J. Biochem. 2012;151(5):511–518 doi:10.1093/jb/mvs011

JB THE JOURNAL OF BIOCHEMISTRY

GPR34 is a receptor for lysophosphatidylserine with a fatty acid at the *sn*-2 position

Received December 8, 2011; accepted January 13, 2012; published online February 16, 2012

Hajime Kitamura[1,*], Kumiko Makide[1,2,*], Akira Shuto[1,*], Masaya Ikubo[3,*], Asuka Inoue[1], Kensuke Suzuki[1], Yusuke Sato[1], Sho Nakamura[3], Yuko Otani[3], Tomohiko Ohwada[3,†] and Junken Aoki[1,4,‡]

[1]Graduate School of Pharmaceutical Sciences, Tohoku University, Aobayama, Aoba-ku, Sendai, 980-8578; [2]Japan Science and Technology Agency, PRESTO, 4-1-8 Honcho, Kawaguchi, Saitama 332-0012, Japan; [3]Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo, 113-0033, Japan; [4]Japan Science and Technology Agency, CREST, 4-1-8 Honcho, Kawaguchi, Saitama 332-0012

[†]Tomohiko Ohwada, Graduate School of Pharmaceutical Sciences, University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo, 113-0033, Japan. Tel: +81-3-5841-4730, Fax: +81-3-5841-4732, email: ohwada@mol.f.u-tokyo.ac.jp

[‡]Junken Aoki, Tohoku University, 6-3, Aoba, Aoba-ku, Sendai, 980-8578, Japan. Tel: +81-22-795-6860, Fax: +81-22-795-6859, email: jaoki@m.tohoku.ac.jp

*These authors contributed equally to this work

GPR34 is a G protein-coupled receptor belonging to the P2Y family. Here, we attempted to resolve conflicting reports about whether it is a functional lysophosphatidylserine (LysoPS) receptor. In HEK293 cells expressing human, mouse or rat GPR34 and Gα chimera between Gαq and Gαi1(Gq/i1), LysoPS quickly elevated intracellular Ca^{2+} ion levels ($[Ca^{2+}]_i$). LysoPS also stimulated alkaline phosphatase (AP)-tagged TGFα (AP-TGFα) release in GPR34-expressing HEK293 cells and induced the migration of CHO-K1 cells expressing GPR34. Other lysophospholipids did not induce these actions. Replacement of the serine residue of LysoPS abolished the reactivity of LysoPS with GPR34, indicating that GPR34 strictly recognizes the serine head group of LysoPS. Recombinant phosphatidylserine-specific phospholipase A_1 (PS-PLA_1) that deacylates fatty acid at the *sn*-1 position of PS and produces 2-acyl-LysoPS, but not catalytically inactive mutant PS-PLA_1, stimulated the release of AP-TGFα from GPR34-expressing cells. Consistent with the result, LysoPS was detected in the cells treated with wild-type PS-PLA_1 but not with the mutant PS-PLA_1. PS treated with PLA_1 was much more effective at stimulating AP-TGFα release than PS treated with PLA_2. In addition, migration-resistant 2-acyl-1-deoxy-LysoPS, a 2-acyl-LysoPS analogue, was much more potent than 1-acyl-2-deoxy-LysoPS. The present studies confirm that GPR34 is a cellular receptor for LysoPS, especially with a fatty acid at the *sn*-2 position.

Keywords: fatty acid/G-protein coupled receptor/phospholipase/phospholipid/trimeric G proteins.

Abbreviations: GPCR, G protein-coupled receptor; LPA, lysophosphatidic acid; LPT, lysophosphatidylthreonine; LysoPS, lysophosphatidylserine.

Lysophosphatidylserine (LysoPS), a deacylated form of phosphatidylserine, has been shown to have lipid mediator-like actions. LysoPS stimulated degranulation of mast cells both *in vivo* and *in vitro* (*1*–*4*). LysoPS is also reported to induce neurite outgrowth in PC12 cells (*5*), suppression of cell proliferation of T lymphocytes (*6*) and migration of fibroblast (*7*) and glioma cells (*8*). Accordingly, it has been postulated that cellular receptors specific to LysoPS account for these actions.

In 2006, Sugo *et al*. (*9*) expressed human, mouse and guinea pig GPR34 in CHO cells, and showed that LysoPS caused the cells to decrease the cAMP level and increase γ-GTP binding, suggesting that GPR34 is a receptor for LysoPS. Iwashita *et al*. (*10*) also showed that CHO cells expressing mouse and rat GPR34 responded to LysoPS to increase the intracellular Ca^{2+} level. Recently, GPR34 deficiency was found to interfere with the immune responses in mice (*11*). However, the authors concluded that LysoPS is not a ligand for human and mouse GPR34 because it did not affect the cAMP and phosphoinositide levels in COS-7 cells expressing the receptors. Interestingly, however, GPR34 from carp, which shows 41–43% amino acid identity to mammalian GPR34, displayed a robust response to LysoPS. Thus, there is not yet a consensus over whether mammalian GPR34 functions as a cellular receptor for LysoPS. A recent sequence analysis and molecular modelling indicated that GPR34 belongs to the P2Y family to which other emerging other newly identified lysophospholipid receptors, such as P2Y9/GPR23/LPA_4, GPR92/LPA_5 and P2Y5/LPA_6 belong (*12*).

In this study, we re-examined whether GPR34 is a functional receptor for LysoPS using several methods for detecting activation of GPCR as well as recently developed LysoPS analogues. Our results confirm that GPR34 responds to LysoPS, especially with a fatty acid at the *sn*-2 position of the LysoPS glycerol backbone.

Materials and Methods

Materials

1-oleoyl (18:1)-lysophosphatidic acid (LPA), 18:1-lysophosphatidylcholine (LPC), 18:1-lysophosphatidylethanolamine (LPE), 18:1-lysophosphatidylglycerol (LPG), 18:1-lysophosphatidylinositol (LPI), 18:1-lysophosphatidylserine (LysoPS), dioleoyl

phosphatidylserine (PS) were purchased from Avanti Polar Lipids. Sphingosine 1-phosphate (S1P) was from Cayman Chemical. LysoPS with various fatty acids [stearic (18:0), palmitic (16:0), palmitoleic (16:1), myristic (14:0), lauric (12:0), capric (10:0)], LysoPS with D-serine and lysophosphatidylthreonine (LPT) were synthesized as previously (*10*).

Cell culture

HEK293 cells and CHO-K1 cells were maintained in DMEM and Ham's F12 medium (Nissui Pharmaceutical), respectively, supplemented with 10% foetal bovine serum (GIBCO), 100 U/ml penicillin (Sigma-Aldrich) and 100 μg/ml streptomycin (GIBCO) in a 37°C incubator with 5% CO_2. Mouse GPR34-expressing stable cells were established as described previously (*10*).

Expression vectors

Open reading frames of human, mouse and rat GPR34 were amplified by PCR using PrimeSTAR HS DNA polymerase (TaKaRa) and HUVEC, mouse tail and rat tail genome as a template DNA, respectively. The sequences of oligonucleotide primers used are as follows: sense primer, 5′-AGTCGAATTCCCACCATGACTACTA CTTCAGTTGACAGC-3′; antisense primer, 5′-GTACACTCGAG TCAATTACCCTTAGTGCTGTAC-3′ (mouse), sense primer, 5′-G CCACCATGAGAAGTCATACCATAACAATGACGACAACTT CAG-3′; antisense primer, 5′-CCGACTCGAGTCAAGTACTTTT AGAACTAGAC-3′ (human, long form), sense primer, 5′-GCCAC CATGACGACAACTTCAGTCAG-3′; antisense primer, 5′-CCGACTCGAGTCAAGTACTTTTAGAACTAGAC-3′ (human, short form), sense primer, 5′-GAAGTTGAATTCCCACC ATGACGACTACAGTTGACAGC-3′; antisense primer, 5′-ACTG CACTCGAGTCAGTTACCCTTAGTGCTGTAC-3′ (rat). Each PCR product was digested with restriction enzymes and ligated into the multiple cloning site of pCAGGS-MCS expression vector.

Construction of PS-PLA$_1$ mutant

cDNAs encoding PS-PLA$_1$ mutants were constructed by PCR method. In the first step, two independent PCR reactions were carried out using mouse PS-PLA$_1$ cDNA as template. One reaction was performed using Primers 1 and 2 to amplify the 5′-half of mutant PS-PLA$_1$ cDNA. The other reaction was performed using Primers 3 and 4 to amplify the 3′-half of mutant PS-PLA$_1$ cDNA. The resulting 3′-half of mutant PS-PLA$_1$ cDNA fragment was subcloned into the EcoRV/XhoI site of a plasmid expression vector pCAGGS-MCS. Next, the resulting 5′-half of mutant PS-PLA$_1$ cDNA fragment was subcloned into the KpnI/EcoRV site of the above pCAGGS. The oligonucleotide DNA primers used for PCR are as follows: Primer 1: CAGGTACCGCCACCATGCGTCCTGG CCTCTGGG; Primer 2: GTGGATTGAGGACTCTGAC; Primer 3: ATCATTGGTGTCGCCCTGGGGGCTCATG; Primer 4: GG TCTCGAGCTACACGCAGGCTATTTTC.

Measurement of calcium influx

The calcium indicator fura-2 AM (Invitrogen) was used to monitor intracellular calcium changes. HEK293 cells were seeded in 10 ml antibiotic-free medium in 10-cm dishes the day before transfection to allow adherence and reach confluence of 70–90% at the time of transfection. The standard co-transfection mix was prepared by adding plasmid DNA encoding GPR34 with Gq/i1 (a chimeric G protein α-subunit consisting of Gq with the C-terminal six amino acids substituted with the corresponding amino acids from Gi1) to 1250 μl Opti-MEM and 12.5 μl Lipofectamine 2000 (Invitrogen) to another 1250 μl Opti-MEM. The two solutions were mixed and incubated at room temperature for 20–30 min before the mix was added to each of 10-cm dishes. The cells were incubated with the transfection mix for 24 h. Cells were harvested and suspended in calcium assay buffer [10 mM HEPES (pH 7.4), 140 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM KCl, 10 mM glucose] and then loaded with 5 μM fura-2 AM. For intracellular calcium measurements, cells were re-suspended in assay buffer at 37°C and fluorescence was monitored at excitation wavelengths of 340 and 380 nm and an emission wavelength of 500 nm by a CAF-110 spectrofluorometer (JASCO). Experiments were calibrated by adding Triton X-100 (final 0.25%) to set the maximum fluorescence value or EDTA (final 50 mM) to establish the minimum fluorescence value.

TGFα shedding assay

TGFα shedding assay was performed as described previously (*13*). Briefly, HEK293 cells were seeded in 6-cm dishes at a density of 8×10^5 cells/dish and cultured at 37°C for 24 h. Then the cells were transfected with cDNAs encoding alkaline phosphatase (AP)-tagged TGFα (AP-TGFα), GPR34 (5 μg each) and chimeric G protein Gq/i1 (0.5 μg) using Lipofectamine 2000 as transfection reagent. The cells were seeded in 96-well plate at a density of 4×10^5 cells/ml (90 μl) in HBSS containing 5 mM HEPES (pH 7.4). After incubation at 37°C for 30 min, the cells were stimulated with LysoPS, other lysophospholipids or LysoPS analogues and incubated at 37°C for 1 h. After 1 h, the cells centrifuged at 190*g* for 3 min and supernatant (80 μl) was moved into a new 96-well plate. An amount of 80 μl of 10 mM *p*-Nitrophenyl phosphate (pNPP) in 2× pNPP buffer [40 mM Tris–HCl (pH 9.5)], 40 mM NaCl and 10 mM $MgCl_2$)/well was added to the supernatant and the cells, and measured at optical density at 405 nm (OD405). After incubation at 37°C for 1 h, both the supernatant and the cells were measured at OD_{405}. TGFα shedding activity was calculated by OD_{405} of 0 and 1 h.

Cell migration assay

Cell migration was assayed using a Boyden chamber equipped with polycarbonate filters (5 μm pore size, Neuroprobe). All polycarbonate filters used were coated with 0.1% fibronectin, 2 h before use in the assay. The lower compartment contained 0.11 ml of Ham's F12 medium containing 0.1% BSA including the test material. Cells were suspended in Ham's F12 medium containing 0.1% BSA (5×10^5 cells/ml) and seeded into the upper compartment of the chamber (total volume 0.2 ml). The chambers were incubated at 37°C for 6 h. Cells attached to the upper side of the filter were mechanically removed. The Filters were subsequently fixed in methanol and stained with a Diff-Quick stain kit (Sysmex). The number of cells that had migrated to the lower surface of the filter was determined by measuring the absorbance of the dye occurs at 590 nm using a microplate reader. Each experiment was performed in triplicate.

Synthesis of 2-oleoyl-1-deoxy LysoPS

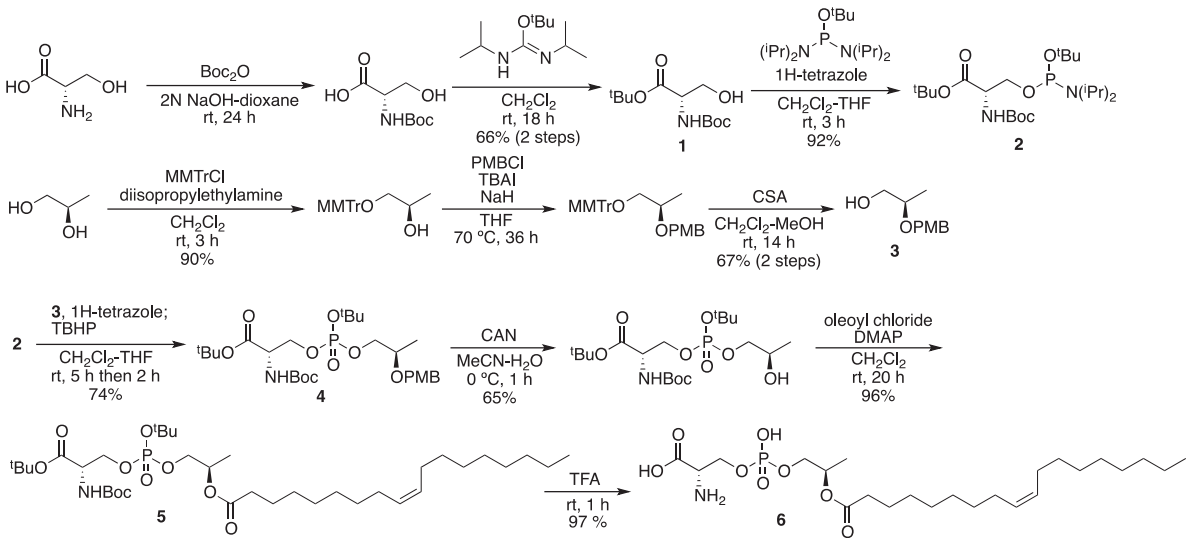
A phosphoramidite method was used to construct the phosphate diester linkage of 2-oleoyl-1-deoxy LPS. First, *N*-Boc (*tert*-butoxycarbonyl)-*L*-serine *tert*-butyl ester **1** was phosphorylated with bis (diisopropylamino)-*tert*-butylphoshine in the presence of 1*H*-tetrazole to give **2**, followed by a second phosphorylation with (*R*)-2-(4-methoxybenzyloxy)propan-1-ol **3**. Subsequent *in situ* oxidation of the resultant phosphite triester intermediate with *tert*-butyl hydroperoxide afforded fully protected phosphate trimester intermediate **4** (68% for 3 steps). Selective oxidative deprotection of the oxygen atom of the secondary alcohol, followed by acylation with oleoyl chloride (to **5**), and complete deprotection by trifluoroacetic acid furnished 2-oleoyl-1-deoxy LPS **6** (61% for 3 steps). **6**:^{1}H-NMR($CDCl_3$/TFA-d = 4:1):δ = 5.377–5.339 (2H, m), 5.153 (1H, m), 4.443 (3H, m), 3.921 (2H, m), 2.318 (2H, m), 2.005 (4H, m), 1.573 (2H, m), 1.259-1.210 (23H, m), 0.875 (3H, t, J = 6.72 Hz). ^{31}P-NMR($CDCl_3$/TFA-d = 4:1): δ = −1.657. HRMS (ESI, $[M-H]^-$): Calcd for $C_{24}H_{45}NO_8P^-$:506.2888. Found: 506.2890. Anal. Calcd for $C_{24}H_{46}NO_8P{\cdot}0.6CF_3COOH$: C, 52.55; H, 8.15; N, 2.43. Found: C, 52.63; H, 8.11; N, 2.32. Mp: 147.0-148.0°C.

Preparation of recombinant PS-PLA$_1$ proteins

HEK293 cells were seeded in 10-cm dishes and cultured at 37°C for 24 h. Then the cells were transfected with cDNAs encoding mouse wild-type PS-PLA$_1$ and mutant PS-PLA$_1$ using Lipofectamine 2000 as transfection reagent. After 72 h, culture supernatant were collected, clarified by low-speed centrifugation and used as a recombinant protein source. Concentrations of the obtained recombinant protein were determined by sandwich enzyme-linked immunosorbent assay for PS-PLA$_1$.

PLA$_1$ and PLA$_2$ reactions

Dioleoyl PS was incubated with *Rhizopus delemar* lipase (Seikagaku-Kogyo) or phospholipase A_2 from porcine pancreas (Sigma-Aldrich) in Hank's balanced salt solution containing 5 mM HEPES (pH 7.4), at 37°C for 30 min. Reaction products were brought to the TGFα shedding assay. The liberated fatty acid was measured using NEFA C-test Wako (Wako) and the concentrations of LysoPS were performed the corrections.



Quantification of lysophospholipids by LC–MS/MS

HEK293 cells (1×10^5 cells) were incubated with recombinant PS-PLA$_1$ for 1 h at 37°C. Then, cells were collected by centrifugation at 1,000*g* for 3 min. An amount of 200 µl of methanol was added into the pellet and vortexed for 30 s. After ultrasonic extraction for 3 min in ice water, the extracts were centrifuged for 3 min (21,500 g). The resulting supernatant was injected into the liquid chromatography–tandem mass spectrometry (LC–MS/MS) system. Measurement of LysoPS, LPC, LPE, LPI, LPA and LPG was performed as described previously (*13*).

Results

GPR34 responds to LysoPS in Ca^{2+}, TGFα shedding and cell migration assays

Previous reports indicated that LysoPS decreased forskolin-induced cAMP accumulation, activated ERK phosphorylation and increased intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in CHO-K1 cells stably expressing human or mouse GPR34 (*9*, *10*). We first examined whether HEK293 cells transiently expressing human, mouse and rat GPR34 respond to LysoPS in a Ca^{2+} assay. Since human GPR34 has two potential translation-initiating methionines in the 5′ region, we constructed two expression vectors, hGPR34 (S) and hGPR34 (L) (Supplementary Fig. S1). When each GPR34 cDNA was transfected into HEK293 cells and stimulated with varying concentrations of LysoPS with oleic acid (18:1), LysoPS induced a rapid increase in $[Ca^{2+}]_i$ (Fig. 1A). All GPR34s including hGPR34 (S), hGPR34 (L), mGPR34 and rGPR34 responded to LysoPS in a similar manner. These responses were specific to LysoPS because none of other lysophospholipids [LPC, LPI, LPG, LPA and S1P] induced the Ca^{2+} response in human and mouse GPR34-expressing HEK293 cells (Fig. 1B). We also tested various LysoPS analogues or lysophospholipids that have slight modifications in the serine moiety of LysoPS, including D-LysoPS, lysophosphatidylthreonine (LPT) and lysophosphatidylethanolamine (LPE) (Fig. 1C). We found that even a slight modification of L-serine abolished the responsiveness to mouse and human GPR34 (Fig. 1D), showing that GPR34 strictly recognizes the structure of the L-serine moiety of LysoPS.

We also examined the reactivity of GPR34 to LysoPS using other systems. We used TGFα shedding assay, in which activation of GPCR is transduced to TACE-dependent release of alkaline phosphatase (AP)-tagged TGFα (AP-TGFα) downstream of G protein and consequent protein kinase C activation. HEK293 cells transfected with GPR34 constructs from various species responded to LysoPS to release AP-TGFα in the cell supernatant (Fig. 2A). The AP-TGFα release by LysoPS was dose-dependent with an EC_{50} of about 100 nM (Fig. 2A). Other lysophospholipids such as LPC, LPI, LPG, LPA and S1P did not induce the release of AP-TGFα (Fig. 2B). None of the LysoPS analogues induced AP-TGFα release from mouse GPR34-expressing HEK293 cells (Fig. 2C), again showing that GPR34 strictly recognizes the structure of serine.

LysoPS produced by PS-PLA$_1$ activates GPR34

Phosphatidylserine-specific phospholipase A$_1$ (PS-PLA$_1$) is a secreted enzyme that specifically hydrolyzes the fatty acid at the *sn*-1 position of PS, yielding 2-acyl-LysoPS. Adding recombinant PS-PLA$_1$ to mouse GPR34-expressing HEK293 cells in the TGFα shedding assay caused a significant AP-TGFα release (Fig. 3A). A catalytically inactive mutant of PS-PLA$_1$ in which serine^{166}, the active centre of the enzyme, was replaced with alanine, failed to induce the AP-TGFα release (Fig. 3A). An LC–MS/MS analysis of HEK293 cells after the addition of wild-type or mutant PS-PLA$_1$ showed that among the various lysophospholipid species, LysoPS with oleic acid (18:1) was dramatically increased in wild-type PS-PLA$_1$-treated cells (Fig. 3B). This result shows that LysoPS (18:1) produced by PS-PLA$_1$ activates GPR34, providing further evidence that GPR34 is a receptor for LysoPS.

Ligand specificity of GPR34

LysoPS species with various fatty acid moieties differently activated mouse GPR34 in the TGFα shedding assay. The rank order potencies

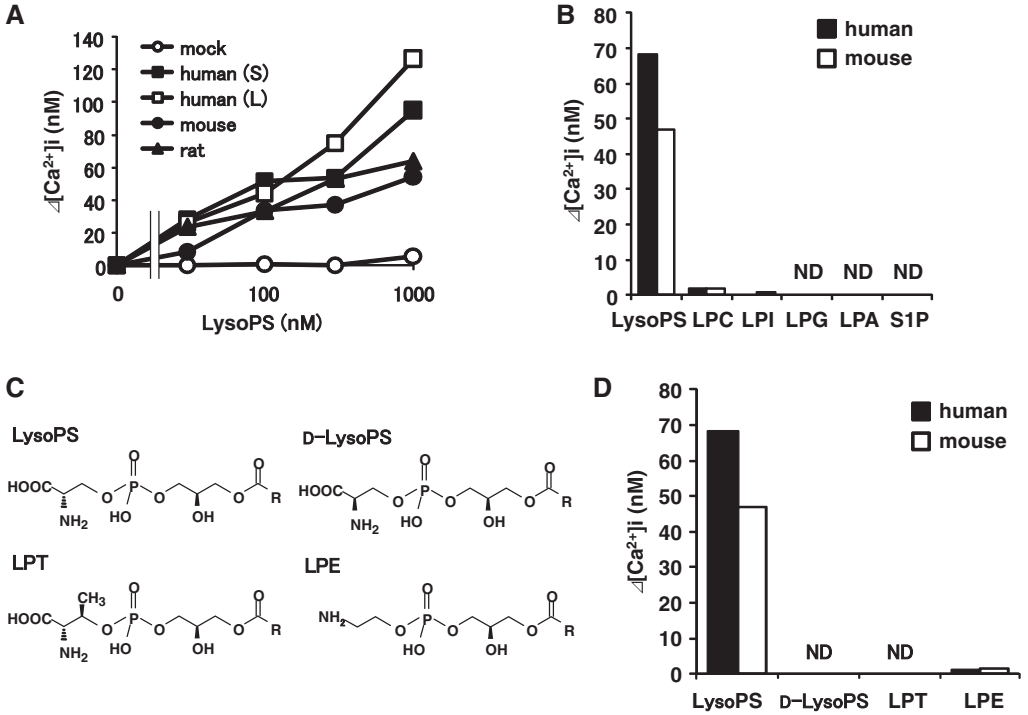


Fig. 1 LysoPS specifically induced calcium mobilization in HEK293 cells expressing GPR34. (A, B and D**)** Effect of LysoPS on calcium mobilization ($[Ca^{2+}]_i$) in HEK293 cells expressing GPR34. HEK293 cells with a transient expression of Gq/i1 and GPR34 were loaded with the fluorescent Ca^{2+} indicator dye fura-2 for 30 min. Fura-2-loaded cells were stimulated with LysoPS (A, B and D), various lysophospholipids including LPC, LPI, LPG, LPA and S1P (100 nM) (B) or LysoPS analogues including D-serine LysoPS (D-LysoPS), LPT and LPE (100 nM) (D), and $[Ca^{2+}]_i$ was determined fluorometrically using CAF-110. HEK293 cells transfected with human long form (L), human short form (S), mouse, rat GPR34 or control vector were tested in Figure 1A. HEK293 cells transfected with human (L) GPR34 or mouse GPR34 were tested in Figure 1B and D. In A, B and D, experiments were performed three times and representative results were shown. ND, not detected. **(C)** Structure of LysoPS analogues.

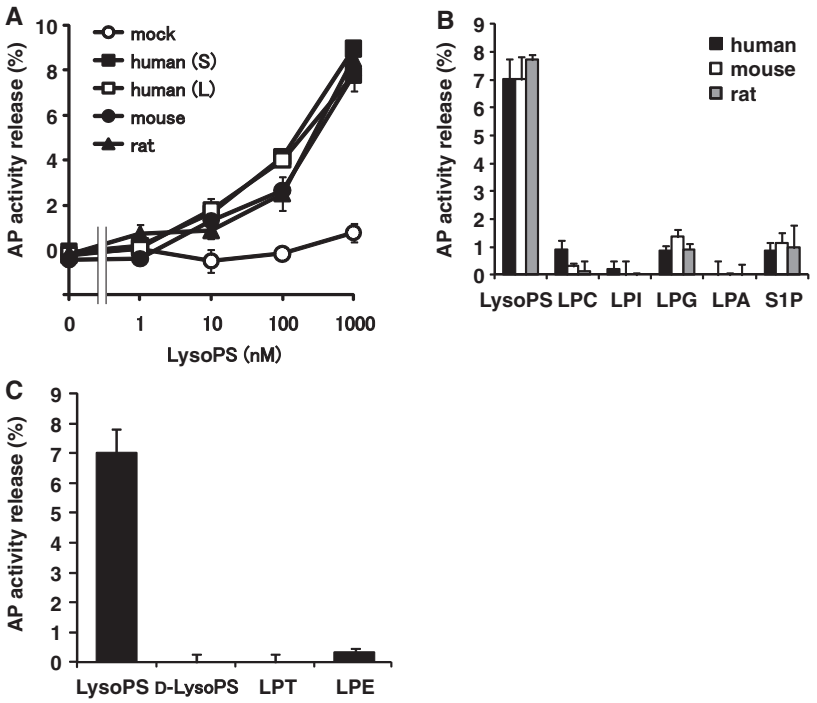


Fig. 2 LysoPS specifically induced AP-TGFα release in HEK293 cells expressing GPR34. (A**)** Effect of LysoPS on release of AP-TGFα in HEK293 cells expressing GPR34. HEK293 cells with a transient expression of AP-TGFα, Gq/i1 and GPR34 were treated for 1 h with LysoPS, and AP activity in conditioned media was quantified with a colorimetric assay. HEK293 cells transfected with human long form (L) GPR34, human short form (S) GPR34, mouse GPR34, rat GPR34 or control vector were tested here. **(B)** Effect of various lysophospholipids including LPC, LPI, LPG, LPA and S1P (1 μM) on release of AP-TGFα. The release of AP-TGFα is measured in HEK293 cells expressing human (left), mouse (centre) and rat (right) in a similar manner shown in Figure 2A. **(C)** Effect of various LysoPS analogues including D-serine LysoPS (D-LysoPS), LPT and LPE (1 μM) on release of AP-TGFα. The release of AP-TGFα is measured in HEK293 cells expressing mouse GPR34 in a similar manner shown in Figure 2A. Each experiment was performed in triplicate and data are the mean ± S.E. ($n = 3$).

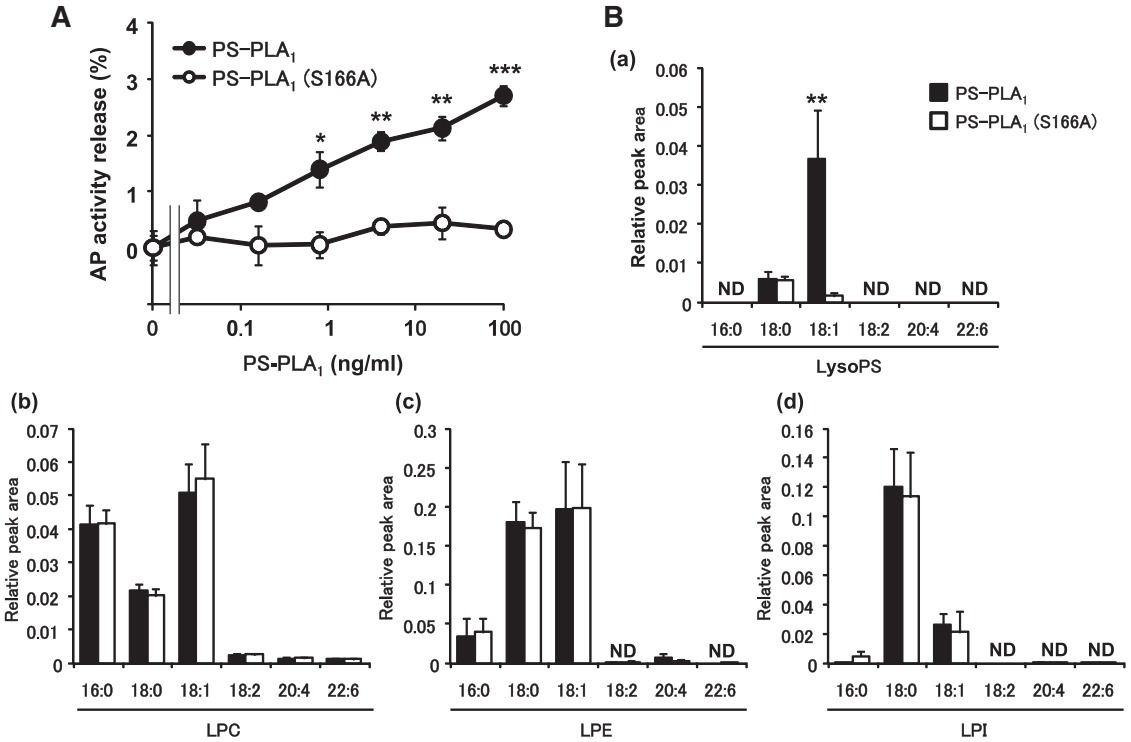


Fig. 3 PS-PLA$_1$ induces enzymatic activity-dependent AP-TGFα release in HEK293 cells expressing GPR34. (A) Effect of PS-PLA$_1$ on release of AP-TGFα in HEK293 cells expressing GPR34. HEK293 cells with a transient expression of AP-TGFα, Gq/i1 and mouse GPR34 were treated for 1 h with the indicated amount of recombinant mouse PS-PLA$_1$ or catalytically inactive PS-PLA$_1$ (S166A), and AP activity in conditioned media was quantified with a colorimetric assay. Each experiment was performed in triplicate and data are the mean ± SE ($n = 3$). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ (Student's *t*-test) versus mutant. **(B)** PS-PLA$_1$ specifically produces LysoPS, but not other lysophospholipids including LPC, LPE and LPI in HEK293 cells. Lysophospholipids in HEK293 cells incubated with recombinant PS-PLA$_1$ were extracted with methanol and analysed by an LC–MS/MS method. Each experiment was performed in triplicate and data are the mean ± SE ($n = 3$). $^{**}P < 0.01$ (Student's *t*-test) versus mutant. ND, not detected.

were 14:0-LysoPS ≥ 16:0-LysoPS ≥ 18:1-LysoPS = 16:1-LysoPS > 12:0-LysoPS > 18:0-LysoPS >> 10:0-LysoPS (Fig. 4A). Thus, GPR34 also recognizes the fatty acid moiety of LysoPS in addition to the serine head group.

To examine the effect of the fatty acid position of LysoPS, dioleoyl PS was digested with either PLA$_1$ or PLA$_2$, which yields 2-acyl-LysoPS and 1-acyl LysoPS, respectively, and the reaction products were subjected to the TGFα shedding assay. We measured the liberated fatty acids in the reaction products and the concentrations of LysoPS were corrected. When the products were subjected to the TGFα shedding assay, PLA$_1$ products of PS induced a much greater AP-TGFα release than PLA$_2$ products, suggesting that GPR34 prefers LysoPS with fatty acid at the *sn*-2 position of the glycerol backbone (Fig. 4B). In general, 2-acyl-LysoPS is unstable and the fatty acid of 2-acyl-LysoPS can easily migrate yielding 1-acyl-LysoPS due to acyl migration. In this study, we synthesized an acyl migration-resistant 2-acyl-LysoPS analogue, 2-oleoyl-1-deoxy-LysoPS, which lacks *sn*-1 hydroxyl group. In the TGFα shedding assay, 2-oleoyl-1-deoxy-LysoPS-induced AP-TGFα release at a concentration 100 times lower than a migration-resistant 1-acyl-LysoPS analogue, 1-oleoyl-2-deoxy-LysoPS, confirming that GPR34 is a receptor for 2-acyl-LysoPS (Fig. 4C). The result also suggests that PS-PLA$_1$ is an enzyme involved in the synthesis of the ligand for GPR34.

LysoPS stimulates cellular migration but not in the presence of PTX

LysoPS is known to induce cell migration of mouse fibroblasts (*7*) and human glioma cells (*8*). To determine whether GPR34 is involved in LysoPS-induced cell migration, we used CHO cells that stably express mouse GPR34. In a Boyden-chamber assay, LysoPS strongly stimulated the migration of CHO cells expressing GPR34 in dose-dependent manner ($EC_{50} = 129$ nM), but it did not stimulate the migration of control CHO cells (Fig. 5A). The LysoPS-induced cell migration was sensitive to pertussis toxin (PTX) (Fig. 5B), indicating that a Gi/o type G protein is involved in the activation of GPR34. LPA-induced migration of both control and GPR34-expressing CHO cells. Other lysophospholipids did not induce the migratory response in GPR34-expressing CHO cells (data not shown).

Discussion

It has been controversial whether GPR34 is a functional receptor for LysoPS. In this study, we used several assay systems in combination with chemically modified LysoPS analogues to confirm that GPR34 responded to LysoPS to elicit various cellular responses including an increase in $[Ca^{2+}]_i$ and TGFα shedding. We also found that LysoPS-stimulated migration of GPR34-expressing cells as a cellular

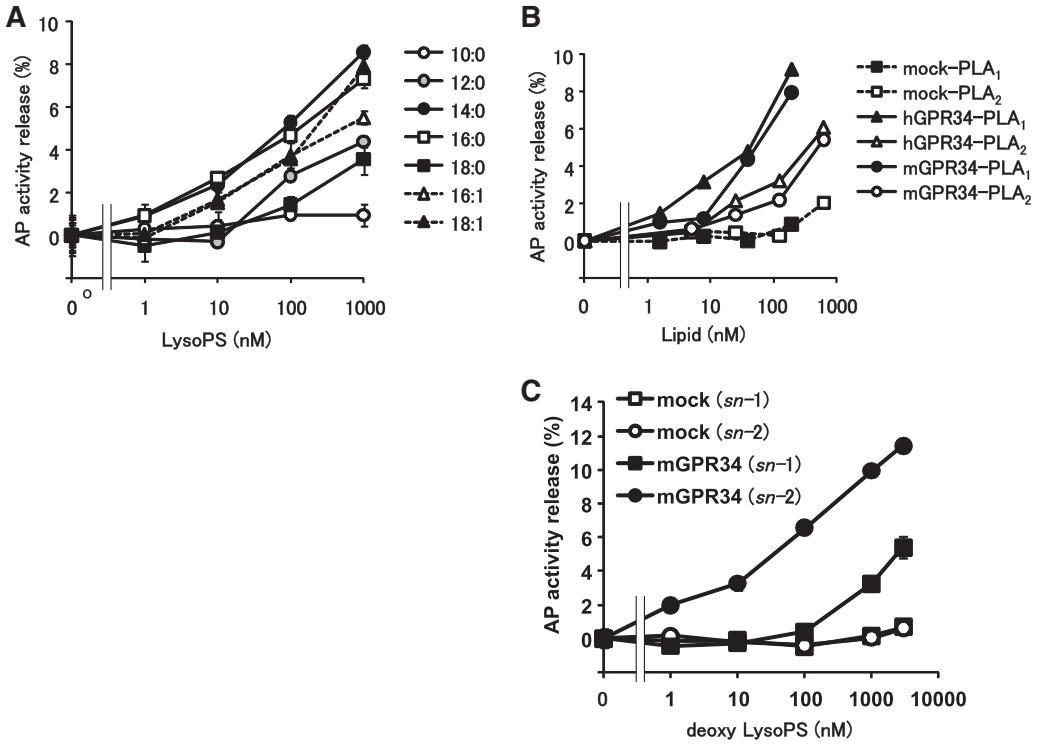


Fig. 4 LysoPS with fatty acid at the *sn*-2 position of glycerol backbone is potent ligand for GPR34. (A) Effect of LysoPS with various fatty acid on release of AP-TGFα in HEK293 cells expressing GPR34. HEK293 cells with a transient expression of AP-TGFα, Gq/i1 and mouse GPR34 were treated with the indicated concentration of LysoPS, and AP activity in conditioned media was quantified with a colorimetric assay. **(B)** Effect of PLA_1 and PLA_2 reaction product on release of AP-TGFα in HEK293 cells expressing GPR34. Dioleoyl PS was incubated with PLA_1 or PLA_2 for 30 min, and the reaction products were immediately used for the TGFα shedding assay. Human or mouse GPR34 expressing HEK293 cells were stimulated with the PLA_1 or PLA_2 reaction products. **(C)** Effect of the position of fatty acyl chains on release of AP-TGFα. HEK293 cells expressing mouse GPR34 were treated with LysoPS, 1-acyl-2-deoxy LysoPS (*sn*-1 deoxy LysoPS) or 1-deoxy-2-acyl-LysoPS (*sn*-2 deoxy LysoPS). The release of AP-TGFα is measured in a similar manner shown in figure 2. Each experiment was performed in triplicate and data are the mean ± SE ($n = 3$).

function of LysoPS. Firstly, we confirmed that GPR34 mediated a Ca^{2+} response in HEK293 cells in a transient expression system. Previously, we showed that CHO-K1 cells stably expressing mouse GPR34 responded to LysoPS to increase $[Ca^{2+}]_i$ (*10*). We also found that LysoPS-stimulated AP-TGFα release in the TGFα shedding assay in GPR34-expressing HEK293 cells. In addition, treatment of GPR34-expressing cells with PS-PLA_1, a presumable LysoPS-producing enzyme, resulted in both production of LysoPS in the cells and activation of the receptor. It should be noted that GPR34 from several mammalian species (human, mouse and rat) responded to LysoPS in the TGFα shedding assay in a similar manner. In addition, we tested GPR34 cDNA constructs with differing initiation methionine and found that both constructs equally responded to LysoPS. We also tested other assays for activation of GPR34 by LysoPS. For example, a widely used reporter assay using the zif268 promoter (*14*) failed to detect activation of mammalian GPR34 in PC12h cells (data not shown). Thus, it is reasonable to assume that activation of GPR34 can be detected only in certain cell types using certain assays. Consistent with this notion, Liebscher *et al.* (*11*) who expressed mammalian GPR34 in COS-7 and yeast cells, reported that LysoPS did not affect cAMP inhibition or $[Ca^{2+}]_i$ in COS-7 cells or the growth of yeast cells. Interestingly, however, they showed that GPR34 is highly conserved in vertebrates and GPR34 from carp (a kind of fish) responded to LysoPS (*11*). Thus it is reasonable to assume that GPR34 is a functional receptor for LysoPS in a wide range of vertebrates.

There is accumulating evidence that the fatty acid species and the manner they are linked to the glycerol backbone determine the affinity of lysophospholipids to their receptors. For example, an LPA receptor, LPA_3/EDG7, is activated fully by LPA with an unsaturated fatty acid at the *sn*-2 position of the glycerol backbone (*15*, *16*). When phosphatidic acid with various fatty acid species was treated with either PLA_1 or PLA_2, reaction products with PLA_1 were by far potent in activating LPA_3 than those with PLA_2. In addition, a 2-acyl-LPA analogue, T13, strongly activated LPA_3 (*17*). Furthermore, the ligand specificity of a recently identified sixth LPA receptor, LPA_6/P2Y5, appears to be similar to that of LPA_3 (*18*). The ligand preference of LPA_6/P2Y5 is reasonable from the viewpoint of the LPA synthetic pathway, because phosphatidic acid-preferential $PLA_1\alpha$ (PA-$PLA_1\alpha$/LIPH), which preferentially produces LPA with an unsaturated fatty acid at the *sn*-2 position, is the enzyme that supplies LPA to LPA_6/P2Y5 (*13*, *19*, *20*). Both LPA_6/P2Y5 and PA-$PLA_1\alpha$ are expressed highly in hair

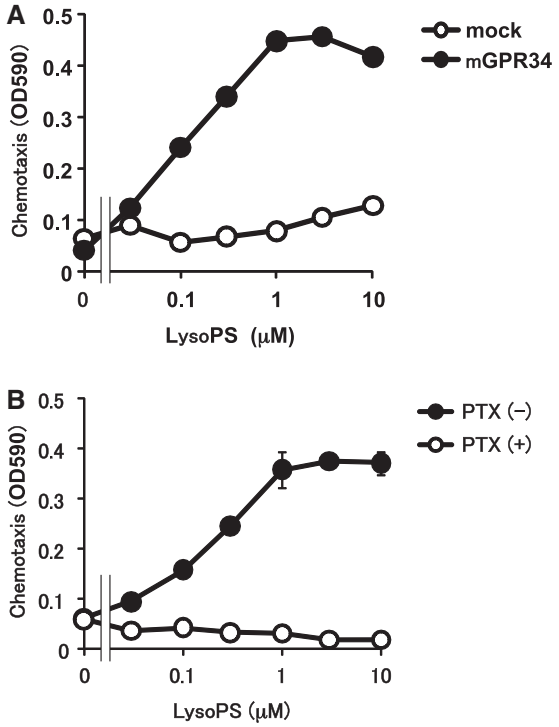


Fig. 5 LysoPS-stimulated chemotaxis of CHO-K1 cells stably expressing mouse GPR34 is mediated via a PTX-sensitive G protein. **(A)** Effect of LysoPS on chemotaxis of CHO-K1 cells stably expressing mouse GPR34 (CHO-GPR34) in the Boyden chamber. The lower compartment contained LysoPS at the concentrations indicated. CHO-GPR34 cells were seeded into the upper compartment of the chamber and incubated at 37°C for 6 h. At the end of the treatments, migrated cells were stained with a Diff-Quick stain kit and the absorbance of the stained cells on the bottom side of the filter was measured with a plate reader. **(B)** CHO-GPR34 cells were pre-treated without or with PTX (25 ng/ml) for 24 h, and the chemotaxis of CHO-GPR34 cells were evaluated in a similar manner shown in Figure 5A. Each experiment was performed in triplicate and data are the mean ± SE ($n = 3$).

follicles and contribute to their formation. Interestingly, mutations in both genes result in similar hair disorders termed LAH2 and LAH3, which are characterized by hereditary woolly hair and/or sparse hair (*20–23*). Both LPA_3 and LPA_6/P2Y5 prefer LPA with an unsaturated fatty acid. The fact that GPR34 has a similar preference for LysoPS with an unsaturated fatty acid at the *sn*-2 position indicates that PLA_1 enzymes are responsible for supplying the ligand to GPR34. PS-PLA_1 is the enzyme that is most likely to supply LysoPS to GPR34 because it activates GPR34 at the cellular level (Fig. 3). A recent study with knockout mice revealed that GPR34 has a role in suppressing the expression of pro-inflammatory cytokines such as TNF-α and IFN-γ (*11*). A remaining question is whether PS-PLA_1 also supplies LysoPS to GPR34 under pathophysiological conditions. This possibility is now being tested in our laboratory.

Supplementary Data

Supplementary Data are available at *JB* Online.

Funding
This study was supported by Japan Science and Technology Agency Precursory Research for Embryonic Science and Technology (PRESTO) (to K.M.), Core Research for Evolutional Science and Technology (CREST) (to J.A.), Grant in aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (to K.M. and J.A.).

Conflict of interest
None declared.

References

1. Martin, T.W. and Lagunoff, D. (1979) Interactions of lysophospholipids and mast cells. *Nature* **279**, 250–252
2. Smith, G.A., Hesketh, T.R., Plumb, R.W., and Metcalfe, J.C. (1979) The exogenous lipid requirement for histamine release from rat peritoneal mast cells stimulated by concanavalin A. *FEBS Lett*. **105**, 58–62
3. Horigome, K., Tamori-Natori, Y., Inoue, K., and Nojima, S. (1986) Effect of serine phospholipid structure on the enhancement of concanavalin A-induced degranulation in rat mast cells. *J. Biochem*. **100**, 571–579
4. Bruni, A., Bigon, E., Battistella, A., Boarato, E., Mietto, L., and Toffano, G. (1984) Lysophosphatidylserine as histamine releaser in mice and rats. *Agents Actions* **14**, 619–625
5. Lourenssen, S. and Blennerhassett, M.G. (1998) Lysophosphatidylserine potentiates nerve growth factor-induced differentiation of PC12 cells. *Neurosci. Lett*. **248**, 77–80
6. Bellini, F. and Bruni, A. (1993) Role of a serum phospholipase A1 in the phosphatidylserine-induced T cell inhibition. *FEBS Lett*. **316**, 1–4
7. Park, K.S., Lee, H.Y., Kim, M.K., Shin, E.H., Jo, S.H., Kim, S.D., Im, D.S., and Bae, Y.S. (2006) Lysophosphatidylserine stimulates L2071 mouse fibroblast chemotactic migration via a process involving pertussis toxin-sensitive trimeric G-proteins. *Mol. Pharmacol*. **69**, 1066–1073
8. Lee, S.Y., Lee, H.Y., Kim, S.D., Jo, S.H., Shim, J.W., Lee, H.J., Yun, J., and Bae, Y.S. (2008) Lysophosphatidylserine stimulates chemotactic migration in U87 human glioma cells. *Biochem. Biophys. Res. Commun*. **374**, 147–151
9. Sugo, T., Tachimoto, H., Chikatsu, T., Murakami, Y., Kikukawa, Y., Sato, S., Kikuchi, K., Nagi, T., Harada, M., Ogi, K., Ebisawa, M., and Mori, M. (2006) Identification of a lysophosphatidylserine receptor on mast cells. *Biochem. Biophys. Res. Commun*. **341**, 1078–1087
10. Iwashita, M., Makide, K., Nonomura, T., Misumi, Y., Otani, Y., Ishida, M., Taguchi, R., Tsujimoto, M., Aoki, J., Arai, H., and Ohwada, T. (2009) Synthesis and evaluation of lysophosphatidylserine analogues as inducers of mast cell degranulation. Potent activities of lysophosphatidylthreonine and its 2-deoxy derivative. *J. Med. Chem*. **52**, 5837–5863
11. Liebscher, I., Muller, U., Teupser, D., Engemaier, E., Engel, K.M., Ritscher, L., Thor, D., Sangkuhl, K., Ricken, A., Wurm, A., Piehler, D., Schmutzler, S., Fuhrmann, H., Albert, F. W., Reichenbach, A., Thiery, J., Schoneberg, T., and Schulz, A. (2011) Altered immune response in mice deficient for the G protein-coupled receptor GPR34. *J. Biol. Chem*. **286**, 2101–2110
12. Schulz, A. and Schoneberg, T. (2003) The structural evolution of a P2Y-like G-protein-coupled receptor. *J. Biol. Chem*. **278**, 35531–35541

13. Inoue, A., Arima, N., Ishiguro, J., Prestwich, G.D., Arai, H., and Aoki, J. (2011) LPA-producing enzyme PA-PLA$_1\alpha$ regulates hair follicle development by modulating EGFR signalling. *EMBO J.* **30**, 4248–4260
14. Noguchi, K., Ishii, S., and Shimizu, T. (2003) Identification of p2y9/GPR23 as a novel G protein-coupled receptor for lysophosphatidic acid, structurally distant from the Edg family. *J. Biol. Chem.* **278**, 25600–25606
15. Bandoh, K., Aoki, J., Hosono, H., Kobayashi, S., Kobayashi, T., Murakami-Murofushi, K., Tsujimoto, M., Arai, H., and Inoue, K. (1999) Molecular cloning and characterization of a novel human G-protein-coupled receptor, EDG7, for lysophosphatidic acid. *J. Biol. Chem.* **274**, 27776–27785
16. Bandoh, K., Aoki, J., Taira, A., Tsujimoto, M., Arai, H., and Inoue, K. (2000) Lysophosphatidic acid (LPA) receptors of the EDG family are differentially activated by LPA species. Structure-activity relationship of cloned LPA receptors. *FEBS Lett.* **478**, 159–165
17. Tamaruya, Y., Suzuki, M., Kamura, G., Kanai, M., Hama, K., Shimizu, K., Aoki, J., Arai, H., and Shibasaki, M. (2004) Identifying specific conformations by using a carbohydrate scaffold: discovery of subtype-selective LPA-receptor agonists and an antagonist. *Angew. Chem. Int. Ed. Engl.* **43**, 2834–2837
18. Yanagida, K., Masago, K., Nakanishi, H., Kihara, Y., Hamano, F., Tajima, Y., Taguchi, R., Shimizu, T., and Ishii, S. (2009) Identification and characterization of a novel lysophosphatidic acid receptor, p2y5/LPA$_6$. *J. Biol. Chem.* **284**, 17731–17741
19. Sonoda, H., Aoki, J., Hiramatsu, T., Ishida, M., Bandoh, K., Nagai, Y., Taguchi, R., Inoue, K., and Arai, H. (2002) A novel phosphatidic acid-selective phospholipase A$_1$ that produces lysophosphatidic acid. *J. Biol. Chem.* **277**, 34254–34263
20. Kazantseva, A., Goltsov, A., Zinchenko, R., Grigorenko, A.P., Abrukova, A.V., Moliaka, Y.K., Kirillov, A. G., Guo, Z., Lyle, S., Ginter, E.K., and Rogaev, E.I. (2006) Human hair growth deficiency is linked to a genetic defect in the phospholipase gene LIPH. *Science* **314**, 982–985
21. Shimomura, Y., Wajid, M., Petukhova, L., Shapiro, L., and Christiano, A.M. (2009) Mutations in the lipase H gene underlie autosomal recessive woolly hair/hypotrichosis. *J. Invest. Dermatol.* **129**, 622–628
22. Pasternack, S.M., von Kugelgen, I., Aboud, K.A., Lee, Y.A., Ruschendorf, F., Voss, K., Hillmer, A.M., Molderings, G.J., Franz, T., Ramirez, A., Nurnberg, P., Nothen, M.M., and Betz, R.C. (2008) G protein-coupled receptor P2Y5 and its ligand LPA are involved in maintenance of human hair growth. *Nat. Genet.* **40**, 329–334
23. Shimomura, Y., Wajid, M., Ishii, Y., Shapiro, L., Petukhova, L., Gordon, D., and Christiano, A.M. (2008) Disruption of P2RY5, an orphan G protein-coupled receptor, underlies autosomal recessive woolly hair. *Nat. Genet.* **40**, 335–339