The heterotrimeric G protein subunits Gαq and Gβ1 have lysophospholipase D activity

Chieko AOYAMA1, Hiroyuki SUGIMOTO1,2, Hiromi ANDO, Satoko YAMASHITA, Yasuhiro HORIBATA, Sayaka SUGIMOTO and Motoyasu SATOU
Department of Biochemistry, Dokkyo Medical University School of Medicine, 880 Kitakobayashi, Mibu, Tochigi, Japan

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 PLA1 (phospholipase A1) [2]. In support of this idea, a PA-selective PLA1, (phospholipase A1) called mPA-PLA1/LIPH (membrane-associated phosphatidic acid-selective phospholipase A1/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 activity, that is strictly dependent on its subfamily and might be important for cellular responses. However, treatment of Hepa-1 cells with Gαq and Gα11 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, Gαq, Gβ1, lysophosphatidic acid (LPA), lysophosphatidylcholine (LPC), lysophospholipase D (lysoPLD).

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 Gαq and Gβ1, respectively. When FLAG-tagged Gαq or Gβ1 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 Mg2+ dependency and substrate specificity of Gαq were similar to those of lysoPLD purified from the rat brain. Mutation of Gαq at amino acids Lys32, Thr96 or Asp98 residues that are predicted to interact with nucleotide phosphates or catalytic Mg2+, 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 Gαq 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 Gαq and Gβ1, as an enzyme with lysoPLD activity. Tag-purified Gα11 also exhibited a high lysoPLD activity, but Gαq and Gα11 did not. The lysoPLD activity of the Gα subunit is strictly dependent on its subfamily and might be important for cellular responses. However, treatment of Hepa-1 cells with Gαq and Gα11 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.

Abbreviations used: Gαq, heterotrimeric G protein α subunit q; GAP, GTPase-activating protein; Gβ1, 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; PLA1, phospholipase A1; PMF, peptide mass fingerprinting; PPARγ, peroxisome proliferation-activator receptor γ; siRNA, small interfering RNA.

1 These authors contributed equally to this work.
2 To whom correspondence should be addressed (email h-sugi@dokkyomed.ac.jp).

© 2011 The Author(s)

The author(s) has paid for this article to be freely available under the terms of the Creative Commons Attribution Non-commercial Licence (http://creativecommons.org/licenses/by-nc/2.5/) which permits unrestricted non-commercial use, distribution and reproduction in any medium, provided the original work is properly cited.
into a non-bioactive saturated free fatty acid and GPC (glycero-phosphorylcholine). 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 in brain that is a Mg\(^{2+}\)-dependent enzyme and utilizes lysoPAF as a substrate. The enzymatic activity was significantly reduced by G\(_{\alpha}\) overexpression using the following primer pairs: 5\(^\prime\)-GACTACAAGGACGACGACGATGACAAGACTCTGGAGTCCATGATG-3\(^\prime\); and 5\(^\prime\)-TTCCAGATCTTGAGGAAG-3\(^\prime\); and G\(_{\gamma}\) complementary sequences: G\(_{\delta}\) 5\(^\prime\)-CTACAAGGACGACGACGATGACAAGGGCTGCACATTGAG-3\(^\prime\) and 5\(^\prime\)-CGATGACAAGGGCTGCCTCGGC-3\(^\prime\).

**CONCLUSION**

This enzyme might be involved in the production of LPA as a bioactive phospholipid that can induce chemotaxis [18], signal-responsive kinase activities [19] and atherosclerosis [20]. As a bioactive phospholipid that can induce chemotaxis [18], signal-responsive kinase activities [19] and atherosclerosis [20].

**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 oxide were purchased from Wako Chemicals. Silica Gel 60 plates and tag-purified G\(_{\alpha}\), G\(_{\beta}\) and G\(_{\gamma}\) subunits were obtained from Santa Cruz Biotechnology, and anti-FLAG antibody, anti-FLAG affinity gels, FLAG peptides, anti-FLAG antibody and anti-FLAG antibody were purchased from Dojindo. Antibodies against G\(_{\sigma}\) (GenBank accession number NM_031036.1), G\(_{\alpha}\) (GenBank accession number NM_031033.1) or G\(_{\beta}\) (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: G\(_{\sigma}\) 5\(^\prime\)-GGGAAGAATGCTGACGCTACAAGGACGACGATGACAAGCTACCTGGATCCTACATGGG-3\(^\prime\) and 5\(^\prime\)-TCACACCAGATTTGATCCTACCTGGG-3\(^\prime\); G\(_{\alpha}\) 5\(^\prime\)-GGACGATGGAAGGACGAGCATGCAAAAGAATTCGCCGCCATGGG-3\(^\prime\) and 5\(^\prime\)-GTCGATGGAAGGACGAGCATGCAAAAGAATTCGCCGCCATGGG-3\(^\prime\); G\(_{\beta}\) 5\(^\prime\)-GCACGATCTACTACAAGGACGAGCATGCAAAAGAATTCGCCGCCATGGG-3\(^\prime\) and 5\(^\prime\)-GTCGATGGAAGGACGAGCATGCAAAAGAATTCGCCGCCATGGG-3\(^\prime\).

**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\(^\circ\)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 diithiothreitol 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 Micronon 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 Cellulose 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 (1 \(\mu\)l) 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\(^\circ\)C with primary antibody diluted 1:1000 (anti-G\(_{\alpha}\), -G\(_{\beta}\), -G\(_{\gamma}\), -actin or -autotaxin) or 1:5000 (anti-FLAG) in TBS-T and then for 1 h at room temperature (20\(^\circ\)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.
5'-CTGCTGCTGGGACAGTGAGGTGCAAG-3'; Gaq (G48A), 5'-CTGCTGCTGGGACAGTGAGGTGCAAG-3'; Gaq (K52A), 5'-GACAGGGGAGTGCGGAGTGACTCTGATAGGAGGCCAAAGG-3'; Gaq (T186A), 5'-GTTCGAGTCCCAGCCAAGGGATCATTTG-3'; Gaq (D205A), 5'-CTCTCAGAATGGTCGCTGAAGGCGCAAAG-3'; Gaq (Q209L), 5'-TGGAAGGGCTAAGGTGAG-3'; and Gβ1 (H311A), 5'-GTCCGCTGAGCTGACACGGTGACGC-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-[14C]palmitoyl-GPC (6000 d.p.m./nmol), 20 mM Tris/HCl (pH 7.0), 1 mM Na3VO4, and 50 mM MgCl2 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 (100:200:1 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 Na3VO4, with or without 50 mM MgCl2, and/or 100 μM 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 21 500 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 1 mM 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 Gaq and Gq11 siRNAs (small interfering RNAs) on lysoPLD activity in Hepa-1 cells

Steady siRNA, a 25-base pair duplex oligoribonucleotide against mouse Gaq or Gq11, was purchased from Invitrogen. The target sense sequences were: Gaq, 5'-CCCCUUGACCUUAC-AAGUGCUAUUU-3' and Gq11, 5'-CCAGUGUGGUGUA-CCAGACACUUU-3'. Hepa-1 cells (1 × 106) 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 ≥ 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 Gaq (Figure 1D) and Gβ1 (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 Gaq11 and Gβ1-4. As shown in Figure 1(B), both Gaq and the 35 kDa band were identified as Gaq11. 

© 2011 The Author(s)

The author(s) has paid for this article to be freely available under the terms of the Creative Commons Attribution Non-Commercial Licence (http://creativecommons.org/licenses/by-nc/2.5/) which permits unrestricted non-commercial use, distribution and reproduction in any medium, provided the original work is properly cited.
Gβ₁ 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 Gα₉ or Gβ₁ 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–Gα₉ was purified with FLAG peptides, endogenous Gβ co-purified and, similarly, endogenous Gα₁₁ co-purified with FLAG-tagged Gβ₁ (Figure 2A). Gγ₅ also co-purified with FLAG–Gα₉ (Figure 2A). These results suggest that heterotrimeric G protein complexes can form with overexpressed FLAG–Gα₉. The density of the immunoreactive bands with anti-Gα₁₁ or anti-Gβ₁-₄ 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 [¹⁴C]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–Gα₉ or FLAG–Gβ₁. 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 Goq and Gβ1

Empty vector (Cont) or a vector encoding FLAG-tagged Goq or Gβ1 was transfected into Hepa-1 cells and FLAG-tagged proteins (F-Goq or F-Gβ1) were purified from one dish. (A) Immunoblot analysis of tag-purified G protein subunits Goq and Gβ1. 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 Goq, Gβ1, Gγ5, or autotaxin proteins (ATX) were detected with anti-Goq, -Gγ5, -Gβ1, or -autotaxin antibodies. As expected, the molecular masses of tagged proteins were approximately 2 kDa larger than endogenous proteins. The immunoreacted Gβ1 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 Goq (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-Goq (black bar) or F-Gβ1 (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 ± S.D. (n = 6). *P < 0.01 compared with the vector control. (D) Fold increase in choline production. (E) LPA production from [14C]-labelled LPC by incubation with the purified enzyme from Hepa-1 cells overexpressing empty vector (open square, n = 6) or FLAG-tagged Goq (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. (A) Empty vector (open square, n = 6) or FLAG-tagged Goq (closed diamonds, n = 6) 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 Goq for up to 6 h of incubation. To identify a preferred substrate, purified FLAG-tagged Goq 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 Goq 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–Goq was dependent on incubation with Mg2+ cations of the lysoPLD activity of tag-purified Goq.

Figure 3 Time dependence, substrate specificity and requirement of Mg2+ cations of the lysoPLD activity of tag-purified Goq

(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 Goq (F-Goq) (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-Goq (40 μl) was incubated with 0.15 mM of various choline lysophospholipids [16:0 (black bar, n = 7), 18:0 (grey bar, n = 3), 16:1 (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-Goq (black bar) were incubated with or without Mg2+ and/or EDTA (n = 3). Relative choline production as compared with F-Goq and Mg2+ was analysed using a colorimetric assay. *P < 0.01 compared with F-Goq and Mg2+ (D) The supernatants of homogenates from Hepa-1 cells overexpressing FLAG–Goq after centrifugation at 105,000 g were applied to immun-affinity chromatography with anti-FLAG antibody, and immunoblot analysis of tag-purified Goq (F-Goq) was performed (top panel). The enzymatic activity in the purified fraction was assayed (bottom panel). Results are means ± S.D. The experiments were repeated several times with similar results. Cont, control.

and lower bands were non-specific bands. LysoPLD activity of autotaxin is not dependent on Mg2+ supplementation [25]. These results suggest that the lysoPLD activities were attributable to Goq or Gβ1 themselves, or to a protein(s) in a complex with these proteins.

Time course, substrate specificity and Mg2+ cation requirement of lysoPLD activity of Goq

To examine the time course of choline production from lysoPAF hydrolysed by purified FLAG-tagged Goq 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 Goq for up to 6 h of incubation. To identify a preferred substrate, purified FLAG-tagged Goq 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 Goq 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–Goq was dependent on incubation with Mg2+...
The lysoPLD activity of Gαq and Gβ1 mutant proteins

As FLAG–Gαq purified from Hepa-1 cells overexpressing the protein co-purified with endogenous Gβ and purified FLAG-Gβ1 co-purified with endogenous Gαqβ1 (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 Gαq subunit might exhibit lysoPLD activity because it hydrolyses GTP to GDP via its intrinsic GTPase activity. The Gβ1 subunit would be the candidate for the protein with lysoPLD activity, as a histidine acid phosphatase consensus motif is present in its C-terminus (V390GILGHDNAVSLGV320, analysed by GENETYX). To test this, we introduced several mutations at amino acid residues thought to be important for Gαq or Gβ1 enzyme activities. The mutant proteins were then purified and their lysoPLD activities were assayed. Tag-purified Gβ1–Gγ2 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 Gαq 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 p21rase, the amino acid sequence motif G105TGESGKS35 is predicted to form a loop in which main-chain amide hydrogens of several amino acids and the ε-amino group of Lys is42 might form bonds with the β- and γ-phosphates of GTP [27]. This site also corresponds to the GTP-binding site. Therefore we speculated that the side chain of Lys might be important for the binding of phosphate at the sn−3 position of LPC, whereas the side chain of Gly34 is not likely to be important for this binding.

This suggests that the GTP- and LPC-binding sites or interacting amino acids of Gαq seem to be not completely the same. Moreover, the side chain of Thr186 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 Mg2+ 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 Asp325 in Gαq might bind catalytic Mg2+ via an intervening water molecule [27,28]. The well-known dominant negative Gαq (Q209L) construct was prepared and its activity was assayed. Only slight reduction of lysoPLD activity of Q209L was detected. The Gβ1 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 Gβ1 subunit (Figure 4B). Tag-purified Gβ1–Gγ2 complex overexpressed in insect cells did not exhibit lysoPLD activity. These results strongly suggest that Gαq exhibits lysoPLD activity and, furthermore, that the predicted nucleotide phosphate and Mg2+−binding sites of the protein are important for lysoPLD enzymatic activity. When we purified lysoPLD from rat brain or culture cells, Gαq and Gβ were always co-purified. It is possible that lysoPLD activity of Gαq is dependent on the presence of Gβ protein.
endogenous $\alpha_{\beta/11}$ 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 (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).

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 of 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 $\mu$M and 0.16 $\mu$mol/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 $\mu$M and 0.29 $\mu$mol/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 (Gly48) 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
activity of G\(\alpha\) possibly rarely occurred in this experiment. As for most of the GTPases, the rate of exchange of GDP to GTP and GTPase activity of G\(\alpha\) 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 G\(\alpha\) subfamily members and the effect of G\(\alpha_4\) and G\(\alpha_{11}\) siRNAs

In the human genome, 16 different G\(\alpha\) subunits have been identified and these can be divided into four subfamilies based on their amino acid similarities and the machinery for their signal transduction. Thus we were interested in the possibility that other G\(\alpha\) protein family members might also exhibit lysoPLD activity. To test this, we first constructed plasmids encoding FLAG-tagged G\(\alpha_{11}\), G\(\alpha_4\), and G\(\alpha_s\). 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–G\(\alpha_{11}\), G\(\alpha_4\), and G\(\alpha_s\) were detected using anti-FLAG and anti-G\(\alpha\) antibodies respectively. Note that G\(\alpha_s\) was incubated with a colorimetric assay after incubation with 0.15 mM lysoPAF for 6 h. Results are means ± S.D. The experiments were repeated at least two times with similar results.

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 $\alpha_q$ and $\beta\gamma$ respectively. When FLAG-tagged $\alpha_q$ or $\beta\gamma$ was overexpressed in Hepa-1 cells and purified, both purified subunits exhibited lysoPLD activity (Figure 2). Mutations in conserved amino acid residues of $\alpha_q$ 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–$\alpha_q$ 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 lysoPAP of the FLAG-purified enzyme obtained from Neuro2A cells were 21 $\mu$M and 0.16 $\mu$mol/min per mg respectively (Figures 7B and 7C). These values were similar to the enzyme purified from the rat brain ($K_m = 26.7 \mu$M and $V_{max} = 0.29 \mu$mol/min per mg) [21]. Although $\beta\gamma$ detected by silver staining was the main protein of purified lysoPLD from rat brain, $\beta\gamma$ is not likely to be the main component for lysoPLD activity because of following four reasons: (i) FLAG–$\beta\gamma$ mutant (H311A) exhibited significant lysoPLD activity (Figure 4B); (ii) FLAG–$\alpha_q\gamma$ co-purified with lower amounts of $\beta\gamma$ exhibited lysoPLD activity comparable with $\alpha_q$ (Figure 8A); (iii) FLAG–$\gamma$ and FLAG–$\alpha_q$ did not exhibit as high a lysoPLD activity as FLAG–$\alpha_q\gamma$ when they were co-purified with $\beta\gamma$ (Figure 8A); and (iv) tagged-purified $\beta\gamma$ and $\gamma$ overexpressed in insect cells did not exhibit lysoPLD activity (Figure 4A). However, the potential importance of $\beta\gamma$ for lysoPLD activity of $\alpha_q$ may be possible because they were always co-purified with respect to lysoPLD activity. These results reveal a new function for $\alpha_q$ and $\beta\gamma$, which are well known as multifunctional signal transduction proteins, as an enzyme with lysoPLD activity.

Although FLAG-purified $\alpha_q$ obtained from the microsomal fraction exhibited significant lysoPLD activity, the purified enzyme from supernatants after 105 000 g centrifugation did not have lysoPLD activity (Figure 3D). The level of lysoPLD activity of purified FLAG–$\alpha_q$ 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 $\alpha_q$. However, it is also possible that $\alpha_q$ proteins in supernatants were misfolded after translation.

Agonist-activated GPCRs enhance GDP/GTP exchange on the G protein $\alpha$ subunit, thereby generating an active GTP-bound $\alpha_q$ subunit, followed by dissociation from the receptor and $\beta\gamma$ 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 of the $\alpha_q$ subunit, resulting in re-association of the $\alpha_q$ subunit with the $\beta\gamma$ subunit and termination of signalling [29]. As for most of GTPases, the rates of exchange of GDP to GTP and GTPase activity of $\alpha_q$ are quite low. However, the GTPase activity of $\alpha_q$ can be dramatically increased by in vitro incubation with ligand-stimulated cholinergic receptor and GAs, 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 $\alpha_q$ is exhibited in the presence of $\beta\gamma$, as endogenous $\beta\gamma$ is co-purified with FLAG–$\alpha_q$ (Figure 2). We speculate that the heterotrimeric form of G protein subunits may exhibit lysoPLD activity.

Previously, GTPyS 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 $\alpha_q$ in the presence of GTPyS (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 $\alpha_q$ and $\beta\gamma$ 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$_2$ 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 PPAR$_\gamma$ acts as a LPA receptor [24]. It seems reasonable to speculate that the $\alpha_q$ and $\beta\gamma$ subunits mediate the signal to these intracellular receptors via lysoPLD activity.

After testing several family members as shown in Figure 8(A), $\alpha_q$ and $\alpha_{11}$ exhibit a high lysoPLD activity, but $\alpha_q$ and $\alpha_{11}$ do not. Therefore we conclude that the lysoPLD activity of the $\alpha_q$ subunit is strictly dependent on its subfamily. However, treatment of Hepa-1 cells with $\alpha_q$ and $\alpha_{11}$ 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 activates $\alpha_{11}$ 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 $\alpha_q$ in signal transduction.

AUTHOR CONTRIBUTION

The project strategy was devised by, and most of the experiments were performed by, Chiho 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.

ACKNOWLEDGEMENTS

We thank Dr Takashi Namatame of the Medical Research Center for help with DNA sequencing and the Research Support Center for allowing us to use the facilities at Dokkyo Medical University School of Medicine. We also thank Dr Junken Aoki (Graduate School of Pharmaceutical Science, Tohoku University, Japan) for providing the monoclonal anti-autotaxin antibody.

FUNDING

This work was supported by a Grant-in-Aid for Young Scientists from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

REFERENCES

1. Aoki, J., Inoue, A. and Ohtsuba, S. (2008) Two pathways for lysophosphatidic acid production. Biochim. Biophys. Acta 1781, 513–518
2. Sonoda, H., Aoki, J., Hiramatsu, T., Ishida, M., Bandoh, K., Nagai, Y., Taguchi, R., Inoue, K. and Arai, H. (2000) A novel phosphatic acid-selective phospholipase A1 that produces lysophosphatic acid. J. Biol. Chem. 275, 34254–34263
3. Kazantseva, A., Gotzov, A., Zinchenko, R., Grigorenko, A. P., Abrukova, A. V., Moliaka, Y. K., Kirillov, A. G., Guo, Z., Lyle, S., Ginter, E. K. and Roganov, E. I. (2006) Human hair growth deficiency is linked to a genetic defect in the phospholipase gene LIPH. Science 314, 982–985
Ali, G., Chisti, M. S., Raza, S. I., John, P. and Ahmad, W. (2007) A mutation in the lipase H (LIPH) gene underlie autosomal recessive hypotrichosis. Hum. Genet. 121, 319–325

Wykle, R. L. and Schremmer, J. M. (1974) A lysophospholipase D pathway in the metabolism of ether-linked lipids in brain microsomes. J. Biol. Chem. 249, 1742–1746

Wykle, R. L., Kramer, W. F. and Schremmer, J. M. (1977) Studies of lysophospholipase D of rat liver and other tissues. Arch. Biochem. Biophys. 184, 145–155

Tokumura, A., Harada, K., Fukuzawa, K. and Tsukatani, H. (1986) Involvement of lysophospholipase D in the production of lysophosphatidic acid in rat plasma. Biochim. Biophys. Acta 875, 31–38

Tokumura, A., Kume, T., Fukuzawa, K. and Tsukatani, H. (1981) Cardiovascular effects of lysophosphatidic acid and its structural analogs in rats. J. Pharmacol. Exp. Ther. 219, 219–224

Smyth, S. S., Cheng, H. Y., Mihaliya, S., Panchatcharam, M. and Morris, A. J. (2008) Roles of lysophosphatidic acid in cardiovascular physiology and disease. Biochim. Biophys. Acta 1781, 563–570

Kobayashi, T., Yamano, S., Murayama, S., Ishikawa, H., Tokumura, A. and Aono, T. (1994) Effect of lysophosphatidic acid on the preimplantation development of mouse embryos. FEBS Lett. 351, 38–40

van Meeteren, L. A., Ruurs, P., Stortelers, C., Bouwman, P., van Rooijen, M. A., Pradere, J. P., Pettit, T. R., Wakeham, M. J., Sautnier-Blaue, J. S., Mumeryy, C. L. et al. (2006) Autotaxin, a secreted lysophospholipase D, is essential for blood vessel formation during development. Mol. Cell. Biol. 26, 5015–5022

Umez-Goto, M., Kishi, Y., Taira, A., Hama, K., Dohmae, N., Takio, K., Yamori, T., Mills, G. B., Inoue, K., Aoki, J. and Arai, H. (2002) Autotaxin has lysophospholipase D activity leading to tumor cell growth and motility by lysophosphatic acid production. J. Cell. Biol. 158, 227–233

Tokumura, A., Majima, E., Kariya, Y., Tominaga, K., Kogure, K., Yasuda, K. and Fukuzawa, K. (2002) Identification of human plasma lysophospholipase D, a lysophosphatidic acid-producing enzyme, as autotaxin, a multifunctional phosphodiesterase. J. Biol. Chem. 277, 39436–39442

Murata, J., Lee, H. Y., Clair, T. M., Krutzsch, H. C., Arestad, A. A., Sobel, M. E., Liotta, L. A. and Stracke, M. L. (1994) cDNA cloning of the human tumor motility-stimulating protein, autotaxin, reveals a homology with phosphodiesterases. J. Biol. Chem. 269, 30479–30484

Six, D. A. and Dennis, E. A. (2000) The expanding superfamily of phospholipase A2 enzymes: classification and characterization. Biochem. Biophys. Acta 1488, 1–19

Weitzman, H. U. (1979) Cytolytic and membrane-perturbing properties of lysophosphatidylcholine. Biochim. Biophys. Acta 559, 259–267

Sugimoto, H., Hayashi, H. and Yamashita, S. (1996) Purification, cDNA cloning, and regulation of lysophospholipase from rat liver. J. Biol. Chem. 271, 7705–7711

Hoffman, R. D., Kliger, M., Sundt, T. M., Anderson, N. D. and Shin, H. S. (1982) Stereospecific chemotaxation of lymphoblastic cells by gradients of lysophosphatidylcholine. Proc. Natl. Acad. Sci. U.S.A. 79, 3285–3289

Fang, X., Gibson, S., Flowers, M., Furuui, T., Bast, Jr, R. C. and Mills, G. B. (1997) Lysosphosphatidylcholine stimulates activator protein 1 and the c-jun N-terminal kinase activity. J. Biol. Chem. 272, 13683–13689

Kugiyama, K., Korns, S. A., Morissett, J. D., Roberts, R. and Henry, P. D. (1990) Impairment of endothelium-dependent arterial relaxation by lysoclethrin in modified low-density lipoproteins. Nature 344, 160–162

Sugimoto, S., Sugimoto, H., Aoyama, C., Aso, C., Mori, M. and Izuim, T. (2006) Purification and characterization of lysophospholipase D from rat brain. Biochim. Biophys. Acta 1761, 1410–1418

Gobeil, J., St. Bernier, S., Gazquez-Tello, A., Braut, S., Beaucamp, M. H., Quiniou, C., Marrache, A. M., Checchin, D., Senilaub, F., Hou, X. et al. (2003) Modulation of pro-inflammatory gene expression by nuclear lysophosphatic acid receptor type-1. J. Biol. Chem. 278, 38875–38883

Waters, C. M., Saatian, B., Moughal, N. A., Zhao, Y., Tigy, G., Natarajan, V., Pyne, S. and Pyne, N. J. (2006) Integrin signalling regulates the nuclear localization and function of the lysophosphatic acid receptor-1 (LPA1) in mammalian cells. Biochem. J. 398, 55–62

McIntyre, T. M., Pontsler, A. V., Silva, A. R., St Hilaire, A., Xu, Y., Hinshaw, J. C., Zimmerman, G. A., Hama, K., Aoki, J., Arai, H. and Prestwich, G. D. (2003) Identification of an intracellular receptor for lysophosphatic acid (LPA): LPA is a transcellular PPARγ agonist. Proc. Natl. Acad. Sci. U.S.A. 100, 131–136

Tokumura, A., Miyake, M., Yoshimoto, D., Shimizu, M. and Fukuzawa, K. (1998) Metal-ion stimulation and inhibition of lysophosphatidic acid D which generates bioactive lysophosphatic acid in rat plasma. Lipids 33, 1009–1016

Clair, T. Lee, H. Y., Liotta, L. A. and Stracke, M. L. (1997) Autotaxin is an exoenzyme possessing 5′-nucleotide phosphodiesterase/ATP pyrophosphatase and ATPase activities. J. Biol. Chem. 272, 996–1001

Bourne, H. R., Sanders, D. A. and McCormick, F. (1991) The GTPase superfamily: conserved structure and molecular mechanism. Nature 349, 117–127

Sprang, S. R. (1997) G protein mechanisms: insights from structural analysis. Annu. Rev. Biochem. 66, 639–678

Caldera-Vera, T. M., Vanhauwe, J., Thomas, T. O., Medkova, M., Preininger, A., Mazzoni, M. R. and Hamm, H. E. (2003) Insights into G protein structure, function, and regulation. Endocr. Rev. 24, 765–781

Berman, D. M., Wilkie, T. M. and Gilman, A. G. (1996) Gα and Gβ subunits. Cell 86, 445–452

Mukhopadhyay, S. and Ross, E. M. (1999) Rapid GTP binding and hydrolysis by Gq isozyme of phosphoinositide-specific phospholipase C. J. Biol. Chem. 274, 18206–18216

Received 24 March 2011/2 August 2011; accepted 3 August 2011

Published as BJ Immediate Publication 3 August 2011, doi:10.1042/BJ20110545

© 2011 The Author(s)