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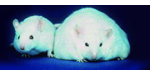

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The heterotrimeric G protein subunits $G\alpha_q$ and $G\beta_1$ have lysophospholipase D activity

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In a previous study we purified a novel lysoPLD (lysophospholipase D) which converts LPC (lysophosphatidylcholine) into a bioactive phospholipid, LPA (lysophosphatidic acid), from the rat brain. In the present study, we identified the purified 42 and 35 kDa proteins as the heterotrimeric G protein subunits $G\alpha_q$ and $G\beta_1$ respectively. When FLAG-tagged $G\alpha_q$ or $G\beta_1$ was expressed in cells and purified, significant lysoPLD activity was observed in the microsomal fractions. Levels of the hydrolysed product choline increased over time, and the Mg^{2+} dependency and substrate specificity of $G\alpha_q$ were similar to those of lysoPLD purified from the rat brain. Mutation of $G\alpha_q$ at amino acids Lys^{52}, Thr^{186} or Asp^{205}, residues that are predicted to interact with nucleotide phosphates or catalytic Mg^{2+}, dramatically reduced lysoPLD activity. GTP does not compete with LPC for the lysoPLD activity, indicating that these substrate-binding sites are not identical. Whereas the enzyme activity of highly purified FLAG-tagged $G\alpha_q$ overexpressed in COS-7 cells was ~4 nmol/min per mg, the activity from Neuro2A cells was 137.4 nmol/min per mg. The calculated K_m and V_{max} values for lysoPAF (1-*O*-hexadecyl-*sn*-glycero-3-phosphocholine) obtained from Neuro2A cells were 21 μM and 0.16 μmol/min per mg respectively, similar to the enzyme purified from the rat brain. These results reveal a new function for $G\alpha_q$ and $G\beta_1$ as an enzyme with lysoPLD activity. Tag-purified $G\alpha_{11}$ also exhibited a high lysoPLD activity, but $G\alpha_i$ and $G\alpha_s$ did not. The lysoPLD activity of the $G\alpha$ subunit is strictly dependent on its subfamily and might be important for cellular responses. However, treatment of Hepa-1 cells with $G\alpha_q$ and $G\alpha_{11}$ siRNAs (small interfering RNAs) did not change lysoPLD activity in the microsomal fraction. Clarification of the physiological relevance of lysoPLD activity of these proteins will need further studies.

Key words: autotaxin, $G\alpha_q$, $G\beta_1$, lysophosphatidic acid (LPA), lysophosphatidylcholine (LPC), lysophospholipase D (lysoPLD).

INTRODUCTION

LPA (lysophosphatidic acid) is a potentially bioactive phospholipid that mediates a number of physiological processes, including cell adhesion, proliferation, differentiation, survival and migration. GPCRs (G-protein-coupled receptors) in the EDG (endothelial differentiation gene) and P2Y families are specific receptors for LPA in the plasma membrane, and intracellular signalling via these GPCRs has been well characterized [1]. However, less is known about the physiological regulation of LPA production or how LPA-specific receptors in the plasma membrane are stimulated *in vivo*. At least two plausible enzymatic pathways for the production of LPA have been described. One of these is the deacylation of PA (phosphatidic acid) by activated PLA (phospholipase A) [2]. In support of this idea, a PA-selective PLA_1 (phospholipase A_1) called mPA-$PLA_1\alpha$/LIPH (membrane-associated phosphatidic acid-selective phospholipase A1α/lipase member H) has been identified that hydrolyses acyl residues at the sn−1 position of PA and enhances release of LPA from the plasma membrane [3,4]. The other pathway for LPA production is direct production of equimolar amounts of LPA and choline from LPC (lysophosphatidylcholine), as catalysed by lysoPLD (lysophospholipase D) [5,6].

The physiological importance of LPA production in serum by lysoPLD was suggested by Tokumura et al. [7] approximately 25 years ago. In circulating blood, LPA modulates blood pressure [8,9], maintenance of pregnancy [10] and maturation of embryonic vessel systems [11]. Previously, a secreted protein with lysoPLD activity was purified from plasma and serum, and was found to be identical with a molecule known as autotaxin [12,13]. Autotaxin [ENPP2 (ectonucleotide pyrophosphatase/phosphodiesterase 2)] was originally cloned as a tumour cell motility-stimulating factor and was recognized as a nucleotide pyrophosphatase on the basis of its primary structure and *in vitro* enzymatic characteristics [14]. LPC, which can bind albumin and lipoproteins, is released into circulation from the liver and may be generated by lecithin/cholesterol acyltransferase. Autotaxin might hydrolyse these LPCs as substrates, and LPA accumulated to levels greater than 50 μM in plasma, presumably due to very low activity of LPA-degrading enzymes in that context [7].

When cells are stimulated by hormones and proliferation factors, equimolar amounts of free fatty acid and LPC are produced from PC (phosphatidylcholine) by the activated PLA_2 (phospholipase A_2) [15]. The free fatty acids that are released, including arachidonic acid, which is present at position sn−2 of PC prior to cleavage, are converted into eicosanoids. Whereas the metabolism of arachidonic acid has been well characterized, the metabolism of LPC is less well understood. Because the accumulation of LPC in cells induces cell lysis [16], it has been thought that LPC was easily hydrolysed by lysophospholipases we cloned in a previous study [17], followed by conversion

Abbreviations used: $G\alpha_q$, heterotrimeric G protein α subunit q; GAP, GTPase-activating protein; $G\beta_1$, heterotrimeric GTP-binding protein β subunit 1; GPC, glycerophosphorylcholine; GPCR, G-protein-coupled receptor; HPPA, 3-(4-hydroxyphenyl) propionic acid; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; lysoPAF, 1-*O*-hexadecyl-*sn*-glycero-3-phosphocholine; lysoPLD, lysophospholipase D; MALDI–TOF-MS, matrix-assisted laser desorption ionization–time-of-flight MS; PA, phosphatidic acid; PC, phosphatidylcholine; PI-PLC, phosphoinositide phospholipase C; PLA_2, phospholipase A_2; PMF, peptide mass fingerprinting; PPARγ, peroxisome proliferation-activator receptor γ; siRNA, small interfering RNA.

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into a non-bioactive saturated free fatty acid and GPC (glycerophosphorylcholine). However, LPC itself was recognized recently as a bioactive phospholipid that can induce chemotaxis [18], signal-responsive kinase activities [19] and atherosclerosis [20].

The first enzyme with lysoPLD activity was isolated from rat brain microsomes by Wykle and Schremmer [5]. The characteristics of this intracellular enzyme, such as substrate specificity, cation requirement and optimal pH, are different from those of autotaxin. We purified a novel lysoPLD from the rat brain that is a Mg^{2+}-dependent enzyme and utilizes lysoPAF (1-*O*-hexadecyl-*sn*-glycero-3-phosphocholine) as a substrate [21], similar to the enzyme reported by Wykle and Schremmer [5]. This enzyme might be involved in the production of LPA from LPC, which is produced from PC hydrolysed by PLA_2 in cells. Previously, it has been reported that LPA receptors are expressed in perinuclear areas [22,23] and, furthermore, that the transcription factor PPARγ (peroxisome proliferation-activator receptor γ) is bound to LPA [24]. Based on these observations, it seems reasonable to propose that intracellular lysoPLD is important for cellular responses.

In the present study, we have identified a purified protein with lysoPLD activity from the rat brain as the heterotrimeric G protein subunits Gα_q and Gβ_1. Mutations predicted to affect the binding of nucleotide phosphates or Mg^{2+} to the Gα_q subunit resulted in a dramatic reduction of lysoPLD activity. Moreover, several types of Gα subunits, including Gα_q and Gα_{11}, exhibit lysoPLD activity when expressed and purified as tagged proteins.

EXPERIMENTAL

Materials

LPC (1-palmitoyl, 16:0; stearoyl, 18:0; and oleoyl, 18:1) was obtained from Sigma–Aldrich. LysoPAF was from Alexis Biochemicals. 1-[^{14}C]Palmitoyl-2-lyso-phosphatidylcholine was purchased from GE Healthcare. HPPA [3-(4-hydroxyphenyl) propionic acid], peroxidase and choline oxidase were purchased from Wako Chemicals. Silica Gel 60 plates and tag-purified G(β_1 and γ_2) complex overexpressed in *Spodoptera frugiperda* insect cells were purchased from Merck, and CHAPS was purchased from Dojindo. Antibodies against G$\alpha_{q/11}$, G$\beta_{1–4}$, and Gγ_5 were obtained from Santa Cruz Biotechnology, and anti-FLAG antibody, anti-FLAG affinity gels, FLAG peptides, anti-actin antibody and GTP were purchased from Sigma. The monoclonal anti-autotaxin antibody was obtained from Dr Junken Aoki (Graduate School of Pharmaceutical Sciences, Tohoku University, Japan).

Identification of purified lysoPLD

Purification of lysoPLD from rat brain tissue was performed exactly as described previously [21]. All steps in enzyme purification were carried out at 4 °C. Briefly, rat brains were minced and homogenized with a Teflon homogenizer in 9 volumes of buffer A [0.3 M sucrose in 50 mM Tris/HCl (pH 7.4), 1 mM EDTA, 1 mM dithiothreitol and 0.1 mM PMSF). Nuclear fractions were obtained by centrifugation at 600 ***g*** for 10 min and resuspended in buffer A. To dissolve lysoPLD in the nuclear pellets, 10 mM CHAPS was added and the suspension was sonicated with a Microson Ultrasonic cell disruptor (Misonix). The suspensions were then centrifuged at 10000 ***g*** for 10 min and the supernatants were used as a source of enzyme. The supernatants were applied to a DEAE Cellulofine A-500 column equilibrated with buffer B [20 mM Tris/HCl (pH 7.5), 0.1 mM PMSF and 5 mM CHAPS), and the enzyme was eluted with a NaCl gradient. Active fractions were sequentially applied to three different types of columns. The enzyme fractions were concentrated using Microcon centrifugal filter devices (Millipore) and then applied to a Superdex 200 10/300 GL column. As a final step, the eluted active fractions were applied to a HiTrap DEAE FF column using a Waters 650 (Millipore). The final eluted fraction was then subjected to SDS/PAGE (10 % gel) and the gel was stained with EZ Stain Silver (Atto). The major protein bands were excised for in-gel digestion with MS grade trypsin (Wako Chemicals). The mass spectra of extracted peptides were analysed by MALDI–TOF MS (matrix-assisted laser desorption ionization–time-of-flight MS) (Voyager Elite/STR Perspective, Life Technologies), and the proteins were subsequently identified using MS-Fit (http://prospector.ucsf.edu).

SDS/PAGE and immunoblotting

Proteins from rat brains and the FLAG tag affinity gel-purified fractions (10 μl) were separated on 10 % acrylamide gels, and stained with a silver staining kit (Wako Chemicals) or transferred on to PVDF membranes using a semi-dry transfer apparatus (Nippon Eido) and blocked with 5 % skimmed milk in TBS-T buffer [150 mM NaCl, 20 mM Tris/HCl (pH 8.0) and 0.05 % Tween 20]. The membranes were incubated overnight at 4 °C with primary antibody diluted 1:1000 (anti-G$\alpha_{q/11}$, -G$\beta_{1–4}$, -Gγ_5, -actin or -autotaxin) or 1:5000 (anti-FLAG) in TBS-T and then for 1 h at room temperature (20 °C) with a peroxide-conjugated secondary antibody diluted 1:5000 in TBS-T. Reactive bands were detected by chemiluminescence using the Bio-Rad Laboratories ChemiDoc XRS+ system.

Plasmid construction

N-terminally FLAG-tagged Gα_q (GenBank® accession number NM_031036.1), Gα_{11} (GenBank® accession number NM_031033.1) or Gβ_1 (GenBank® accession number NM_030987.2) cDNAs were amplified from cDNAs from a rat brain cDNA library using a forward primer containing the FLAG tag sequence, then subcloned into the pcDNA3.1 V5/His TOPO TA vector (Invitrogen). PCR was performed with the following pairs of primers: Gα_q, 5′-GGAAGAATGGACTACAAGGACGACGATGACAAGACTCTGGAGTCCATCATGG-3′ and 5′-TCACACCAGATTGTACTCCTTCAGG-3′; Gα_{11}, 5′-GCGACGATGGACTACAAGGACGACGATGACAAGACTCTGGAGTCCATGATG-3′ and 5′-TCACACCAGATTGTACTCCTTCAGG-3′; and Gβ_1, 5′-GTGAAGATGGACTACAAGGACGACGATGACAAGAGTGAACTTGACCAGCTGC-3′ and 5′-GTTCCAGATCTTGAGGAAG-3′. The Gα_i (GenBank® accession number NM_010305.1) and Gα_s (GenBank® accession number AK168996.1) cDNAs were amplified from a mouse brain cDNA library with the primer pairs 5′-AAAGAATTCGCCACCATGGGCTGCAC-3′ and 5′-AAACTCGAGTTCGAAGAGACCACAGTCTTT-3′, and 5′-AAAGAATTCGCCGCCATGGGCTGCCTC-3′ and 5′-AAACTCGAGTTAGAGCAGCTCGTATTGGCG-3′ respectively. The amplicons were cloned into pcDNA3.1 His/V5 after digestion with EcoRI and XhoI. The FLAG tag was added via a site-directed mutagenesis approach using the following primers paired with their complimentary sequences: Gα_i, 5′-GAATTCGCCACCATGGACTACAAGGACGACGATGACAAGGGCTGCACATTGAG-3′; and Gα_s, 5′-GAATTCGCCGCCATGGACTACAAGGACGACGATGACAAGGGCTGCCTCGGC-3′. Mutations were introduced by site-directed mutagenesis using the following primers paired with complimentary sequences: Gα_q (G48V),

5′-CTGCTGCTGGGGACAGTCGAGAGTGGCAAG-3′; $G\alpha_q$ (G48A), 5′-CTGCTGCTGGGGACAGCGGAGAGTGGCA-AG-3′; $G\alpha_q$ (K52A), 5′-GACAGGGGAGAGTGGCGCGAGTA-CCTTCATAAG-3′; $G\alpha_q$ (T186A), 5′-GTTCGAGTCCCCGCCA-CAGGGATCATTG-3′; $G\alpha_q$ (D205A), 5′-TCTTCAGAATGGT-CGCTGTAGGAGGCCAAAGG-3′; $G\alpha_q$ (Q209L), 5′-TGTAGG-AGGCCTAAGGTCAGAGA-3′; and $G\beta_1$ (H311A), 5′-GTC-CTAGCTGGAGCTGACAACCGAGTCAGC-3′. The sequences of the constructs were verified by direct DNA sequencing (ABI PRISM 377-XL, Applied Biosystems).

LysoPLD assay

The isotopic lysoPLD activity assay was performed as described previously [21]. Briefly, 30 μl of the source of enzyme was incubated at 37 °C for 6 h in a reaction mixture containing 0.15 mM 1-[^{14}C]palmitoyl-GPC (6000 d.p.m./nmol), 20 mM Tris/HCl (pH 7.0), 1 mM Na_3VO_4, and 50 mM $MgCl_2$ with or without 1 mM GTP in a final volume of 75 μl. The reactions were terminated by the addition of 15 μl of 2 M HCl and 187.5 μl of chloroform/methanol/HCl [100:200:1 (v/v)]. The lipids were extracted by the addition of 93.75 μl each of chloroform and 2 M KCl, followed by centrifugation at 500 ***g*** for 10 min at 20 °C. The extracted lipids were subjected to two-dimensional TLC [chloroform/methanol/28 % ammonia at 65:35:5 (v/v) for the first dimension and chloroform/acetone/methanol/acetic acid/water at 45:20:10:13:5 (v/v) for the second dimension]. The LPA spots were visualized and quantified using the Fuji BAS2000 system (Fujifilm).

For the colorimetric assay to detect lysoPLD activity, the amount of choline released from choline lysophospholipids was used as a measure of enzyme activity. Purified proteins (40 μl) were incubated at 37 °C with 0.15 mM (0.0375–0.6 mM) choline lysophospholipids in the presence of 20 mM Tris/HCl (pH 7.0) and 1 mM Na_3VO_4 with or without 50 mM $MgCl_2$ and/or 100 mM EDTA in a total reaction volume of 200 μl. After incubation for the given lengths of time, the reactions were terminated by boiling. To determine the amount of choline released, a second reaction was performed at 37 °C for 15 min in a 500 μl reaction mixture containing the first assay mixture plus 50 mM Tris/HCl (pH 8.5), 0.5 mM HPPA, 0.033 unit/ml horseradish peroxidase and 1 unit/ml choline oxidase. The fluorescence intensity of each mixture was determined by excitation at 320 nm and collection at 404 nm using an RF5300 instrument (Shimadzu).

Purification of FLAG-tagged proteins

Mouse hepatocytoma cells (Hepa-1 cells), COS-7 cells or Neuro2A cells were seeded into 100 mm dishes in DMEM (Dulbecco's modified Eagle's medium; Wako Chemicals) with 10 % fetal bovine serum (Gibco) 1 day prior to transfection. A total of 24 μg of empty vector or FLAG-tagged protein expression vector were transfected into cells using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions. At 1 day (24 h) post-transfection, the cells in one or five dishes were collected in 800 μl/dish of buffer C [0.25 M sucrose in 50 mM Tris/HCl (pH 7.4), 1 mM EDTA and 1 mM PMSF] and disrupted by sonication (three to eight pulses of 10 s each) with a Microson Ultrasonic cell disruptor. The homogenates were then centrifuged at 105 000 ***g*** for 1 h at 4 °C and the supernatants were removed or used for FLAG-tagged protein purification as indicated below. The pellets were resuspended in 400 μl/dish of buffer C with 10 mM CHAPS, and homogenized with an Ultrasonic cell disruptor. The suspensions were diluted to 1600 μl/dish of buffer C, then centrifuged at 21 500 ***g*** for 10 min at 4 °C and the supernatants were mixed with 250 μl of anti-FLAG M2 affinity gel (Sigma–Aldrich) suspended in 1.6 ml/dish of TBS buffer [50 mM Tris/HCl (pH 7.4), 150 mM NaCl and 1mM PMSF]. After incubation with gentle agitation at 4 °C for several hours, the gel was washed with 5 ml of wash buffer (TBS with 0.1 % CHAPS), and then tagged proteins were eluted with 250 μl of 100 μg/ml FLAG peptide in wash buffer four times. The viability of cells transfected with control or FLAG-tagged protein expression vectors did not change over 24 h and nearly equal amounts of protein were obtained in each sample.

Effect of mouse $G\alpha_q$ and $G\alpha_{11}$ siRNAs (small interfering RNAs) on lysoPLD activity in Hepa-1 cells

Stealth siRNA, a 25-base pair duplex oligoribonucleotide against mouse $G\alpha_q$ or $G\alpha_{11}$, was purchased from Invitrogen. The target sense sequences were: $G\alpha_q$, 5′-CCCUUUGACUUAC-AAAGUGUCAUUU-3′; and $G\alpha_{11}$, 5′-CCAAGUUGGUGUA-CCAGAACAUCUU-3′. Hepa-1 cells (1×10^5) were cultured in 60 mm dishes for 24 h, and then transfected with both siRNAs by using Lipofectamine™ 2000 (Invitrogen) according to manufacturer's instructions. After 48 h, the cells were collected in 400 μl/dish of buffer C and disrupted by sonication (three to eight pulses of 10 s each) with a Microson Ultrasonic cell disruptor. The homogenates were then centrifuged at 105 000 ***g*** for 1 h at 4 °C and the supernatants were removed. The pellets were resuspended in 300 μl/dish of buffer C with 10 mM CHAPS, and homogenized with an Ultrasonic cell disruptor. The suspensions were centrifuged at 21 500 ***g*** for 10 min at 4 °C, and the supernatants were used as microsomal fractions for lysoPLD assay with 10 μg of the protein for a 30 min incubation.

Statistical analysis

All values are expressed as the means ± S.D. ($n \geqslant 3$). Group means were compared using the Student's t test or ANOVA to determine the significance of the differences among the individual means. Statistical significance was assumed at $P < 0.05$. Each experiment was repeated at least twice with similar results.

RESULTS

A protein with lysoPLD activity is highly associated with heterotrimeric G protein

In a previous study, we purified a novel protein with lysoPLD activity from rat brain tissue and analysed its enzymatic characteristics, which were different from those of the secreted lysoPLD autotaxin. Initially, a 35 kDa protein was the main band in purified lysoPLD from rat brain and did not react with an anti-autotaxin antibody [21]. However, as shown in Figure 1(A), two bands (faint 42 kDa and 35 kDa bands, indicated by arrows on the right-hand side) were observed following repeated purifications as reported previously [21], separation by SDS/PAGE and silver staining. The 42 kDa protein was present at lower levels and was not recognized as potentially important at first. To identify their molecular species, the bands were analysed by PMF (peptide mass fingerprinting). The 42 kDa and 35 kDa proteins were identified as $G\alpha_q$ (Figure 1D) and $G\beta_1$ (Figure 1E) respectively.

To confirm this result, the fractions obtained from the final step of enzyme purification with HiTrap DEAE FF (Figure 1C) were subjected to immunoblot analysis with antibodies specific against $G\alpha_{q/11}$ and $G\beta_{1-4}$. As shown in Figure 1(B), both $G\alpha_q$ and

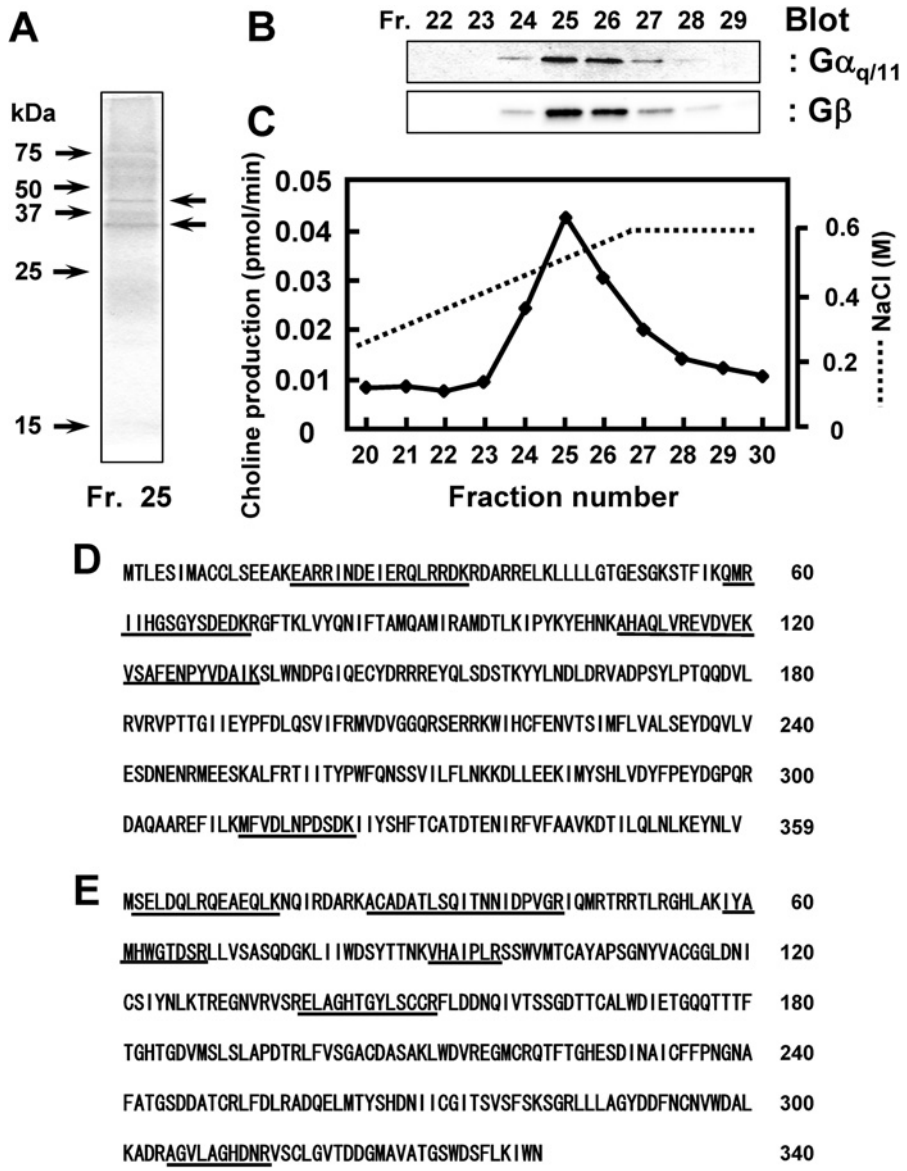

Figure 1 Identification of G protein subunits $G\alpha_q$ and $G\beta_1$ in purified lysoPLD fractions from the rat brain

(**A** and **B**) Fractions eluted after HiTrap DEAE FF purification, the final step in a previously reported protocol for purification of a lysoPLD activity from rat brain tissue, were subjected to SDS/PAGE and detected by silver staining (**A**) and immunoblotting (**B**). The right-hand side arrows in (**A**) indicate protein bands at 42 kDa (upper) and 35 kDa (lower). (**C**) LysoPLD activities of the same purified samples obtained from rat brain tissue as determined using a colorimetric assay. (**D** and **E**) Identification of the purified 42 kDa and 35 kDa proteins from rat brain tissues by PMF. The 42 kDa (**D**) and 35 kDa (**E**) proteins were identified as $G\alpha_q$ and $G\beta_1$ respectively. Amino acid sequences identified by PMF analysis are indicated with underlining. Fr., fraction.

$G\beta_1$ proteins were detected in those fractions and the levels of those proteins correlated well with lysoPLD activities in each fraction. On the basis of these results, we speculated that the heterotrimeric G protein complex is highly associated with lysoPLD activity.

Tag-purified G protein exhibits lysoPLD activity

FLAG-tagged $G\alpha_q$ or $G\beta_1$ proteins were overexpressed in the mouse hepatocytoma cell line Hepa-1 and then purified via immune-affinity chromatography with an anti-FLAG antibody. When overexpressed, FLAG–$G\alpha_q$ was purified with FLAG peptides, endogenous $G\beta$ co-purified and, similarly, endogenous $G\alpha_{q/11}$ co-purified with FLAG-tagged $G\beta_1$ (Figure 2A). $G\gamma_5$ also co-purified with FLAG–$G\alpha_q$ (Figure 2A). These results suggest that heterotrimeric G protein complexes can form with overexpressed FLAG–$G\alpha_q$. The density of the immunoreactive bands with anti-$G\alpha_{q/11}$ or anti-$G\beta_{1\text{-}4}$ antibody and lysoPLD activities coincided well (Figures 2A and 2B); thus we used fraction 4 for subsequent analyses. The significant increase in lysoPLD activity was not observed in fractions prepared from cells transfected with empty vector. The lysoPLD activity was confirmed by choline production from lysoPAF (Figure 2C) and LPA production from [^{14}C]palmitoyl-labelled LPC, (Figures 2D and 2E). Significant increases in both choline and/or LPA production via lysoPLD activity were detected in the eluates prepared from cells overexpressing FLAG–$G\alpha_q$ or FLAG–$G\beta_1$. Autotaxin was not detected in the tag-purified fraction by immunoblot analysis (Figure 2A, indicated by the arrow),

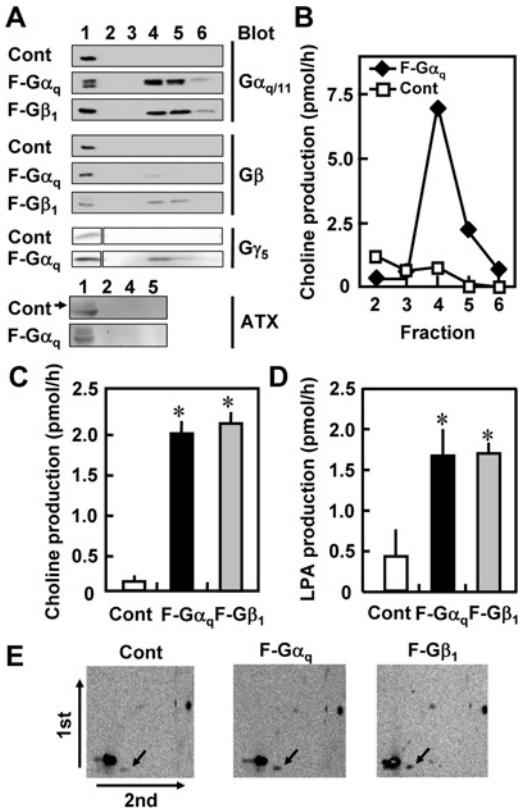

Figure 2 LysoPLD activity of tag-purified G protein subunits $G\alpha_q$ and $G\beta_1$

Empty vector (Cont) or a vector encoding FLAG-tagged $G\alpha_q$ or $G\beta_1$ was transfected into Hepa-1 cells and FLAG-tagged proteins (F-$G\alpha_q$ or F-$G\beta_1$) were purified from one dish. (**A**) Immunoblot analysis of tag-purified G protein subunits $G\alpha_q$ and $G\beta_1$. A total of 10 μl of each sample prior to purification (lane 1), eluted with a wash buffer (lane 2) or eluted with elution buffers containing FLAG-peptides (lanes 3–6) was separated by SDS/PAGE (10 % gel) and transferred on to PVDF membranes. The immunoreacted $G\alpha_q$, $G\beta_1$, $G\gamma_5$, or autotoxin proteins (ATX) were detected with anti-$G\alpha_{q/11}$, -Gβ, -$G\gamma_5$ or -autotoxin antibodies. As expected, the molecular masses of tagged proteins were approximately 2 kDa larger than endogenous proteins. The immunoreacted $G\gamma_5$ in lane 1 and other lanes were determined in separated lanes, but on the same membrane. The experiments were repeated several times and similar results were obtained each time. (**B**) A total of 40 μl of each fraction (lanes 2–6 in **A**) from Hepa-1 cells overexpressing empty vector (open square) or FLAG-tagged $G\alpha_q$ (closed diamond) was incubated with 0.15 mM lysoPAF and then lysoPLD activities were calculated using a colorimetric assay. (**C**) A total of 40 μl of the second fractions eluted with FLAG peptides (lane 4 in **A**) obtained from Hepa-1 cells overexpressing empty vector (white bar), F-$G\alpha_q$ (black bar) or F-$G\beta_1$ (grey bar) was incubated with 0.15 mM lysoPAF for 6 h and then lysoPLD activities were calculated using a colorimetric assay. Results are means $\pm$ S.D. ($n = 6$). *$P < 0.001$ compared with the vector control. (**D** and **E**) LPA production from [^{14}C]-labelled LPC by incubation with the purified enzyme from Hepa-1 cells overexpressing empty vector (Cont) (white bar), FLAG-tagged $G\alpha_q$ (F-$G\alpha_q$) (black bar) or FLAG-tagged $G\beta_1$ (F-$G\beta_1$) (grey bar) for 6 h was visualized after lipids were extracted and subjected to TLC analysis. Results are means $\pm$ S.D ($n = 3$). *$P < 0.012$ compared with the vector control. Arrows (**E**) indicate the positions of LPA. The experiments were repeated more than three times with similar results.

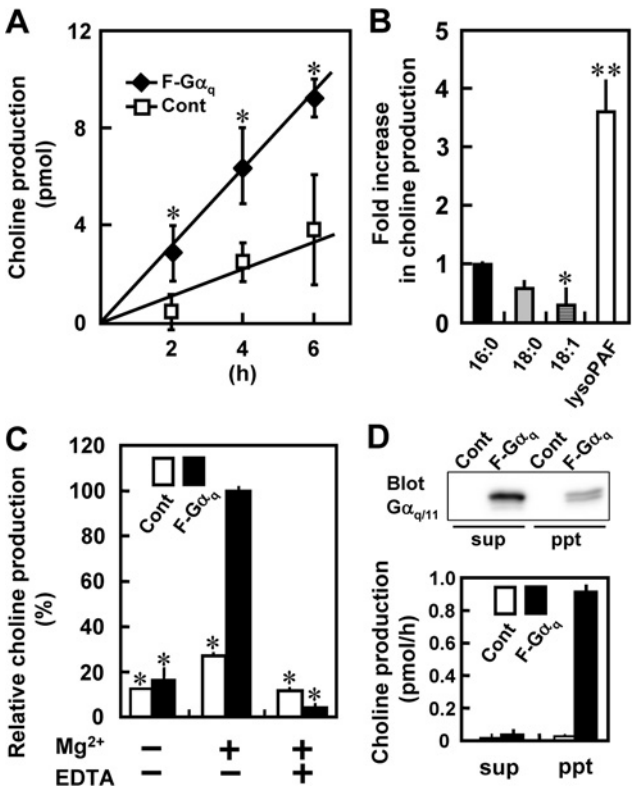

Figure 3 Time dependence, substrate specificity and requirement of Mg^{2+} cations of the lysoPLD activity of tag-purified $G\alpha_q$

(**A**) A total of 40 μl of the tag-purified fractions obtained from Hepa-1 cells overexpressing empty vector (open squares, $n = 6$) or FLAG-tagged $G\alpha_q$ (F-$G\alpha_q$) (closed diamonds, $n = 6$) was incubated with 0.15 mM lysoPAF for the indicated times and then subjected to a colorimetric lysoPLD assay. *$P < 0.01$ compared with the vector control. (**B**) The purified F-$G\alpha_q$ (40 μl) was incubated with 0.15 mM of various choline lysophospholipids [16:0 (black bar, $n = 7$), 18:0 (grey bar, $n = 3$), 18:1 (horizontally striped bar, $n = 6$) or lysoPAF (white bar, $n = 5$)] for 6 h. Relative choline production as compared with lysoPC (16:0) was analysed using a colorimetric assay. *$P < 0.01$ compared with 16:0; **$P < 0.001$ compared with the other substrates. (**C**) The lysoPLD activities of the fractions (40 μl) obtained from Hepa-1 cells overexpressing empty vector (white bar) or F-$G\alpha_q$ (black bar) were incubated with or without Mg^{2+} and/or EDTA ($n = 3$). Relative choline production as compared with F-$G\alpha_q$ and Mg^{2+} was analysed using a colorimetric assay. *$P \ll 0.001$ compared with F-$G\alpha_q$ and Mg^{2+}. (**D**) The supernatants of homogenates from Hepa-1 cells overexpressing FLAG–$G\alpha_q$ after centrifugation at 105 000 ***g*** were applied on to immune-affinity chromatography with anti-FLAG antibody, and immunoblot analysis of tag-purified $G\alpha_q$ (F-$G\alpha_q$) was performed (top panel). The enzymatic activity in the purified fraction was assayed (bottom panel). Results are means $\pm$ S.D. The experiments were repeated several times with similar results. Cont, control.

and lower bands were non-specific bands. LysoPLD activity of autotoxin is not dependent on Mg^{2+} supplementation [25]. These results suggest that the lysoPLD activities were attributable to $G\alpha_q$ or $G\beta_1$ themselves, or to a protein(s) in a complex with these proteins.

Time course, substrate specificity and Mg^{2+} cation requirement of lysoPLD activity of $G\alpha_q$

To examine the time course of choline production from lysoPAF hydrolysed by purified FLAG-tagged $G\alpha_q$ subunit, the enzyme was incubated with 0.15 mM lysoPAF for 0–6 h and activities were tested using a colorimetric assay. As shown in Figure 3(A), the levels of the hydrolysed product, choline, significantly increased in the presence of purified FLAG-tagged $G\alpha_q$ for up to 6 h of incubation.

To identify a preferred substrate, purified FLAG-tagged $G\alpha_q$ was incubated for 6 h with several different choline lysophospholipid substrates (Figure 3B). The preferred substrate identified in this assay is lysoPAF ($P < 0.01$). The enzyme was more efficient at hydrolysis of 1-palmitoyl-2-lyso-GPC (16:0) than hydrolysis of 1-oleoyl-2-lyso-GPC (18:1) ($P < 0.01$). The substrate specificity of $G\alpha_q$ for lysophospholipids is very similar to what was observed for the enzyme activity previously purified from the rat brain [21]. Choline production from lysoPAF by the purified FLAG–$G\alpha_q$ was dependent on incubation with Mg^{2+}

(Figure 3C), similar to what was observed for the enzyme purified from rat brain tissue [21]. Although after centrifugation at 105000 ***g*** high levels of FLAG–Gα_q could be purified from the supernatants of homogenates from cells overexpressing the protein, enzymatic activity was not detected (Figure 3D).

The lysoPLD activity of Gα_q and Gβ_1 mutant proteins

As FLAG–Gα_q purified from Hepa-1 cells overexpressing the protein co-purified with endogenous Gβ and purified FLAG-Gβ_1 co-purified with endogenous G$\alpha_{q/11}$ (Figure 2A), we next tried to clarify which subunit is responsible for the lysoPLD activity we observed. Autotaxin had been identified previously as a member of the ecto-nucleotide PDE (pyrophosphatase/phosphodiesterase) family due to similarity in its primary structure [14,26]. Later, it became clear that the ester bond between phosphate and choline in LPC was also hydrolysed by autotaxin [12,13]. Therefore we speculated that Gα_q subunit might exhibit lysoPLD activity because it hydrolyses GTP to GDP via its intrinsic GTPase activity. The Gβ_1 subunit would be the candidate for the protein with lysoPLD activity, as a histidine acid phosphatase consensus motif is present in its C-terminus (V^{305}GILSGHDNAVSCLGV^{320}, analysed by GENETYX). To test this, we introduced several mutations at amino acid residues thought to be important for Gα_q or Gβ_1 enzyme activities. The mutant proteins were then purified and their lysoPLD activities were assayed. Tag-purified Gβ_1–Gγ_2 complex overexpressed in *S. frugiperda* insect cells was also obtained, and its lysoPLD activity was determined.

As shown in Figure 4(A), the K52A, T186A, D205A, G48A and Q209L mutant forms of Gα_q showed a significant reduction of lysoPLD activity, whereas G48V did not. The decrease in lysoPLD activity of G48A and Q209L was low. Based on amino acid sequence similarity to $p21^{ras}$, the amino acid sequence motif G^{46}TGESGKS^{53} is predicted to form a loop in which main-chain amide hydrogens of several amino acids and the ε-amino group of Lys^{52} might form bonds with the β- and γ-phosphate of GTP [27]. This site also corresponds to the GTP-binding site. Therefore we speculated that the side chain of Lys^{52} might be important for the binding of phosphate at the sn−3 position of LPC, whereas the side chain of Gly^{48} is not likely to be important for this binding. This suggests that the GTP- and LPC-binding sites or interacting amino acids of Gα_q seem to be not completely the same. Moreover, the side chain of Thr^{186} may point away from the bound nucleotide in the GDP-bound form, but flip towards the nucleotide in the GTP-bound form, where it might directly interact with a Mg^{2+} ion coordinated with oxygen molecules in the β- and γ-phosphates of GTP. On the basis of crystal structure analysis, it has been suggested that the side chain of Asp^{205} in Gα_q might bind catalytic Mg^{2+} via an intervening water molecule [27,28]. The well-known dominant negative Gα_q (Q209L) construct was prepared and its activity was assayed. Only slight reduction of lysoPLD activity of Q209L was detected. The Gβ_1 mutant H311A, which disrupts a conserved amino acid residue in the histidine acid phosphatase consensus, did not show a significant change in lysoPLD activity as compared with the wild-type Gβ_1 subunit (Figure 4B). Tag-purified Gβ_1–Gγ_2 complex overexpressed in insect cells did not exhibit lysoPLD activity. These results strongly suggest that Gα_q exhibits lysoPLD activity and, furthermore, that the predicted nucleotide phosphate and Mg^{2+}-binding sites of the protein are important for lysoPLD enzymatic activity. When we purified lysoPLD from rat brain or culture cells, Gα_q and Gβ were always co-purified. It is possible that lysoPLD activity of Gα_q is dependent on the presence of Gβ protein.

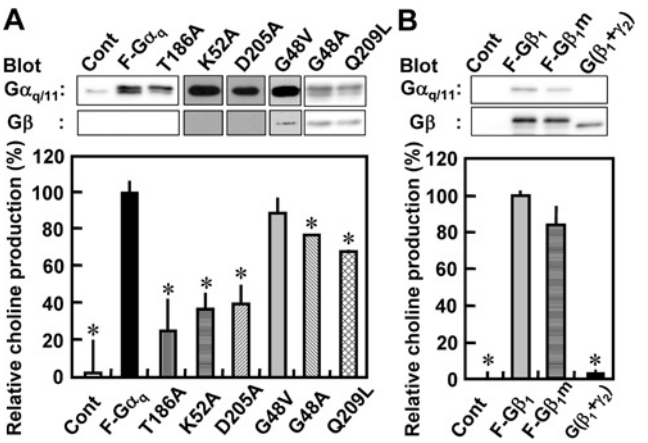

Figure 4 LysoPLD activity of mutant forms of the G protein subunits Gα_q and Gβ_1, and tag-purified Gβ_1–Gγ_2 complex

Empty vector (Cont), FLAG-tagged wild-type Gα_q (**A**, F-Gα_q) or Gβ_1 (**B**, F-Gβ_1), or mutant forms (**A**, T186A, K52A, D205A, G48V, G48A, Q209L mutations of Gα_q, and **B**, F-Gβ_1m for Gβ_1) were expressed in Hepa-1 cells and purified from one dish. Top panels: the levels of unmodified or mutant FLAG-tagged Gα_q and Gβ_1 subunits in 10 μl of the purified fractions, and tag-purified Gβ_1–Gγ_2 complex (3 ng). The immunoreacted wild-type Gα_q and mutant forms in (**A**) were not determined in the same experiments, but all experiments were repeated several times and almost equivalent levels of proteins compared with F-Gα_q were obtained each time. (**A**) Bottom panel: relative choline production by tag-purified enzymes (40 μl) from cells overexpressing empty vector (Cont; white bar, $n = 6$), T186A (vertically striped bar, $n = 5$), K52A (horizontally striped bar, $n = 3$), D205A (left oblique striped bar, $n = 3$), G48V (grey bar, $n = 4$), G48A (right oblique striped bar, $n = 3$) or Q209L (crosswise striped bar, $n = 3$) as compared with unmodified FLAG-tagged Gα_q (black bar, $n = 8$) was analysed with a colorimetric assay after incubation with 0.15 mM lysoPAF for 6 h. $^*P < 0.001$ compared with FLAG-tagged Gα_q. (**B**) Bottom panel: relative choline production by tag-purified enzymes from cells overexpressing empty vector (Cont; white bar, $n = 6$), F-Gβ_1m (horizontally striped bar; $n = 6$) or tag-purified Gβ_1–Gγ_2 complex (12 ng) [G(β_1 and γ_2); black bar, $n = 3$] as compared with F-Gβ_1 (grey bar; $n = 6$). $^*P < 0.001$ compared with F-Gβ_1. Results are means ± S.D. The experiments were repeated more than three times with similar results.

LysoPLD activity of Gα_q protein and a mutant form, T186A, highly purified from COS-7 cells

To eliminate the possibility that the detection of lysoPLD activity from purified tagged Gα_q protein is cell-line dependent, we performed the same experiment with COS-7 cells, which do not exhibit autotaxin activity and express relatively low endogenous levels of G proteins. As shown in Figure 5(A), highly purified wild-type FLAG–Gα_q protein and a mutant form, T186A, could be detected following ectopic expression in COS-7 cells. The levels of the hydrolysed product, choline, significantly increased over time in the presence of purified FLAG-tagged Gα_q (Figure 5B). The activity was dependent on incubation with Mg^{2+} cations (Figure 5C). The presence of lysoPLD activity was confirmed by the significant production of choline from lysoPAF (Figures 5B–5D) and LPA from LPC (Figures 5E and 5F). The T186A mutant form of Gα_q showed significantly less lysoPLD activity than the wild-type FLAG–Gα_q protein. These results clearly show that Gα_q has lysoPLD activity. As shown in Figure 5(A) (bottom panel), a small amount of endogenous Gβ in COS-7 cells was also co-purified with intact or mutated FLAG-tagged Gα_q.

LysoPLD activity of Gβ_1 protein highly purified from COS-7 cells

To examine the possibility that purified FLAG–Gβ_1 obtained from COS-7 cells also has lysoPLD activity, we repeated the experiment described above with these cells. A small amount of

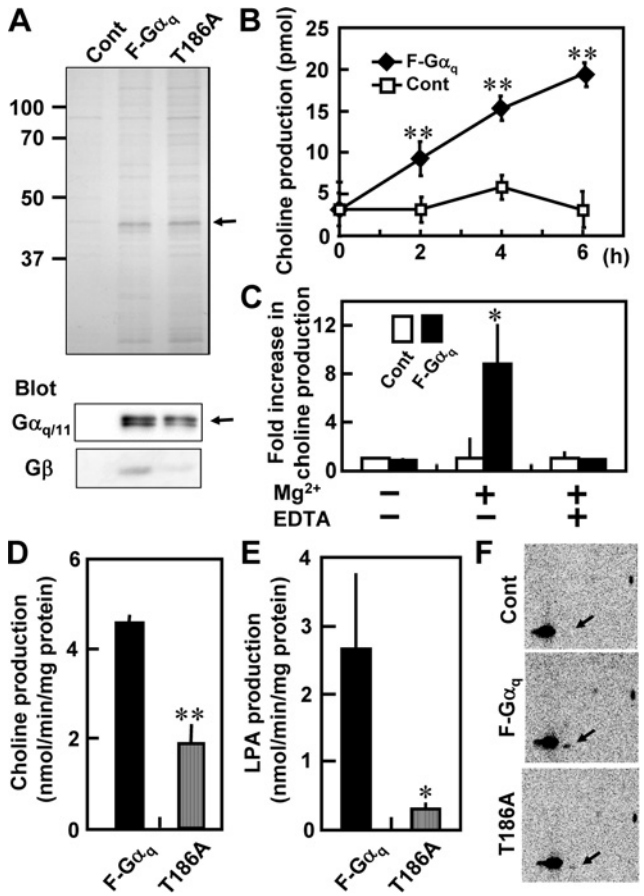

Figure 5 LysoPLD activity of highly purified $G\alpha_q$ protein and a mutant form, T186A, from COS-7 cells

Empty vector (Cont), FLAG-tagged wild-type $G\alpha_q$ (F-$G\alpha_q$) or a FLAG-tagged mutant form (T186A) was expressed in COS-7 cells and purified from five dishes. (**A**) Silver staining (top panel) or immunoblot analysis (bottom panel) with anti-$G\alpha_{q/11}$ and $G\beta$ of vector control (Cont), tag-purified wild-type $G\alpha_q$ (F-$G\alpha_q$) or T186A in the purified fractions. (**B**) A total of 40 μl of the tag-purified fractions obtained from COS-7 cells overexpressing empty vector (Cont) (open squares, $n=3$) or FLAG-tagged $G\alpha_q$ (F-$G\alpha_q$) (closed diamonds, $n=3$) was incubated with 0.15 mM lysoPAF for the indicated times and then subjected to a colorimetric lysoPLD assay. $^*P<0.001$ compared with the vector control. (**C**) The lysoPLD activities of the purified fractions (40 μl) obtained from COS-7 cells overexpressing empty vector (open bars) or F-$G\alpha_q$ (closed bars) were incubated with or without Mg^{2+} and/or EDTA ($n=3$). Relative choline production as compared with the vector control was analysed using a colorimetric assay. $^*P<0.05$ compared with the vector control and F-$G\alpha_q$ with or without Mg^{2+} and EDTA. (**D**) Choline production by tag-purified enzymes (40 μl) from cells overexpressing unmodified FLAG-tagged $G\alpha_q$ (F-$G\alpha_q$) (black bar, $n=3$) or T186A (vertically striped bar, $n=3$) was analysed with a colorimetric assay after incubation with 0.15 mM lysoPAF for 6 h. $^{**}P<0.001$ compared with F-$G\alpha_q$. (**E** and **F**) LPA production by tag-purified enzymes (30 μl) from cells overexpressing unmodified FLAG-tagged $G\alpha_q$ (F-$G\alpha_q$) (black bar, $n=3$) or T186A (vertically striped bar, $n=3$) was analysed with an isotopic assay after incubation with 0.15 mM [^{14}C]-labelled LPC for 6 h. LPA production was visualized after lipids were extracted and subjected to TLC analysis. (**E**) $^*P<0.03$ compared with F-$G\alpha_q$. (**F**) Arrows indicate the positions of LPA. Results are means $\pm$ S.D. The experiments were repeated at least twice with similar results.

endogenous $G\alpha_{q/11}$ in COS-7 cells was co-purified with FLAG–$G\beta_1$ (Figure 6A). Almost the same lysoPLD activity as for purified FLAG–$G\alpha_q$ protein was observed, in FLAG–$G\beta_1$ with the production of choline from lysoPAF (Figure 6B) and LPA from LPC (Figure 6C). When purified FLAG–$G\alpha_q$ and FLAG–$G\beta_1$ proteins were incubated together *in vitro*, an additive effect of lysoPLD activity was observed, as specific activity did not change (Figure 6B).

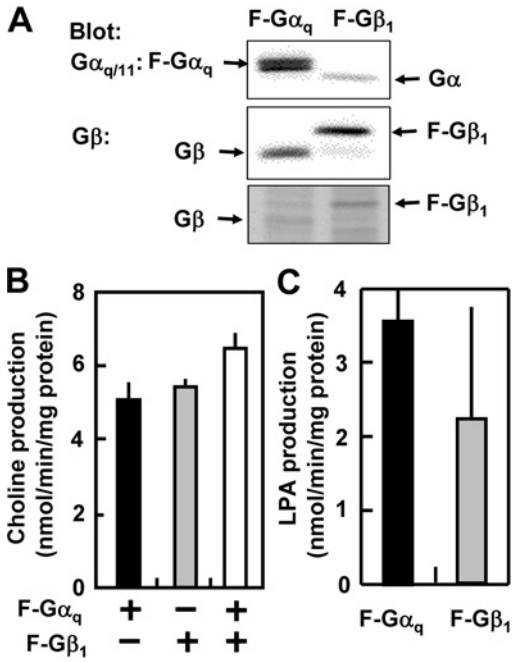

Figure 6 LysoPLD activity of highly purified $G\beta_1$ protein from COS-7 cells

FLAG-tagged wild-type $G\alpha_q$ (F-$G\alpha_q$) or a FLAG-tagged wild-type $G\beta_1$ (F-$G\beta_1$) was expressed in COS-7 cells and purified from five dishes. (**A**) Immunoblot analysis with anti-$G\alpha_{q/11}$ (top panel) and anti-$G\beta$ (middle panel), or silver staining (bottom panel) of tag-purified wild-type $G\alpha_q$ (F-$G\alpha_q$) or $G\beta_1$ (F-$G\beta_1$) in the purified fractions. (**B**) A total of 40 μl of the tag-purified fractions obtained from COS-7 cells overexpressing FLAG-tagged $G\alpha_q$ (F-$G\alpha_q$) (black bar, $n=3$), FLAG-tagged $G\beta_1$ (F-$G\beta_1$) (grey bar, $n=3$) or equal amounts of both F-$G\alpha_q$ and F-$G\beta_1$ (white bar, $n=3$) was incubated with 0.15 mM lysoPAF for 6 h and then subjected to a colorimetric lysoPLD assay. (**C**) The lysoPLD activity of the purified fraction (30 μl) obtained from COS-7 cells overexpressing F-$G\alpha_q$ (black bar, $n=3$) or F-$G\beta_1$ (grey bar, $n=3$) was analysed with an isotopic assay after incubation with 0.15 mM [^{14}C]-labelled LPC for 6 h. LPA production was visualized after lipids were extracted and subjected to TLC analysis. Results are means $\pm$ S.D. The experiments were repeated at least twice with similar results.

Effect of substrate concentration and GTP on the activity of FLAG-purified lysoPLD from Neuro2A cells overexpressing FLAG–$G\alpha_q$

Because the lysoPLD enzyme that we have purified previously was from the brain, we also tested overexpression and tag-affinity purification of FLAG–$G\alpha_q$ using Neuro2A cells. As shown in Figures 7(A) and 7(B), the level of lysoPLD activity of purified FLAG-$G\alpha_q$ was dependent on cell type (COS-7<Hepa-1<Neuro2A cells). Notably, the enzyme activity of purified FLAG-tagged $G\alpha_q$ expressed in COS-7 cells was approximately 4 nmol/min per mg, whereas the activity from Neuro2A cells was much higher, i.e. 137.4 nmol/min per mg. The calculated K_m and V_{max} values for lysoPAF using Neuro2A cells were 21 μM and 0.16 μmol/min per mg respectively (Figures 7B and 7C). The K_m and V_{max} values of lysoPLD obtained from rat brain for lysoPAF were 26.7 μM and 0.29 μmol/min per mg respectively. These results using Neuro2A cells and rat brain were almost equivalent with each other. The slightly lower V_{max} value in Neuro2A cells is probably due to the purity of the enzyme or the disturbance of enzyme activity by N-terminal FLAG peptides.

As shown in Figure 7(D), 1 mM GTP did not disturb the lysoPLD activity (0.15 mM LPC) of FLAG–$G\alpha_q$ obtained from Neuro2A cells. As the mutation at the GTP-binding region (Gly^{48}) did not decrease lysoPLD activity of $G\alpha_q$ much (Figure 4A), the GTP- and LPC-binding sites seem to not be completely the same. Moreover, the exchange of GDP to GTP and activation of GTPase

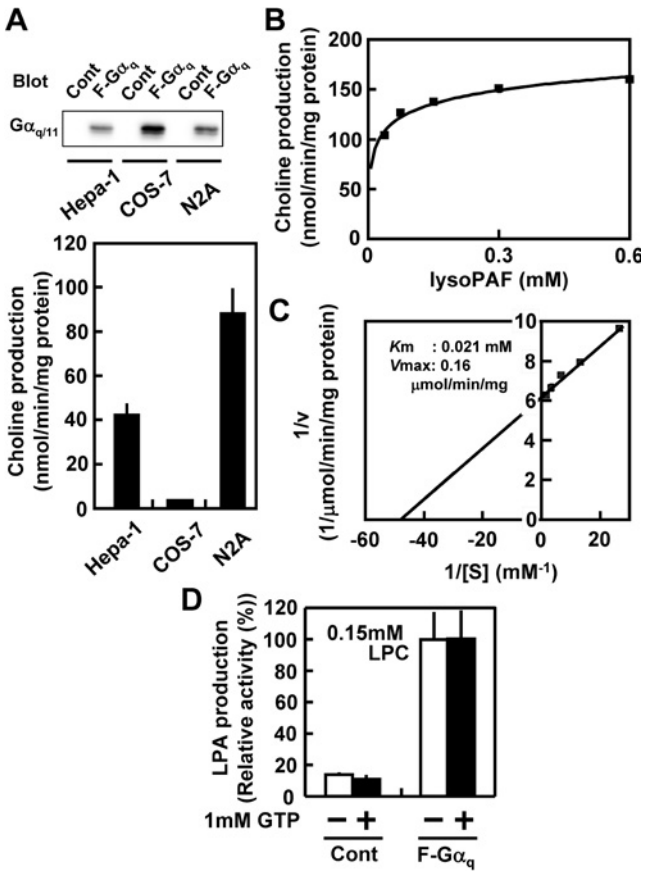

Figure 7 Effect of substrate concentration and GTP on the activity of FLAG-purified lysoPLD obtained from Neuro2A cells overexpressing FLAG–Gα_q

Empty vector (Cont) or FLAG-tagged wild-type Gα_q (F-Gα_q) was expressed in Hepa-1, COS-7 or Neuro2A (N2A) cells and purified from five dishes. (**A**) Immunoblot analysis with anti-G$\alpha_{q/11}$ antibody (upper panel) of tag-purified Gα_q (F-Gα_q) obtained from Hepa-1, COS-7 or Neuro2A cells overexpressing FLAG-Gα_q. Choline production (lower panel) by tag-purified enzyme (40 μl) obtained from cells overexpressing unmodified FLAG-tagged Gα_q (F-Gα_q) ($n = 3$) was analysed with a colorimetric assay after incubation with 0.15 mM lysoPAF for 6 h. Results are means $\pm$ S.D. The experiments were repeated at least two times with similar results. Tag-purified enzyme from Neuro2A cells overexpressing unmodified FLAG-tagged Gα_q was incubated with various concentrations from 0.0375 to 0.6 mM of lysoPAF for 6 h, and choline production was analysed with a colorimetric assay. An untransformed plot (**B**) and Lineweaver–Burk (double-reciprocal) plot (**C**) are shown. (**D**) The lysoPLD activity of the purified fraction (30 μl) obtained from Neuro2A cells overexpressing empty vector or F-Gα_q was analysed with an isotopic assay after incubation with 0.15 mM [^{14}C]-labelled LPC for 6 h with (black bar, $n = 3$) or without (white bar, $n = 3$) 1 mM GTP. Relative LPA production was determined as compared with FLAG-tagged Gα_q without GTP. LPA production was visualized after lipids were extracted and subjected to TLC analysis. Results are means $\pm$ S.D. The experiments were repeated at least two times with similar results.

activity of Gα_q possibly rarely occurred in this experiment. As for most of the GTPases, the rate of exchange of GDP to GTP and GTPase activity of Gα_q are quite low without ligand-stimulated receptor and GAPs (GTPase-activating proteins) [29–31]. LysoPLD activity of autotaxin may be inhibited by incubation with GTP because autotaxin catalyses the hydrolysis of GTP as well as ATP.

LysoPLD activity of other Gα subfamily members and the effect of Gα_q and Gα_{11} siRNAs

In the human genome, 16 different Gα subunits have been identified and these can be divided into four subfamilies based on their amino acid similarities and the machinery for their signal

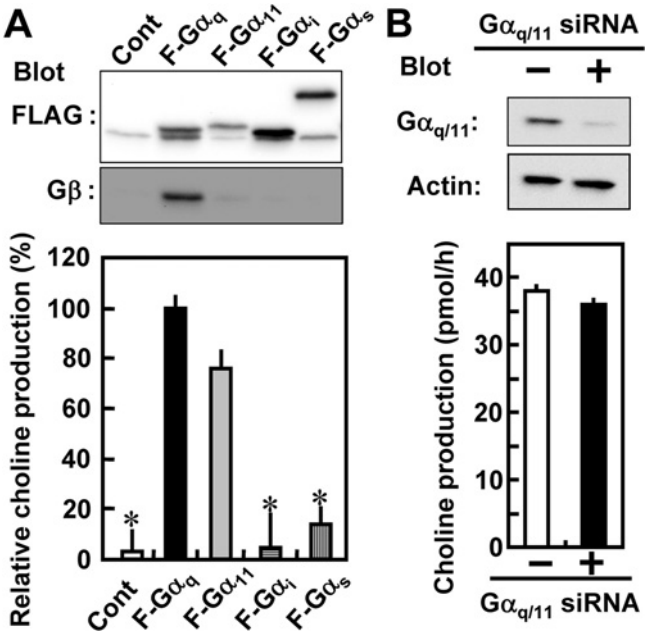

Figure 8 Testing LysoPLD activity of other Gα subfamily members and effect of Gα_q and Gα_{11} siRNAs

(**A**) FLAG-tagged Gα subtypes F-Gα_q (black bar, $n = 18$), F-Gα_{11} (grey bar, $n = 6$), F-Gα_i (horizontally striped bar, $n = 6$), F-Gα_s (vertically striped bar, $n = 6$) or empty vector (Cont; white bar, $n = 12$) were overexpressed in Hepa-1 cells and tagged proteins were purified from one dish. Levels of tag-purified Gα subfamily proteins (top panel) and co-purified Gβ proteins (middle panel) detected using anti-FLAG and anti-Gβ_{1-4} antibodies respectively. Note that Gα_s migrates at a higher molecular mass than the others. The bands of the same size present in the control and experimental samples are non-specific. Bottom panel: relative choline production by 40 μl of purified enzyme incubated with lysoPAF as compared with FLAG-tagged Gα_q, detected with a colorimetric assay. $^*P < 0.001$ compared with F-Gα_q. Results are means $\pm$ S.D. The experiment was repeated several times with similar results. (**B**) Hepa-1 cells were pretreated with control siRNA or mouse Gα_q and Gα_{11} siRNAs for 48 h. Levels of endogenous Gα_q and Gα_{11} (top panel) and actin (middle panel) detected using anti-G$\alpha_{q/11}$ and anti-actin antibodies respectively. Bottom panel: lysoPLD activity in a crude microsomal fraction (10 μg) from Hepa-1 cells pretreated with control siRNA (white bar, $n = 3$) or Gα_q and Gα_{11} siRNAs (black bar, $n = 3$) was analysed with a colorimetric assay after incubation with 0.15 mM lysoPAF for 30 min.

transduction. Thus we were interested in the possibility that other Gα protein family members might also exhibit lysoPLD activity. To test this, we first constructed plasmids encoding FLAG-tagged Gα_{11}, Gα_i and Gα_s. The plasmids were introduced into Hepa-1 cells and the activities of the purified overexpressed proteins were analysed. As shown in Figure 8(A), purified FLAG–Gα_{11}, which has 89 % sequence similarity with Gα_q and belongs to the same subfamily, exhibited a high lysoPLD activity ($P < 0.01$), whereas FLAG–Gα_i and FLAG–Gα_s did not. However, a slight increase in lysoPLD activity of FLAG–Gα_s was observed.

As shown in Figure 8(B), pretreatment of Gα_q and Gα_{11} siRNAs clearly decreased the endogenous Gα_q and Gα_{11} in Hepa-1 cells. However, lysoPLD activity in the crude microsomal fraction did not changed compared with the control. In the crude microsome, we speculated that other undetermined G proteins or contaminated autotaxin may be responsible for the high endogenous lysoPLD activity and that the lysoPLD activity of the interested protein may be masked.

DISCUSSION

We were interested to molecularly identify a lysoPLD activity that had been reported previously. Two proteins from the rat brain exhibiting lysoPLD activity (faint 42 kDa and 35 kDa bands by

gel electrophoresis) can be observed following a method reported previously for purification [21]. As shown in Figure 1(A), the 42 kDa protein was present at lower levels and, at first, was not recognized as potentially important. The enzymatic properties of the fractions containing both proteins, including optimum pH, substrate specificity and cation requirement, were different from those of a secreted protein with lysoPLD activity, autotaxin. In the present study, we analysed the 42 kDa and 35 kDa proteins by MS and identified them as the heterotrimeric G protein subunits $G\alpha_q$ and $G\beta_1$ respectively. When FLAG-tagged $G\alpha_q$ or $G\beta_1$ was overexpressed in Hepa-1 cells and purified, both purified subunits exhibited lysoPLD activity (Figure 2). Mutations in conserved amino acid residues of $G\alpha_q$ known to be important for GTPase activity and interaction with nucleotide phosphates or Mg^{2+} significantly reduced lysoPLD activity (Figure 4). Furthermore, highly purified FLAG–$G\alpha_q$ protein ectopically expressed in COS-7 cells clearly hydrolysed LPC to LPA, whereas a mutant form, T186A, showed significantly less lysoPLD activity (Figure 5). The calculated K_m and V_{max} values for lysoPAF of the FLAG-purified enzyme obtained from Neuro2A cells were 21 μM and 0.16 μmol/min per mg respectively (Figures 7B and 7C). These values were similar to the enzyme purified from the rat brain ($K_m = 26.7$ μM and $V_{max} = 0.29$ μmol/min per mg) (21). Although $G\beta_1$ detected by silver staining was the main protein of purified lysoPLD from rat brain, $G\beta_1$ is not likely to be the main component for lysoPLD activity because of following four reasons: (i) FLAG–$G\beta_1$ mutant (H311A) exhibited significant lysoPLD activity (Figure 4B); (ii) FLAG–$G\alpha_{11}$ co-purified with lower amounts of $G\beta_1$ exhibited lysoPLD activity comparable with $G\alpha_q$ (Figure 8A); (iii) FLAG–$G\alpha_i$ and FLAG–$G\alpha_s$ did not exhibit as high a lysoPLD activity as FLAG–$G\alpha_q$ when they were co-purified with $G\beta$ (Figure 8A); and (iv) tag-purified $G\beta_1$ and $G\gamma_2$ overexpressed in insect cells did not exhibit lysoPLD activity (Figure 4A). However, the potential importance of $G\beta_1$ for lysoPLD activity of $G\alpha_q$ may be possible because they were always co-purified with respect to lysoPLD activity. These results reveal a new function for $G\alpha_q$ and $G\beta_1$, which are well known as multifunctional signal transduction proteins, as an enzyme with lysoPLD activity.

Although FLAG-purified $G\alpha_q$ obtained from the microsomal fraction exhibited significant lysoPLD activity, the purified enzyme from supernatants after 105000 ***g*** centrifugation did not have lysoPLD activity (Figure 3D). The level of lysoPLD activity of purified FLAG–$G\alpha_q$ from the microsomal fraction was dependent on cell type (COS-7<Hepa-1<Neuro2A cells) (Figure 7A). These results suggest that post-translational modification(s), or some kind of activator(s) present in the microsome, is important for lysoPLD activity of $G\alpha_q$. However, it is also possible that $G\alpha_q$ proteins in supernatants were misfolded after translation.

Agonist-activated GPCRs enhance GDP/GTP exchange on the G protein α subunit, thereby generating an active GTP-bound $G\alpha$ subunit, followed by dissociation from the receptor and $G\beta\gamma$ subunit. The onset and termination of G protein signalling is determined by the length of time spent in the active GTP-bound state. GTP is hydrolysed to GDP by the intrinsic GTPase activity of the $G\alpha$ subunit, resulting in re-association of the $G\alpha$ subunit with the $G\beta\gamma$ subunit and termination of signalling [29]. As for most of GTPases, the rates of exchange of GDP to GTP and GTPase activity of $G\alpha_q$ are quite low. However, the GTPase activity of $G\alpha_q$ can be dramatically increased by *in vitro* incubation with ligand-stimulated cholinergic receptor and GAPs, such as the RGS (regulator of G protein signalling) proteins [30] or phospholipase C [31]. In the present study, we found evidence that the lysoPLD activity of FLAG-tagged $G\alpha_q$ is exhibited in the presence of $G\beta$ and $G\gamma_5$, as endogenous $G\beta$ and $G\gamma_5$ co-purified with FLAG–$G\alpha_q$ (Figure 2). We speculate that the heterotrimeric form of G protein subunits may exhibit lysoPLD activity.

Previously, GTPγS was found to be bound to the purified activators [32] and, additionally, that purified PI-PLC (phosphoinositide phospholipase C) from the bovine brain could be activated by purified $G\alpha_q$ in the presence of GTPγS (0.1–1 mM) [33]. On the basis of our results, we propose that a possible role of the novel lysoPLD identified in the present study for $G\alpha_q$ and $G\beta_1$ is the production of LPA inside cells in response to ligand stimulation, which is then followed by sequential activation of PI-PLC (Ca^{2+} influx) and PLA_2s that hydrolyse PC to free fatty acid and LPC [15]. It was reported previously that LPA receptors can be detected on the nuclear membrane [22,23] and that the transcription factor PPARγ acts as a LPA receptor [24]. It seems reasonable to speculate that the $G\alpha_q$ and $G\beta_1$ subunits mediate the signal to these intracellular receptors via lysoPLD activity.

After testing several family members as shown in Figure 8(A), $G\alpha_q$ and $G\alpha_{11}$ exhibit a high lysoPLD activity, but $G\alpha_i$ and $G\alpha_s$ do not. Therefore we conclude that the lysoPLD activity of the $G\alpha$ subunit is strictly dependent on its subfamily. However, treatment of Hepa-1 cells with $G\alpha_q$ and $G\alpha_{11}$ siRNAs did not affect lysoPLD activity in the microsomal fraction (Figure 8B). It may be possible that other undetermined G proteins and/or different kinds of lysoPLD, such as autotaxin, are responsible for the high endogenous lysoPLD activity and that they masked the reduced activity. Furthermore, our preliminary experiments showed that the stimulation of radio-labelled cells by carbachol that activates $G\alpha_{q/11}$ through the muscarinic receptor did not affect the basal amount of LPA (results not shown). We speculate that the degradation of LPA in cells was rapid or that the stimulation system was inadequate. Further investigation is required to help elucidate the importance of LPA production by $G\alpha_q$ in signal transduction.

AUTHOR CONTRIBUTION

The project strategy was devised by, and most of the experiments were performed by, Chieko Aoyama and Hiroyuki Sugimoto. Purification of proteins was helped by Hiromi Ando, Satoko Yamashita and Sayaka Sugimoto. siRNA experiments and PMF analysis were performed by Yasuhiro Horibata and Motoyasu Satou respectively.

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