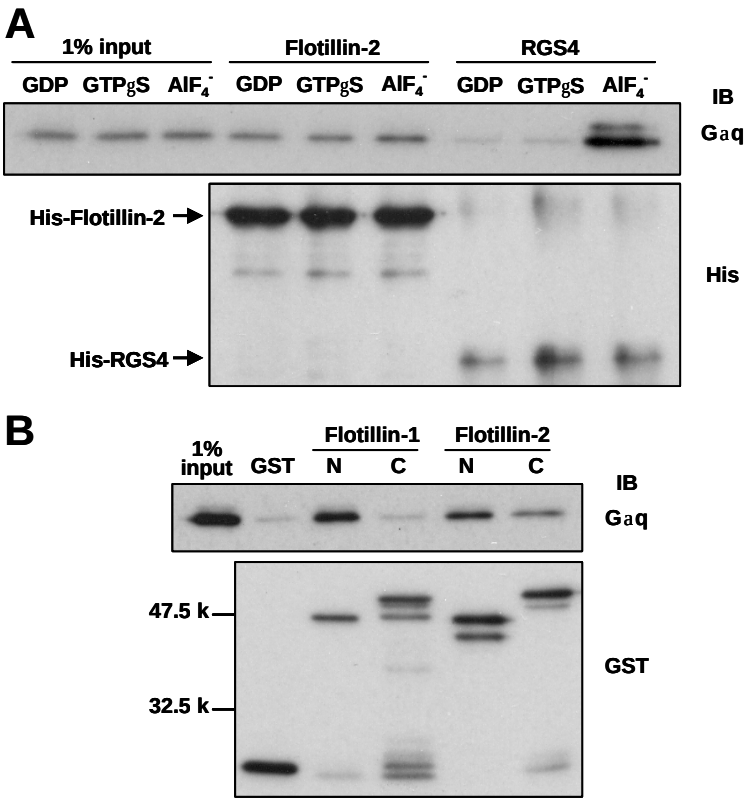

Fig. 2. Interaction of recombinant Gαq and flotillins. (A) Recombinant Gαq (25 nM) was treated with 100 μM of GDP, GTPγS, or GDP plus AlF_4^- (AlF_4^-) at 25 ℃ for 2 h and then pulled down by hexahistidine-tagged flotillin-2 (500 nM) or RGS4 immobilized to Ni-NTA agarose. Hexahistidine-tagged proteins and Gαq bound to the resin were detected by the anti-His antibody and the anti-Gαq antibody, respectively. (B) Recombinant Gαq (25 nM) was pulled down by GST or GST-fused flotillin deletion mutants (500 nM) immobilized to glutathione-Sepharose. GST-fused proteins and Gαq bound to the resin were detected by the anti-GST antibody and the anti-Gαq antibody, respectively.

Fig. 3

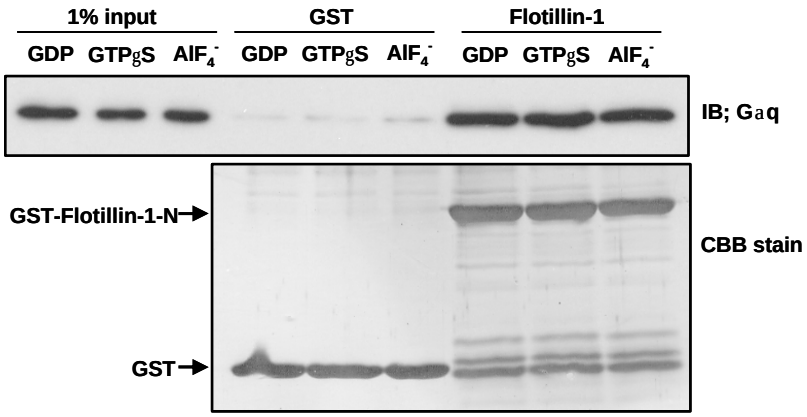

Fig. 3. Interaction of recombinant Gαq and flotillin-1 *in vitro*. Recombinant Gαq (25 nM) was treated with 100 μM of GDP, GTPγS, or GDP plus AlF_4^- (AlF_4^-) at 25 ℃ for 2 h and then pulled down by GST or GST-fused N-terminal half of flotillin-1 (GST-Flotillin-1-N) which harbors 1-192 amino acid residues of flotillin-1 (500 nM) immobilized to glutathione-Sepharose. Gαq bound to the resin were detected by the anti-Gαq antibody. GST and GST-Flotillin-1-N immobilized to GST-Sepharose were stained with Coomassie Brilliant Blue (CBB stain).

Fig. 4

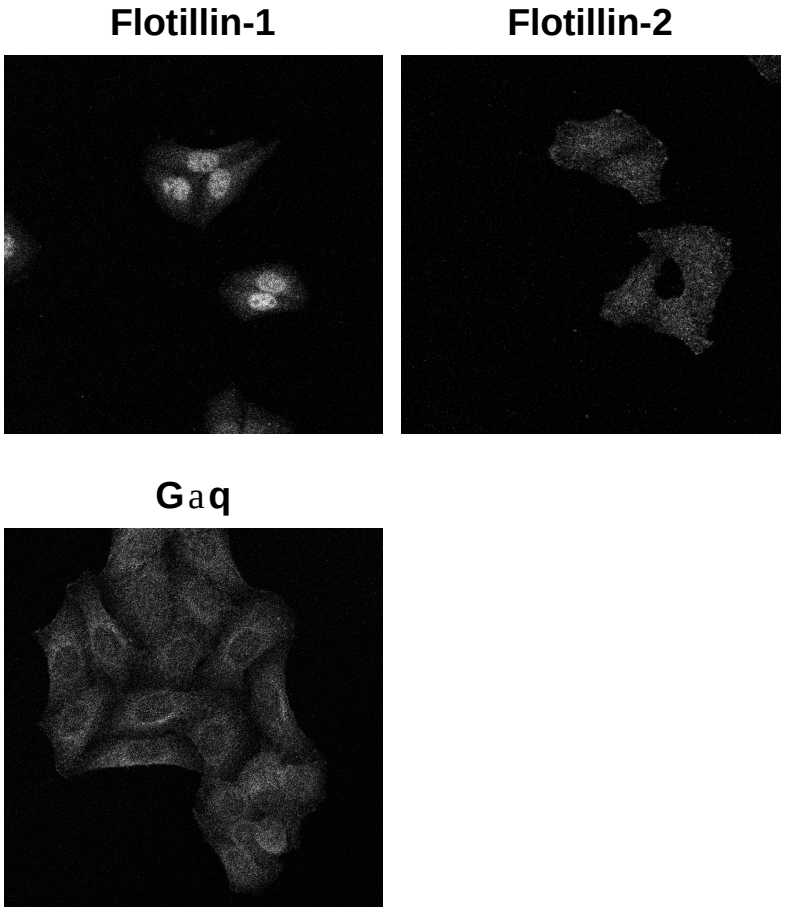

Fig. 4. Localizaion of flotillins and Gαq in HeLa cells. HeLa cells were fixed and subsequently subjected to confocal immunofluorescent microscopy using antibodies for indicated proteins.

Fig. 5

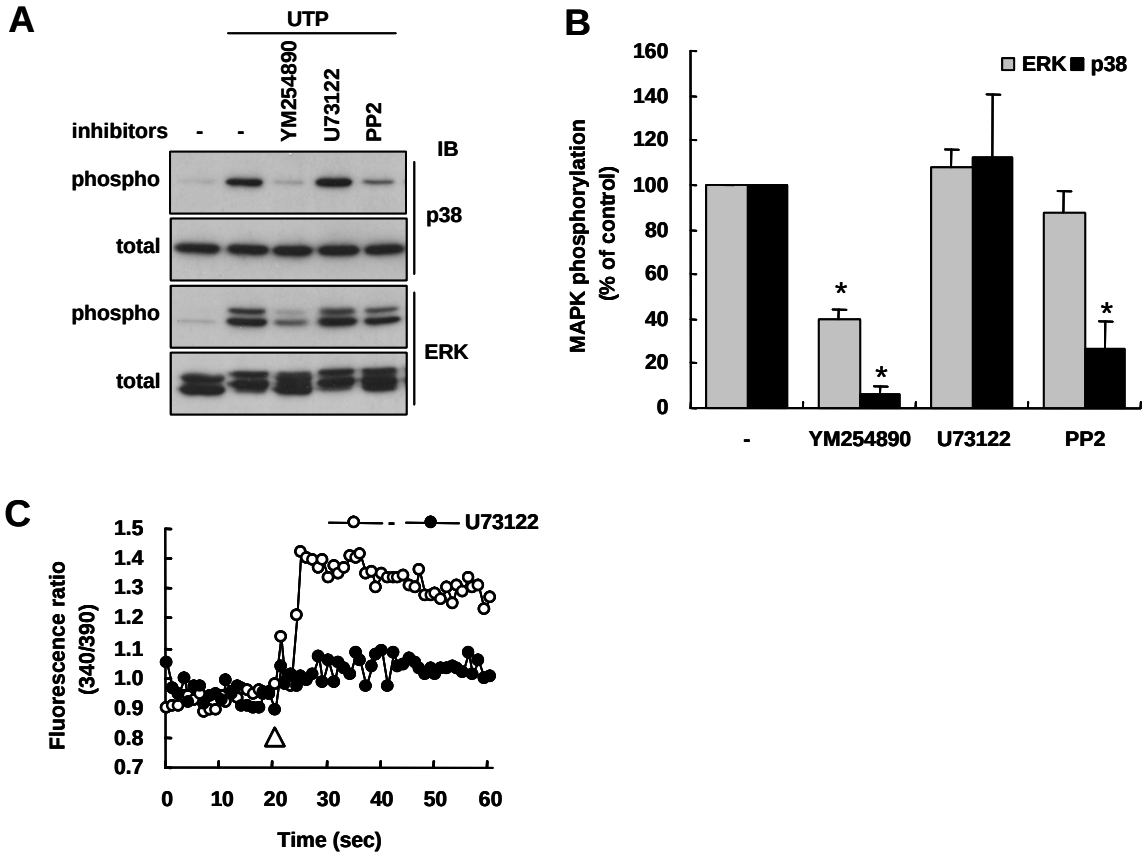

Fig. 5. Effects of inhibitors on UTP-induced MAPK activation. (A, B) HeLa cells were pretreated with the indicated inhibitors (10 μM YM-254890, 10 μM U-73122, or 10 μM PP2) for 30 min before a 5 min treatment with 10 μM UTP. Each cell lysate was prepared and subjected to SDS-PAGE and immunoblot analysis. Phosphorylated MAPKs were detected by a phospho-specific antibody for each MAPK. The band intensity of phosphorylated p38 MAPK (black) and ERK (gray) was quantified and compared to that of a UTP-stimulated sample in the absence of inhibitors. Each value represents the mean ± S.D. of three independent experiments [*P<0.01]. (C) HeLa cells were pretreated with (closed circles) or without (open circles) 10 μM U-73122 and were stimulated with 10 μM UTP (arrowhead). Changes in intracellular free calcium were determined by the ratio of fluorescence emission at 510 nm after excitation at 340 and 390 nm. Results shown here are representative data from three independent experiments.

Fig. 6

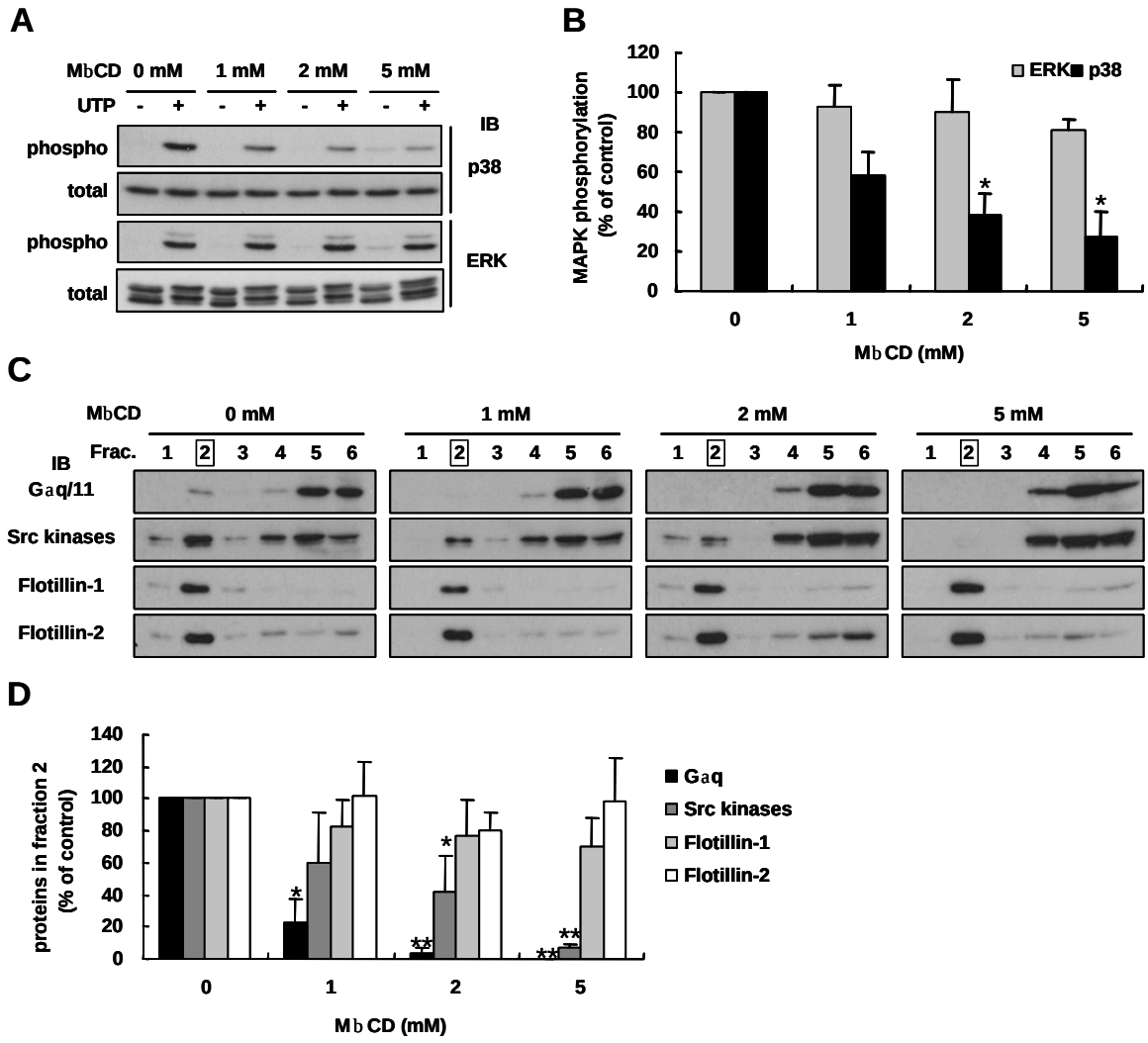

Fig. 6. Effects of methyl-β-cyclodextrin on UTP-induced MAPK activation and DRM localization of Gαq and Src kinases. (A, B) HeLa cells were pretreated with the indicated concentration of methyl-β-cyclodextrin (MβCD) for 1 h followed by a 5 min treatment with 10 μM UTP. Each cell lysate was prepared and subjected to SDS-PAGE and immunoblot analysis. Phosphorylated MAPKs were detected by a phospho-specific antibody for each MAPK. The band intensity of phosphorylated p38 MAPK (black) and ERK (gray) was quantified and compared to that of a UTP-stimulated sample in the absence of MβCD. Each value represents the mean ± S.D. of three independent experiments [**P*<0.01]. (C, D) HeLa cells were treated with an indicated concentration of MβCD for 1 h and thereafter subjected to sucrose density gradient as described in Materials and Methods. Each fraction was subjected to SDS-PAGE and immunoblot analysis using antibodies against the indicated proteins. DRM was mainly included in fraction number 2. The band intensity was quantified and the percentage of proteins in fraction number 2 was evaluated. The acquired values were compared to that of a MβCD-untreated sample. Each value represents the mean ± S.D. of three independent experiments [**P*<0.05, ***P*<0.005].

Fig. 7

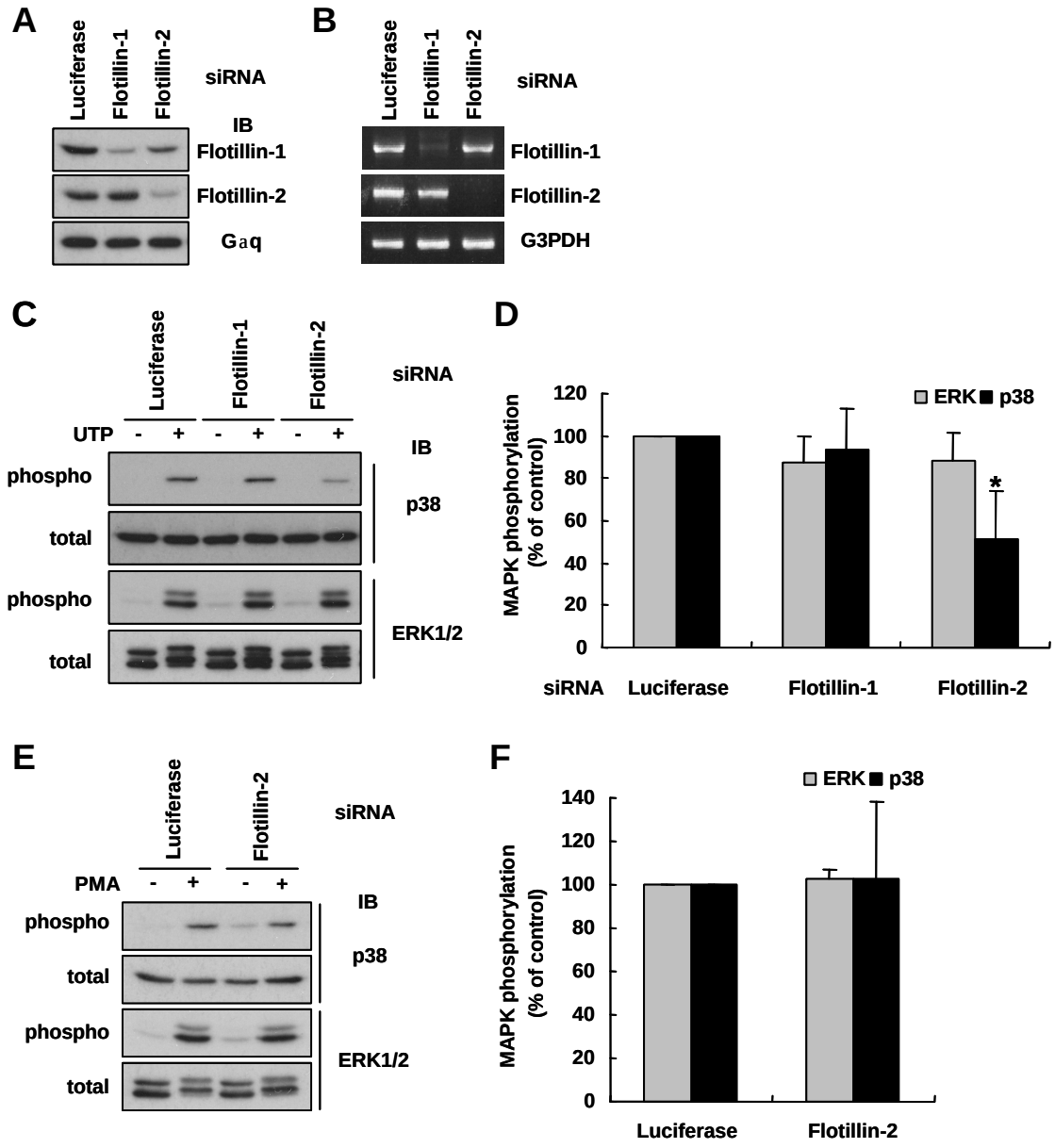

Fig. 7. Effects of flotillin-1/2 knockdown on UTP-induced MAPK activation. (A) HeLa cells were transfected with 5 nM of small interfering RNA (siRNA) targeted for flotillin-1, flotillin-2, or luciferase. Forty-eight h after transfection, each cell lysate was prepared and then subjected to SDS-PAGE and immunoblot analysis. (B) mRNAs for flotillins were detected by RT-PCR. The total RNA extracted from each siRNA transfectant was used as the template. A primer pair for glyceraldehyde 3-phosphate dehydrogenase (G3PDH) was used as the control. (C, D) HeLa cells were transfected with siRNA as described in A. Forty-eight h after transfection, the transfectants were treated with 10 μM UTP for 5 min. Phosphorylated MAPKs were detected by phospho-specific antibodies against each MAPK. The band intensity of phosphorylated p38 MAPK (black) and ERK (gray) was quantified and compared to that of a siLuciferase-transfected and UTP-treated sample. Each value represents the mean ± S.D. of six independent experiments [*P<0.005]. (E, F) HeLa cells were transfected with siRNA as described above. Forty-eight hours after transfection, the transfectants were treated with 100 nM phorbol 12-myristate 13-acetate (PMA) for 30 min. Phosphorylated MAPKs were detected and quantified as described above. Each value represents the mean ± S.D. of three independent experiments.

Fig. 8

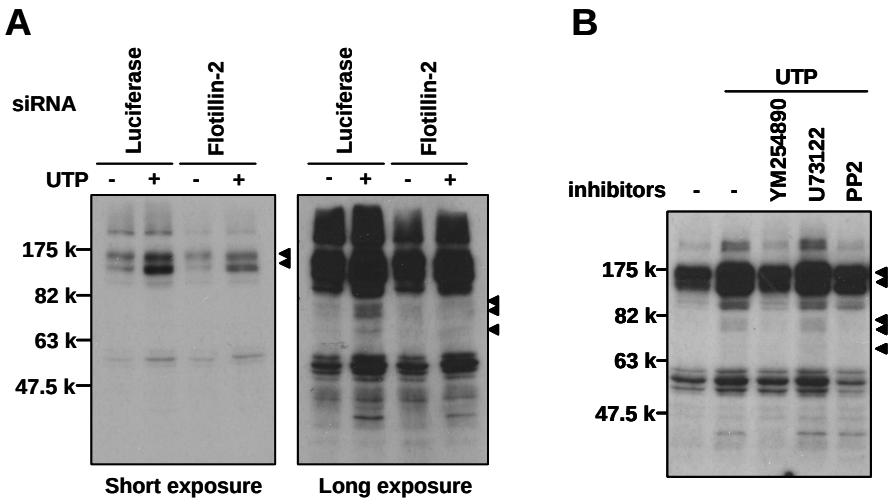

Fig. 8. Effects of flotillin-2 knockdown on UTP-induced protein tyrosine phosphorylation. (A) HeLa cells were transfected with 5 nM of small interfering RNA (siRNA) targeted for flotillin-2 or luciferase. Forty-eight hours after transfection, the transfectants were treated with 10 μM UTP for 5 min. The total cell lysates were prepared and immunoblotted with an anti-phosphotyrosine antibody (PY99). The bands whose intensity was increased upon UTP addition and reduced by flotillin-2 knockdown are indicated by arrowheads. (B) HeLa cells were pretreated with 100 nM YM-254890, 10 μM U-73122, or 10 μM PP2 for 30 min before a 5 min treatment with 10 μM UTP. Each cell lysate was prepared and subjected to SDS-PAGE and immunoblotted with PY99.

Fig. 9

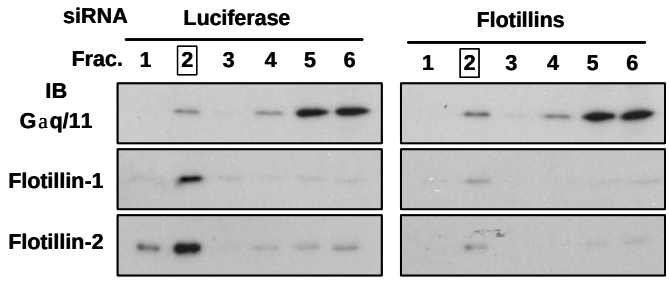

Fig. 9. Effects of flotillin knockdown on DRM localization of Gαq. HeLa cells were transfected with 5 nM of small interfering RNA (siRNA) targeted for flotillin-1 and flotillin-2, or 10 nM of siRNA targeted for luciferase. Forty-eight hours after transfection, the transfectants were subjected to sucrose density gradient as described in Materials and Methods. Each fraction was subjected to SDS-PAGE and immunoblot analysis using antibodies against the indicated proteins. DRM was mainly included in fraction number 2.

Fig. 10

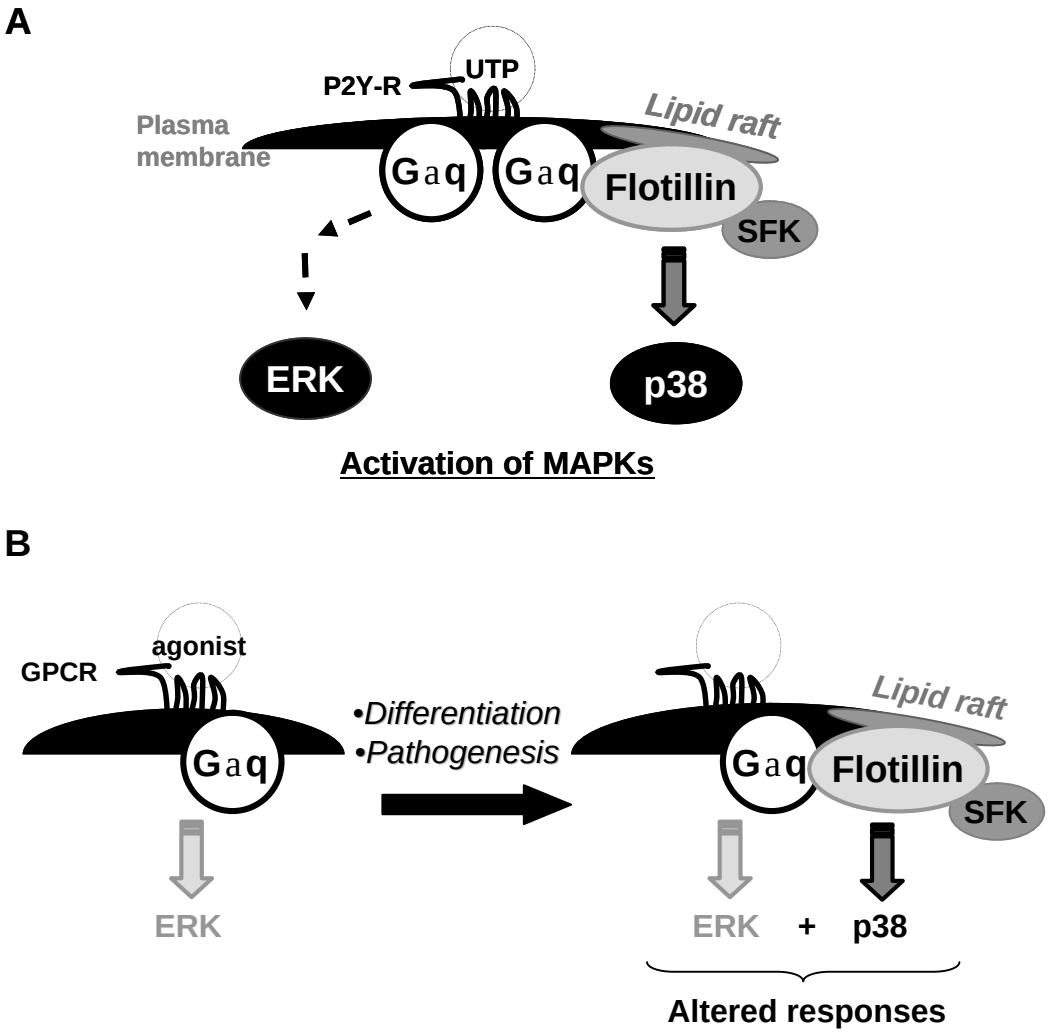

Fig. 10. Schematic representation of Gq-coupled GPCR-induced MAPK activation. Details are described in Discussion. SFK; Src family tyrosine kinases.

Discussion

In this report, I showed that (1) flotillins interacted with Gαq independently of its guanine nucleotide-binding state and (2) the knockdown of flotillins, particularly flotillin-2, attenuated the Gq-coupled GPCR-mediated activation of p38 MAPK but not that of ERK1/2. Moreover, (3) the lipid raft disrupter MβCD induced the separation of both Gαq and Src from flotillins and the inhibition of Gq-mediated p38 MAPK activation.

Three regulators for the GTPase cycle of Gα are well characterized, namely, guanine nucleotide dissociation inhibitors (G protein βγ subunit, GoLoco proteins), guanine nucleotide exchange factors (GPCRs and Ric-8), and GTPase-activating proteins (RGS proteins) [30]. All of them interact with Gα depending on its nucleotide-binding state and affect a particular step of the GTPase cycle. On the other hand, flotillins bound to Gαq independently of the nucleotide-binding state. Therefore, flotillins may have other functions than the control of the GTPase cycle. Flotillins have been suggested to function as scaffold proteins in some signaling systems, such as T cell receptor signaling [31, 32], IgE receptor signaling [33], and insulin signaling [34]. Flotillins might act in the same way in Gq-coupled GPCR signaling.

I showed that the knockdown of flotillins attenuated p38 MAPK activation but not ERK1/2 activation in HeLa cells. UTP-induced p38 MAPK activation was mediated by the Gq-coupled receptor and dependent on Src family tyrosine kinase and lipid raft integrity (Figs. 5, 6). The activation of ERK1/2 by UTP was also mediated by Gq but independent of Src kinases and lipid rafts. These results suggest that flotillins selectively regulate the pathway in which Src kinases and lipid rafts are involved. The differential regulation of those two types of MAPKs activation elicited by Gαq was also observed by Zhang *et al*. [35]. They found that RGS16, but not RGS1, RGS2, or RGS5, attenuated Gq/11-induced p38 MAPK activation but not that of ERK. Interestingly, RGS16 was reported to be palmitoylated and localized in lipid

rafts [36]. This report supports the hypothesis that Gq/11-mediated p38 MAPK activation is regulated in lipid rafts. Therefore, it was conceivable that flotillins might have a role in Gαq targeting to lipid rafts. However, the knockdown of flotillins did not affect the DRM localization of Gαq (Fig. 9). Additionally, when HeLa cells were pretreated with MβCD before DRM preparation, flotillins remained in DRM fraction although Gαq was completely depleted (Fig.6C, D). These results suggest that cholesterol but not flotillins is required for the targeting of Gαq to lipid rafts. Kato *et al*. also reported that the knockdown of flotillin-1 did not affect the DRM localization of its direct interacting protein Lyn [33]. Since the knockdown of flotillins was incomplete, it would be possible that the residual flotillins and cholesterol have a role in the recruitment of Gαq to lipid rafts. Nonetheless, lipid raft localization of Gαq was insufficient for the maximal activation of p38 MAPK, for which other functions of flotillins might be required.

It was previously described that Gαq stimulates p38 MAPK through Src family tyrosine kinases [28]. Src family tyrosine kinases are known to be activated under the stimulation of GPCRs [37]. The mechanism by which GPCRs activate Src kinases varies between cell types. Although the detailed mechanism of Src kinase activation by Gαq remains to be elucidated, at least its activation was not dependent on PLCβ because the PLC inhibitor, U-73122, did not inhibit p38 MAPK activation (Fig. 5A, B). This result was consistent with a previous report [38]. Recently, Kato *et al*. reported that flotillin-1 interacted with Src family kinase Lyn and positively regulated its kinase activity in mast cells [33]. They demonstrated a direct interaction between flotillin-1 and Lyn. The interaction of Src kinases with not only flotillin-1 [39] but also flotillin-2 [40] has been reported by other groups. Gαq might influence the flotillin-Src complex independently of PLCβ or might constitute a signaling complex with these two proteins in lipid rafts. These complexes appear to be essential for p38 MAPK activation by the Gq-coupled receptor (Fig. 10A).

The effect of flotillin knockdown on p38 MAPK activation was remarkable for flotillin-2 but not for flotillin-1. Therefore, there seems to be some functional difference between flotillin-1 and flotillin-2 in HeLa cells. Binding studies revealed that the N-terminal hydrophobic portion of both flotillins (1-34 amino acids for flotillin-1, 1-40 amino acids for flotillin-2) is essential for the interaction with Gαq. However, flotillin-2 has an additional binding region in the C-terminus. Therefore, the interaction between the C-terminal region of flotillin-2 and Gαq might be important for Gq-mediated signaling in lipid rafts. Moreover, the differential localization of flotillins might account for the functional difference. Santamaría *et al*. reported the nuclear localization of flotillin-1 in PC-3 cells [26]. They described that flotillin-2 was exclusively localized to the cell membrane. I also observed the differential localization of flotillin-1 and flotillin-2 in HeLa cells by using immunofluorescence microscopy (Fig. 4). Therefore, flotillin-2 may be a major binding partner of Gαq in HeLa cells and function in Gq signaling even though both flotillins are capable of interacting with Gαq.

Finally, it is conceivable that the Gq-mediated signal should acquire the p38 MAPK activation pathway during differentiation or pathogenesis that is concomitant with the up-regulation of flotillins (Fig. 10B). p38 MAPK is known to mediate inflammation and apoptosis. Besides these well-known roles, p38 MAPK has been described to function in some other cellular processes, such as the regulation of the cell cycle, differentiation, cardiomyocyte hypertrophy, and endocytosis [41, 42, 43]. Some of these processes could be regulated under Gq-coupled receptor activation. Further study is needed to clarify the role of Gq-mediated p38 MAPK activation involving flotillins.

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