Role of phospholipase D in agonist-stimulated lysophosphatidic acid synthesis by ovarian cancer cells

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Abstract  Lysophosphatidic acid (LPA) is a receptor-active lipid mediator with a broad range of biological effects. Ovarian cancer cells synthesize LPA, which promotes their motility, growth, and survival. We show that a murine homolog of a human protein previously reported to hydrolyze LPA is a highly selective detergent-stimulated LPA phosphatase that can be used to detect and quantitate LPA. Use of this protein in novel enzymatic assay demonstrates that SK-OV-3 ovarian cancer cells release physiologically relevant levels of biologically active LPA into the extracellular space. LPA release is markedly increased by nucleotide agonists acting through a P2Y4 purinergic receptor. Promotion of LPA formation by nucleotides is accompanied by stimulation of phospholipase D (PLD) activity. Overexpression of both PLD1 and PLD2 in SK-OV-3 cells produces active enzymes, but only overexpression of PLD2 results in significant amplification of both nucleotide-stimulated PLD activity and LPA production. SK-OV-3 cells express and secrete a phospholipase A2 activity that can generate LPA from the lipid product of PLD, phosphatidic acid. Our results identify a novel role for nucleotides in the regulation of ovarian cancer cells and suggest an indirect but critical function for PLD2 in agonist-stimulated LPA production.—Luquain, C. A. Singh, L. Wang, V. Natarajan, and A. J. Morris. Role of phospholipase D in agonist-stimulated lysophosphatidic acid synthesis by ovarian cancer cells. J. Lipid Res. 2003. 44: 1963–1975.

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Lyosphosphatidic acid (LPA) is a naturally occurring lysophospholipid with diverse actions on a broad range of cell types. Responses to LPA include stimulation of cell growth, differentiation, survival, and alterations in cell morphology and motility. LPA binds to cell surface G-protein-coupled receptors termed LPA1-3. These receptors are widely expressed and signal through actions of members of both the Gα and Gβγ families of heterotrimeric G-proteins. Many, but possibly not all, of the effects of LPA on cells are mediated by these receptors (1, 2).

While the signaling actions of LPA have been well characterized, much less is known about the pathways and enzymes involved in the synthesis and inactivation of this mediator. LPA is a normal constituent of serum, where it is produced and released by activated platelets (3–5). LPA production has also been demonstrated for a small number of other cell types (6, 7). LPA can be synthesized both de novo and through pathways that are initiated by phospholipase-catalyzed degradation of precursor glycerolipids (8). Hydrolysis of lysophospholipids could generate LPA directly, and enzymes that catalyze this reaction with selectivity for lysophosphatidylcholine (lysoPC) have been reported (9, 10). LPA can also be generated by phospholipase A (PLA)–catalyzed hydrolysis of phosphatidic acid (PA), which can be formed in stimulated cells through actions of inositol lipid-specific phospholipase C (PLC), diacylglycerol kinase, or phosphatidylcholine-specific phospholipase D (PLD) (11). How LPA that is formed from cellular lipids accumulates in the extracellular space is not clear. Studies with platelets implicate membrane microvesicles released in response to agonist activation as key intermediates in LPA production, but the relevance of this pathway to the process of LPA production by other cell types is unknown (4).

LPA plays a central role in the growth invasiveness and resistance to chemotherapeutics of ovarian cancer cells (12–14). Ovarian surface epithelial cells, from which these cancers are commonly derived, are resistant to many of these effects of LPA, suggesting that acquisition of enhanced LPA

Abbreviations:  ARF, ADP-ribosylation factor; C1P, ceramide 1-phosphate; LPA, lysophosphatidic acid; LPA1-3, lysophosphatidic acid phosphatase D; LPP, lipid phosphatase phosphatase; PA, phosphatidic acid; PC, phosphatidylcholine; PI(4,5)P2, phosphatidylinositol 4,5-bisphosphate; PLAs, phospholipase A2; PLC, phospholipase C; PLD, phospholipase D; PtdBut, phosphatidylbutanol; S1P, sphingosine 1-phosphate.

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responsiveness is associated with transformation and that targeting LPA synthesis or signaling might provide a novel treatment strategy for the disease (14). Ovarian tumors grow in the peritoneal cavity, where patients accumulate ascites fluid. Ovarian cancer ascites contain physiologically significant levels of LPA, and it is likely that ovarian cancer cells themselves are the source of this LPA (14, 15). Certain ovarian cancer cell lines have been shown to release LPA constitutively and in response to pharmacological agents, including Ca^{2+} ionophore, phorbol esters, and LPA itself. The use of primary alcohols that divert PA produced by PLD to biologically inactive phosphatidylalcohols implicates PLD in the pathway of LPA synthesis by ovarian cancer cells, but the mechanism involved is unclear (16).

Sensitive measurement of LPA is presently a complicated process that generally involves either radiolabeling approaches or combined liquid chromatography mass spectrometry. Here we show that a murine homolog of a human enzyme previously reported to hydrolyze LPA is a highly specific LPA phosphatase that can be used to detect and quantitate LPA. We have used this validated assay to identify a novel role for nucleotides and a specific PLD enzyme, PLD2, in the control of LPA synthesis by SK-OV-3 ovarian cancer cells.

MATERIALS AND METHODS

General reagents

McCoy’s tissue culture medium, penicillin, streptomycin, and fetal bovine serum were obtained from the tissue culture facility, UNC Lineberger Comprehensive Cancer Center, Chapel Hill, NC. Nucleotide agonists, ATP, ADP, uridine 5’ triphosphate (UTP), UDP, 2-methyl thio-adenosine triphosphate (2MeSATP), diadenosine tetraphosphate (Ap4A), and suramin were obtained from Sigma Chemical Co. (St. Louis, MO). Phospholipids, sphingosine 1-phosphate (SIP), and ceramide 1-phosphate (CIP) were from Avanti Polar Lipids (Alabaster, AL, or BIOMOL, Inc., Plymouth Meeting, PA), and phosphoinositides were purified from brain lipid extracts as described previously. Other reagents were from previously identified sources (17-19).

Cell culture

The ovarian cancer cell line SK-OV-3 was a generous gift from Dr. Gordon Mills (University of Texas, MD Anderson Cancer Center, Houston, TX). These cells were also obtained from the American Type Culture Collection (Manassas, VA). SK-OV-3 cells from both sources behaved identically in the assays reported in this paper. Cells were propagated in McCoy’s medium supplemented with 10% fetal bovine serum, 1.5 mM l-glutamine, and 100 U/ml of penicillin/streptomycin. Cells were generally used at low passage number and resuscitated from frozen stocks frequently during this study.

PLD assays

In vivo PLD activity was measured by transphosphatidylation as described previously (17-19). Cells were seeded in 12-well dishes (5 x 10^5 cells/well) and allowed to attach in the complete growth medium. The medium was replaced 24 h later with McCoy’s media containing 10% FCS and 2 µCi/well [3H]palmitic acid. After 24 h, the medium was removed, and cells were incubated in McCoy’s medium without serum for 1 h. Butan-1-ol was added to a final concentration of 0.3%. The cells were incubated at 37°C for a further 10 min, and then reactions were initiated by the direct addition of the appropriate nucleotides from concentrated stock solutions. For investigations into the antagonist action of ATP on UTP-stimulated PLD activity, cells were preincubated with varying concentrations of ATP for 10 min and then incubated for 10 min with different concentrations of UTP in the continuing presence of ATP. Unless otherwise noted, assays were for 10 min, after which the incubations were terminated by removal of the culture medium and addition of ice-cold MeOH-0.1 M HCl (1:1; v/v). Lipids were extracted using acidified organic solvents before resolution by TLC in the organic phase of a solvent comprised of 2,2,4-trimethylpentane-ethyl acetate-acetic acid-water (5:11:2:10; v/v/v/v) using an unlined, unequilibrated chromatography tank. [3H]phosphatidylbutanol (PtdBut) bands, identified by unlabeled or [14C]PtdBut standards, were scraped and quantitated by liquid scintillation counting. PLD activity was determined in vitro using previously described methods (17-19).

Lipid extractions from cells and culture medium

LPA release by SK-OV-3 cells was measured by both an enzymatic procedure and a bioassay. For sample preparation, cells were seeded in 100-mm-diameter dishes and allowed to attach in the basic growth medium. The cells were grown to ~75% confluence, and the medium was replaced 24 h later with McCoy’s medium without serum. After 2 h of incubation, the medium was then removed, cells were incubated in McCoy’s medium containing 1 mg/ml BSA, and cells were treated with vehicle or nucleotide agonists. The medium was collected and centrifuged at 100 g for 10 min to remove any unattached cells. Lipids were then extracted from the medium using acidified organic solvents with back extraction using a synthetic lower phase followed by three washes with a synthetic upper phase. The lower phases, which were removed by evaporation, were collected. Samples were stored in a small volume of CHCl_3 at ~20°C.

Cloning and expression of murine lysophosphatidic acid phosphatase

A full-length cDNA encoding of this enzyme, identified by blast searches of the IMAGE ConsortiumExpressed Sequence Tag database, was obtained from Research Genetics, Inc. (Birmingham, AL) and sequenced. The sequence has been deposited in GenBank with accession number AF216223. Murine lysophosphatidic acid phosphatase (mLAP) was expressed in insect cells using a baculovirus vector and purified using an N-terminally appended His6 epitope tag. In general, purifications were from a 225 cm^2 flask of sf9 cells that generated sufficient enzyme for 100 to 200 assays. Cells were lysed in 5 ml ice-cold 5 mM Tris (pH 7.4) containing protease inhibitors by brief sonication, and the lysate was cleared by centrifugation. The purification was conducted at 4°C. Proteins were bound to 1 ml of Talon Superflow resin (Clontech, Inc.) in a capped, 10 ml disposable column for 1 h, and then the caps were removed and the column was drained and washed with 5 x 10 ml of extraction buffer. For the final stages of the purification, the column was washed with 10 ml of 0.1 M Tris (pH 7.5), and then bound proteins were eluted with sequential 1 ml applications of 0.1 M Tris (pH 7.5) containing 150 mM imidazole. LAP activity was determined using assay buffer containing 0.1 M Tris (pH 7.5), 100 µM lipid substrate, and 0.3 mM Triton X-100 (22, 23). In some experiments, the Triton X-100 concentration was varied.

Coupled enzymatic assay for LPA

Recombinant LPAP was expressed and purified as described above. Lipids extracted from SK-OV-3 cell culture medium were
dried and resuspended in 50 μl of 0.1 M Tris (pH 7.5) containing 0.6 mM Triton X-100 by vortexing followed by brief bath sonication, and then an equal volume of purified LPAP (~1 μg) was added to a final volume of 100 μl. Standard amounts of LPA (0–100 pmol) were run in parallel to unknown samples. The phosphatase reactions were run to completion (generally 30–60 min) as determined by a separate assay with [32P]LPA. Released phosphate was quantitated by a highly sensitive cycling reaction in which maltose phosphorylase generates glucose substrate for glucose oxidase, and peroxide formed by this latter reaction reacts with Amplex Red to generate resorufin, which is quantitated spectrophotometrically. The enzymes and reagents required for the phosphate detection step of the assay were obtained from Molecular Probes, Inc. (Eugene, OR), and the assays were performed exactly as described by the manufacturer. For the quantitation step of the assay, 50 μl of each dephosphorylation reaction was transferred directly to individual wells of a 96-well microtiter plate. Fifty microliters of 0.1 M Tris (pH 7.5) containing Amplex Red, maltose, glucose oxidase, and horseradish peroxidase was added to each well. The reactions were incubated at room temperature in a plate-reading spectrophotometer (BioTek, Inc.), which shook the plate and took an absorbance reading at 563 nM every 30 min for 12 h. Progress curves for the reactions with known quantities of LPA generated a series of standard curves for the assay, which in turn were used to determine LPA mass in the unknown samples.

Measurement of LPA activity using recombinantly expressed LPA2 receptor

We used cultured insect cells expressing the LPA2 (Edg4) receptor by means of a recombinant baculovirus constructed with a cDNA encoding human LPA2 vector, generously provided by Kevin Lynch (University of Virginia), to detect LPA activity. In these cells, stimulation of the LPA2 receptor by agonist binding produces rapid increases in intracellular Ca2+, which can be readily detected using fluorescent indicators (20). In brief, serum-free adapted S9 cells were cultured in suspension in SF900 II serum-free medium. Monolayer cultures of these cells were infected with baculoviruses at a multiplicity of 10 and cultured for 48 h. Cells were dislodged by gentle shaking, recovered by centrifugation, and resuspended at 105 cells/ml in Grace’s medium without serum. The cells were loaded by incubation with purified LPAP or heat-inactivated LPAP using a SPEX Fluromax-2 fluorimeter. Excitation was at 340 nm and 380 nm, and the fluorescence signal was detected at 510 nm. The fluorescence ratio was used to monitor intracellular Ca2+ levels, which time the cells were gently mixed periodically. The cells were then washed with 70% ice-cold ethanol, air dried, and resuspended in 50 μl of 0.1 M Tris (pH 7.5) containing 0.6 mM Triton X-100 and incubated at a multiplicity of 10 and cultured for 48 h. Cells were dislodged by gentle shaking, recovered by centrifugation, and resuspended at 105 cells/ml in Grace’s medium without serum. The cells were loaded by incubation with purified LPAP or heat-inactivated LPAP using a SPEX Fluromax-2 fluorimeter. Excitation was at 340 nm and 380 nm, and the fluorescence signal was detected at 510 nm. The fluorescence ratio was used to monitor intracellular Ca2+ levels.

Analysis of [32P]LPA released by SK-OV-3 cells

After washing with PBS, SK-OV-3 cells were incubated at 37°C in phosphate-free DMEM containing 100 μCi/ml [32P]HPO4. After 90 min, cells were washed three times with phosphate-free DMEM to remove unincorporated [32P]HPO4 and they were then incubated at 37°C with nucleotide agonists and other pharmacological agents for 10 min in phosphate-free DMEM containing 1% BSA. In experiments using adenovirus vectors (see below), cells were infected 48 h before the experiment. The culture medium was collected and treated as described above to extract lipids, and the lower phases were dried under N2 and then dissolved in chloroform-methanol (1:1; v/v). Lipids were then separated by two-dimensional TLC using chloroform-methanol-28% ammonia (65:25:5; v/v/v) for the first dimension and chloroform-methanol-acetic acid-water (45:20:5:0.5; v/v/v/v) for the second dimension. Labeled lipids were detected and quantitated using a PhosphorImager (Image Quant software, Molecular Dynamics, Sunnyvale, CA) and quantitated as arbitrary units. The identity of each [32P]-labeled phospholipid was achieved by comigration with unlabelled internal standards visualized with iodine vapor.

Reverse transcription-polymerase chain reaction

Total cellular RNA was extracted using Trizol reagent (Life Technologies, Grant Island, NY) according to the manufacturer’s instructions. Confluent SK-OV-3 cells were directly lysed by adding Trizol in a 75 cm2 flask. The resulting RNA pellet was finally washed with 70% ice-cold ethanol, air dried, and resuspended in 100 μl of diethyl pyrocarbonate-treated water. Reverse transcription-polymerase chain reaction (RT-PCR) was carried out using the Titan-One Tube RT-PCR Kit (Roche Diagnostics Corp., Indianapolis, IN). Specific primers for the human PLD1 and PLD2, and P2X1, P2Y1, P2Y2, P2Y4, and P2Y6 receptors were constructed based on cDNA sequences previously determined by us or obtained from GenBank: PLD1 forward (5’-CCGATCCCCATTTCCGACAGTAGAAGAGGATGGGG-3’) and PLD1 reverse (5’-CACAGGAATTCACCGCTGTTG’-3’) (313 bp); PLD2 forward (5’-CGGTTCCTGACATGATGCTCA-3’) and PLD2 reverse (5’-TGCCCTGATGCTGCTGAG-3’) (358 bp); P2X1 forward (5’-TCTTCAGAGACGCGGAGGAACT-3’) and P2X1 reverse (5’-GGTACGGTGTCGCTTCCGC’) (580 bp); P2X2 forward (5’-TCTGGCCGTGCTGCTGCTCA-3’) and P2X2 reverse (5’-TGATAGAAGCTGGGCG-3’) (380 bp); P2X2 forward (5’-GGCCCTGGATGACACCATT-3’) and P2X2 reverse (5’-GGCTGCTGGTGTTGAAAAGGTTA-3’) (512 bp); P2X2 forward (5’-GTGGTACCCGGCTGTTCA-3’) and P2X2 reverse (5’-CTGGTACACAGAGAGGTGGG-3’) (380 bp); P2X4 forward (5’-GAGGCTCTGGAGAAGCTGG-3’) (363 bp); P2X6 forward (5’-GACGGCCGGCTTGACACCCTTAA-3’) and P2X6 reverse (5’-TACACACTGAGCCAGGCGC-3’) (269 bp).

First-strand cDNA synthesis was carried out in a 50 μl volume at 50°C for 30 min. Amplification was performed using the following profile: 2 min at 94°C followed by 35 cycles of 30 s at 94°C, 30 s at 50–70°C gradient, 1 min at 68°C, and a final extension step of 7 min at 72°C. The products were separated in a 1.5% agarose gel containing 1 mg/ml ethidium bromide.

Lysis and fractionation of SK-OV-3 cells

To prepare lysates and subcellular fractions for in vitro enzyme assays, SK-OV-3 cells were washed in PBS and harvested by scraping in ice-cold lysis buffer containing 20 mM Tris (pH 7.5), 1 mM EGTA, 0.1 mM benzamidine, and 0.1 mM PMSF. Nuclei and broken cells were removed by centrifugation at 500 g for 10 min. The supernatant obtained was separated into a total membrane and cytosolic fraction by centrifugation at 35,000 g for 30 min. The membrane fraction was resuspended in lysis buffer.
**Adenovirus-mediated and transient expression of PLD1 and PLD2**

Adenovirus vectors for expression of wild-type and catalytically inactive mutants of PLD1 and PLD2 were generated using the AdEasy system (Stratagene, Inc., La Jolla, CA). In brief, PLD1 and PLD2 cDNAs were subcloned into pShuttle-cytomegalovirus (CMV), and the PLD cDNAs and CMV promoter were transferred into the adenovirus genome by homologous recombination in an adenovirus-packing cell line according to the manufacturer's instructions. The adenoviruses were propagated in HEK293 cells and high titer preparations generated. Subconfluent monolayers of SK-OV-3 cells in 6-well plates were infected with virus control, wild-type PLD1, or wild-type PLD2 adenoviruses at a multiplicity of ~10. Viral infection was allowed to proceed for 6 h, and then the medium was changed and the cells incubated in McCoy's medium containing 10% serum for a further 48 h. For determinations of intact cell PLD activity, cells were labeled with [3H]palmitic acid for the final 24 h as described above.

Vectors for expression of PLD1 and PLD2 with N-terminally appended enhanced green fluorescent protein (EGFP) tags (EGFP-PLD1 and EGFP-PLD2) have been described previously (18). SK-OV-3 cells were cultured in McCoy's medium supplemented with 10% FBS. Thirty-five millimeter-diameter dishes of 50% confluent cells were transfected with 1 μg pCGN-hPLD1 or pCGN-hPLD2 using lipofectamine in Opti-MEM (Life Technologies, Inc.). The transfection medium was removed after 24 h and replaced with complete McCoy's medium. The cells were harvested 24 h later by washing in PBS followed by scraping into ice-cold lysis buffer containing 20 mM Tris (pH 7.5), 5 mM EGTA, 0.1 mM benzamidine, and 0.1 mM PMSF. The lysate was disrupted by sonication on ice with a probe-type sonicator, and the material was used in assays within 24 h.

**Measurement of PLA activity**

PLA activity was measured by monitoring conversion of [32P]PA or 1- or 2-[3H]palmitoyl PA into [32P]LPA or [3H]LPA using minor adaptations of previously described methods (21). Briefly, the substrate preparation used contained final concentrations of 100 μM radiolabeled PA (0.2 μCi/assay) and 1 mM 1-palmitoyl,2-oleyl-PC in 400 mM Triton X-100 mixed micelles in 100 mM HEPES (pH 7.5), 2 mM DTT, 5 mM EDTA, and 1 mM ATP. The assay volume was 100 μL and reactions were carried out at 37°C for 30 min. At the end of the incubation, lipids were extracted using acidified organic solvents. Lipid extracts were analyzed by TLC with a solvent system of chloroform-methanol-20% NH₄OH (60:35:5; v/v/v). Radiolabeled LPA and PA were localized by reference to unlabeled standards run in parallel. The appropriate regions were scraped from the plate and associated radioactivity quantitated by liquid scintillation counting.

**Other methods**

SDS-PAGE, Western blotting, and protein determination were performed as described previously (18). PLD1 and PLD2 were expressed in sf9 cells using baculovirus vectors and purified as described previously (18).

**RESULTS**

**Identification and characterization of murine LPAP**

An LPA-selective phosphatase was previously purified from rat liver. Expression of a cDNA encoding as a likely human homolog of this enzyme in COS-7 cells resulted in increased rates of hydrolysis of LPA in cell extracts; however, the recombinant protein was not purified, and its selectivity for a range of phospholipid monoester substrates was not evaluated (22, 23). Because of the obvious potential of this enzyme as a tool to detect and manipulate LPA levels, we sought to define its catalytic properties and substrate selectivity more fully. We identified a murine homolog of human LPAP by BLAST searches of the GenBank expressed sequence tag database. The cDNA obtained encodes a protein of 418 amino acids with a predicted molecular mass of 47.6 kDa. The amino acid sequence is 75% identical (85% conserved) to that of human LPAP. Because we did not identify murine cDNA sequences with higher homology, we presume that this is the murine homolog of the previously identified human gene (23). Like the human homolog, mLPA contains a putative mitochondrial import sequence at the N-terminus (residues 1–9), followed immediately by a postimport proteolytic processing motif (marked with an arrowhead in Fig. 1). The sequence contains a histidine phosphatase catalytic motif, RHGXRRXP. An alignment of human and murine LPAP with lysosomal histidine acid phosphatase highlighting these sequence motifs is shown in Fig. 1.

**mLPAP is a highly specific LPA phosphatase**

MLPAP was expressed in insect cells using recombinant baculovirus vectors. The protein was engineered to contain an N-terminal His6 tag to facilitate purification. The protein was predominantly cytosolic and could be readily purified by metal ion affinity chromatography (Fig. 2A). Purified preparations of LPAP actively hydrolyzed LPA. With 100 μM substrate, LPAP activity was stimulated over 100-fold by the Triton X-100, with maximal activity observed at 0.3 mM Triton X-100 (Fig. 2B). LPAP exhibited considerable activity in the presence of 5 mM EDTA with no added divalent cations. Inclusion of Mg²⁺ to give free Mg²⁺ concentrations in excess of the chelator produced a very modest 1.4-fold increase in LPAP activity that was maximal at 0.5 mM Mg²⁺. Ca²⁺ did not stimulate LPAP activity and actually produced a small decrease in LPAP activity at low concentrations (data not shown). Using optimal assay conditions (i.e., 0.3 mM Triton X-100 and 100 μM substrate), we examined the specificity of purified LPAP for a number of glycerophospholipid substrates (Fig. 2C). We estimated the specific activity of the purified His6-tagged LPAP with LPA as substrate to be ~13 μmol/min/mg, which is ~10-fold higher than reported for a purified native preparation of a rat homolog of the enzyme (22). S1P, C1P, and the phosphoinositides phosphatidylinositol 4-phosphate (PI4P) and phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂) were not substrates for mLPA. Activity against PA was 1.6% of that observed with LPA. We were unable to exclude the possibility that this low level of activity actually reflected hydrolysis of minor quantities of LPA contaminating these preparations, and in support of this idea we were unable to detect concurrent formation of diglyceride when the enzyme was incubated with [3H]PA. Activity against freshly prepared HPLC-purified [32P]PA was less than 0.2% of that observed with LPA. Activities with S1P and C1P were less than 0.7% and 0.3%, re-
spectively, of that observed with LPA, while no hydrolysis of PI4P or PI(4,5)P₂ could be detected (Fig. 2C). LPAP did not discriminate between 10:0 (v/v), 14:0 (v/v), 16:0 (v/v), 18:0 (v/v), and 18:1 (v/v) LPA species (data not shown).

Coupled enzymatic assay for LPA

We exploited the high selectivity of LPAP for LPA to develop an enzymatic assay for LPA, in which phosphate released by the enzyme is utilized by a sensitive coupled-enzyme reaction to generate a product that can be quantitated by spectrophotometric or fluorescence measurements. A schematic overview of the assay is shown in Fig. 3A. Standard quantities of LPA were dried in microcentrifuge tubes, resuspended in Triton X-100 (final concentration 0.3 mM), and incubated with purified LPAP for 1 h at 37°C. The phosphate released was quantitated by combining the dephosphorylation reaction with the phosphate assay reagents in a microtiter plate, which was incubated at room temperature in a shaking plate-reading spectrophotometer. It was not necessary to stop the phosphatase reaction, and the constituents of the phosphatase reaction mixture did not interfere with the phosphate detection step. The phosphate detection reaction was moni-

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**Fig. 1.** Sequence alignment of human and murine lysophosphatidic acid phosphatase (mLPAP) with human lysosomal histidine acid phosphatase precursor. Sequences were analyzed using AlignX (Informax, Inc.), and the alignment shown generated using BOXSHADE. The putative mitochondrial localization sequence is bracketed and the site of post-import proteolysis marked with an arrowhead. The consensus histidine acid phosphatase active site is boxed.
tored spectrophotometrically for 12 h, with data collection every 30 min and a series of progress curves generated (Fig. 3B). For measurements of LPA in the range of 1–20 pmol, we generated a standard curve for the reaction at times between 5 h and 10 h.

**Nucleotide agonists stimulate production of biologically active LPA by SK-OV-3 cells**

Certain breast and ovarian cancer cell lines have been reported to produce LPA both constitutively and in response to phorbol esters and Ca\(^{2+}\) ionophore, but aside from LPA itself, no physiological agonists that regulate this process have been identified (16). We used our assay to test the ability of a number of different agonists to promote LPA production by the ovarian cancer cell line SK-OV-3. We found that these cells released LPA into the culture medium constitutively, and that after 30 min extracellular LPA levels reached \(\approx 3\) pmol/ml. Production of LPA was increased 4-fold by stimulation with 10 nM PMA. We used the LPA assay described above to screen agonists for their ability to stimulate LPA production by SK-OV-3 cells and found that ADP, ATP, and to a greater extent UTP (approximately a 2-fold increase) promoted LPA production by these cells (Fig. 4A). Production of LPA in both the presence and absence of UTP was time dependent. In the absence of a stimulus, LPA concentrations reached a level of \(\approx 4\) pmol/ml after 15 min, and this was increased to \(\approx 17\) pmol/ml in the presence of UTP. LPA accumulation in the medium was not linear with time. Intact SK-OV-3 cells actively dephosphorylate exogenously provided LPA, which is likely due to endogenous expression of lipid phosphate phosphatases (LPPs) (41), so it is likely that the accumulation of LPA in the medium of unstimulated and UTP-stimulated SK-OV-3 cells reflects an equilibrium between rates of synthesis and degradation (Fig. 4B). The effects of UTP on LPA production by SK-OV-3 cells were concentration dependent. One-tenth micromolar UTP produced a small but significant increase in LPA after 15 min of stimulation. LPA accumulation was increased further by 1 \(\mu\)M and 10 \(\mu\)M UTP (Fig. 4C). We were not able to determine whether the effects of UTP on LPA production were saturable because, for reasons that are presently unclear, concentrations of UTP in excess of 30 \(\mu\)M produced an inhibition of LPA production by the cells (data not shown, but see Fig. 6).

To validate the results obtained using the enzymatic assay, we also measured LPA production by SK-OV-3 cells after radiolabeling the cells with \([32P]PO_4^{2-}\). In these experiments, LPA was quantitated in the culture medium after two-dimensional TLC. The plates were analyzed using a PhosphorImager and lipid species quantitated as arbitrary units. In a 30 min assay, unstimulated SK-OV-3 cells produced 46 ± 8 units of LPA, and this was increased to 245 ± 28 units (mean ± SD, \(n = 3\)) upon stimulation with 10 \(\mu\)M UTP. These experiments confirm that SK-OV-3 cells produce LPA both constitutively and in response to stimulation with UTP.

**LPA produced by SK-OV-3 cells is receptor active**

To examine the biological activity of LPA produced by SK-OV-3 cells, we determined the ability of lipids ex-
cells were incubated with vehicle (closed triangle) or 10 μM uridine 5′ triphosphate (UTP), 1 mM ATP or 1 mM ADP. After 15 min, the medium was collected and processed for determination of LPA mass. The data are means ± SD of triplicate determinations. A: SK-OV-3 cells were incubated in a serum-free medium containing no agonist, 10 nM PMA, or 10 μM uridine 5′ triphosphate (UTP), 1 mM ATP or 1 mM ADP. After 15 min, the medium was collected and processed for determination of LPA mass. The data are means ± SD of triplicate determinations. The activity responsible for activation of LPA2 receptor-mediated Ca²⁺ mobilization was abolished by pretreating the material with purified LPAP, but not by pretreatment with LPAP that had been inactivated by heating (Fig. 5B). The experiments shown in Fig. 5 used lipids isolated from 10 ml of culture medium from SK-OV-3 cells that were stimulated with 10 μM UTP for 15 min. On the basis of LPA measurements made in parallel, we estimate that the final concentration of LPA derived from this lipid extract in the cuvette is 0.1–0.2 μM, which is well within the range of LPA concentrations reported to activate the LPA2 receptor when expressed in sF9 cells (20). Taken together, these results show that LPA produced by SK-OV-3 cells is capable of activating cell surface LPA receptors.

Identification of a P2Y₁ purinergic receptor coupled to PLD in SK-OV-3 cells

Having established an involvement of nucleotide agonists in regulation of LPA production by ovarian cancer cells, we were interested in determining the receptor subtype involved and the signaling pathways that it regulates. Nucleotide agonists have been reported to stimulate PLD activity in a number of systems, and as this enzyme converts membrane phospholipids to PA, it has been implicated in LPA production by platelets and ovarian cancer cells (16). We found that ATP, UTP, and ADP all induced a rapid and robust increase in PLD activity when applied to serum-starved SK-OV-3 cells. Concentration response curves revealed that at low concentrations in the range of 10⁻⁸ to 10⁻⁴ M of UTP, ATP, and ADP, each increased intracellular PLD activity in a concentration-dependent manner with a rank over a potency of UTP > ATP > ADP. The maximal increase in PLD activity-evoked UTP was greater than that observed for ATP and ADP (Fig. 6A). Surprisingly, although at concentrations below 10⁻³ M, UTP produced a concentration-dependent increase in PLD activity, at concentrations above 10⁻² M, UTP was inhibitory to PLD activity (Fig. 6B). UDP had no effect on PLD activity over the range of concentrations tested (data not shown). This agonist pharmacology is consistent with the involvement of a G-protein-coupled P2 purinergic receptor in SK-OV-3 cell responses to nucleotides; therefore, we examined effects of two P2Y-receptor subtype selective agonists on PLD activity. 2MeSATP, a specific P2Y₁-receptor agonist, had no effect on PLD activity at concentrations of up to 10⁻² M. Together with the agonist pharmacology observed for ATP and UTP, this is consistent with the involvement of a P2Y₂ or P2Y₁ receptor (24, 25). To discriminate between these two subtypes of receptor, we examined effects of Ap4A, which has been reported to be a selective agonist at P2Y₁ and P2Y₂ receptors (26, 27). Ap4A did not have any effect on PLD activity at concentrations up to 10⁻² M and produced a very modest stimulation of PLD activity at higher concentrations, suggesting that relevant receptor was not of the P2Y₂ subtype. Consistent with this finding, we observed that suramin,
which has been reported to block P2Y1 and P2Y2 receptor-mediated responses (28), had little effect on the stimulation of PLD by ATP in SK-OV-3 cells (Fig. 7A). ATP appears to act as a partial agonist at P2Y4 receptors, and in a recombinant expression system, ATP has been shown to produce a rightwards shift in the concentration-response curve to UTP (29). We observed that preincubation with ATP resulted in a substantial decrease in PLD activation in response to a subsequent challenge with UTP, and that this effect was most pronounced at low UTP concentrations (Fig. 7B).

Effective antibodies for the purinergic receptors are not available, so we used RT-PCR with P2Y receptor-specific primers to selectively detect receptor transcripts in RNA isolated from SK-OV-3 cells. This analysis resulted in amplification of products with the expected sizes for P2Y1, P2Y2, P2Y4, and P2Y6 receptors. PCR products amplified from P2Y4 and P2Y6 receptor mRNAs were especially prominent (Fig. 6C). The PCR primer sets used were designed to amplify cDNA fragments of their intended targets containing unique restriction-enzyme sites. Digestion with these restriction enzymes was used to confirm the identity of the PCR products generated in these experiments. No products were detected in control experiments, where the RT step or one or both of the primers was omitted (data not shown). Thus, RNA analysis and pharmacological experiments together suggest that the actions of nucleotides on SK-OV-3 cell PLD activity are most likely mediated through a P2Y4 purinergic receptor.

Identification of PLD isoforms coupled to purinergic receptors in SK-OV-3 cells

Two PLD enzymes, PLD1 and PLD2, have been identified in mammalian cells. While there is evidence that both can be regulated by cell surface receptors, very little is known about the selectivity with which different G-protein-coupled receptors activate specific PLD isoforms (30). RNA analysis by RT-PCR using PLD isoform-specific primers revealed the presence of transcripts for both PLD1 and PLD2 in SK-OV-3 cells (data not shown). In agreement with these results, Western blotting using PLD isoenzyme-specific antibodies resulted in detection of proteins with the expected mobilities of both PLD1 and PLD2 in extracts from SK-OV-3 cells (Fig. 8A, B). As discussed below, membrane fractions of SK-OV-3 cells contain PLD activities with the properties of PLD1 and PLD2 that can be readily measured in vitro. Taken together, these results suggest that both PLD1 and PLD2 are expressed in SK-OV-3 cells. We used recombinant adenovirus vectors to overexpress PLD1 and PLD2 in SK-OV-3 cells and investigate the consequences of this overexpression on purinergic receptor signaling and LPA production. Infection of SK-OV-3 cells with these viruses resulted in a substantial increase in the appropriate PLD proteins measured by Western blotting (Fig. 8A, B). Using an exogenous substrate assay in vitro consistent with the RNA analysis and Western blotting experiments, SK-OV-3 cell membranes contained readily measurable PLD activity that was dependent on PI(4,5)P2 and modestly increased by guanosine 5'-O-(3-
thio)-triphosphate (GTP\_S)-preactivated ADP ribosylation factor 1 (ARF1). Infection of the cells with these PLD1 or PLD2 adenoviruses produced substantial increases in PI(4,5)P2-dependent PLD activity. PLD1 activity was sensitive to stimulation by ARF, while PLD2 activity was much less responsive to this activator (Fig. 8C).

Intact cell PLD activity was determined after labeling substrate phospholipids with [\( ^{3}H \)] palmitic acid by a transphosphatidylation assay. Infection of the cells with adenovirus for expression of PLD2 produced a modest increase in basal PLD activity, and the increase in PLD activity was more dramatic when the cells were stimulated with nucleotide agonists and PMA. By contrast, adenovirus-mediated expression of PLD1 produced a more modest increase in UTP-stimulated PLD activity and much smaller increases in PLD activity in response to stimulation with ATP and PMA. These results suggest that PLD2 is much more responsive to activation of the P2Y4 receptor than is PLD1. SK-OV-3 cells express LPA receptors (12). We found that adenovirus-mediated expression of PLD1 but not PLD2 in these cells produced a 2-fold increase in LPA-stimulated PLD activity (data not shown). Together with our demonstration that adenovirus-expressed PLD1 is active in SK-OV-3 cells when measured in vitro, these results suggest that the apparent preference shown by the P2Y4 receptor for activation of PLD2 activity does not arise from some undefined impairment of recombinantly expressed PLD1.

A role for PLD2 in LPA synthesis

We used the adenovirus expression system described above to explore the role of PLD1 and PLD2 in constitutive and purinergic agonist-regulated LPA production by SK-OV-3 cells. Catalytically inactive mutants of PLD1 and PLD2 have been reported to have interfering effects on endogenous PLD activities and PLD-regulated processes in a number of different cell types (31, 32). We found that adenovirus-mediated expression of an inactive PLD2 mutant (PLD2K898R), but not of an inactive PLD1 mutant (PLD1K556R), produced a significant (\( P < 0.1 \)) 30–40% inhibition of both ATP- and UTP-stimulated increases in PLD activity in SK-OV-3 cells (Fig. 9A). We therefore examined the effects of adenovirus-mediated overexpression
of wild-type or inactive mutants of PLD1 and PLD2 on basal- and UTP-stimulated LPA production by SK-OV-3 cells. Overexpression of PLD1 had no significant effect on basal- or UTP-stimulated LPA production by the cells. By contrast, overexpression of PLD2 produced a robust and significant (P < 0.1) 2.2-fold increase in UTP-stimulated LPA production by these cells and a more modest rise in basal LPA production. Expression of inactive PLD1 had no significant effect on basal- or agonist-stimulated LPA production by SK-OV-3 cells, but we found that adenovirus-mediated expression of the catalytically inactive PLD2 mutant produced a modest but not statistically significant inhibition of basal- and nucleotide-stimulated LPA production (Fig. 9B). Taken together, these data suggest that PLD2 is selectively coupled to activation by nucleotide receptors and has a role in agonist and possibly also constitutive LPA production by SK-OV-3 cells.

**PLA and lysophospholipase activities in SK-OV-3 cells**

The simplest way in which PLD2 could participate in LPA production by SK-OV-3 cells would be through direct hydrolysis of lysoPC. To test the activity of PLD1 and PLD2 against this substrate, both enzymes were purified and incubated with [3H]choline-labeled lysoPC under assay conditions that support high activity against PC. In the case of PLD1, assays contained 1 mM GTPγS-preactivated ARF1. While both enzymes could readily hydrolyze PC, no activity was observed with lysoPC as substrate (Table 1). Interestingly, though, we were able to detect a lysoPC PLD activity in SK-OV-3 cell membranes, and more dramatically in SK-OV-3-cell culture medium (Table 1). PLD2 could also participate indirectly in production of LPA by generating PA substrate for the actions of PLA enzymes. We used a combination of [32P]PA and PA that was selectively labeled in either the 1- or 2-acyl chains in an attempt to measure such activities in extracts and culture medium from SK-OV-3 cells. We found that SK-OV-3 cell lysates, subcellular fractions, and culture medium contained a PA-specific PLA activity that was selective for release of the sn-2-acyl chain. Although we have not identified the enzyme responsible, the properties of this activity are similar to those of a Ca2+-independent PA-prefering phospholipase A2 (PLA2) that has been reported in other cell types (11). Our results identify a potential pathway for formation of LPA from PA in SK-OV-3 cells, in which PA generated by the action of PLD is a substrate for LPA production by a substrate-selective PLA2.

**DISCUSSION**

Although possibly all mammalian cell types are responsive to LPA, only a few cells have been shown to actively release this mediator into extracellular space. Extensive investigations into the enzymes and pathways involved in LPA production by cells have been hampered by the technical challenge of measuring LPA mass. LPA is a minor constituent of cells and biological fluids, so quantitation of LPA in these materials requires both resolution of LPA from more abundant lipids and an effective detection step. Chemical

![Fig. 9. Effects of adenovirus-mediated expression of wild-type and inactive mutants of PLD1 and PLD2 on PLD activity, and constitutive- and agonist-stimulated LPA production by SK-OV-3 cells. A: SK-OV-3 cells were infected with adenovirus control or with viruses for expression of catalytically inactive mutants of PLD1 or PLD2. PLD activity was determined in cells treated with vehicle (white bars), 10 μM UTP (gray bars), or 1 mM ATP (black bars) for 10 min. The data are means ± SD from a representative experiment. B: SK-OV-3 cells were infected with control adenovirus or with adenoviruses for expression of wild-type or catalytically inactive mutants of PLD1 and PLD2. Serum-starved cells were treated with vehicle or with 10 μM UTP for 10 min, and LPA production quantitated as described in the text. The data are means ± SD of triplicate determinations.

| Source of Enzyme | Substrate | Product(s) | % substrate |
|------------------|-----------|------------|-------------|
| Purified PLD1    | 1-[3H]palmitoyl-PC | PA 78     |
| Purified PLD2    | 1-[3H]palmitoyl-lysoPC | lysoPA 0 |
| SK-OV-3 cells    | Particulate 1-[3H]palmitoyl-lysoPC | LPA 0.8 |
| SK-OV-3 cells    | Soluble 1-[3H]palmitoyl-lysoPC | LPA 0.3 |
| SK-OV-3 cells    | Medium 1-[3H]palmitoyl-lysoPC | LPA 17   |
| SK-OV-3 cells    | Particulate [32P]dipalmitoyl-PA | LPA 0.1 |
| SK-OV-3 cells    | Soluble [32P]dipalmitoyl-PA | LPA 2.5 |
| SK-OV-3 cells    | Medium [32P]dipalmitoyl-PA | LPA 5.5 |

LPA, lysophosphatidic acid; PA, phosphatidic acid; PLD, phospholipase D; PC, phosphatidylycholine; lysoPC, lysophosphatidylycholine.

The indicated purified proteins or material derived from SK-OV-3 cells or culture medium was incubated with the indicated radiolabeled substrates under conditions described in the text. The reactions were terminated and analyzed by TLC followed by autoradiography, fluorography, or liquid scintillation counting. Products were identified by chromatography with authentic standards and are expressed as a percentage of the total amount of substrate added to each reaction.
analysis of LPA can be accomplished by tandem chromatography/mass spectrometry or by radiolabeling cellular phospholipids with a suitable precursor and chromatographic separation of LPA from other labeled metabolites. Alternatively, bioassays for LPA have been developed that exploit the selectivity of LPA receptors or LPA-metabolizing enzymes for the lipid. These include the use of LPA acyltransferase with radiolabeled fatty acyl CoA of known specific radioactivity or the combination of phospholipase-B and coupled detection of the glycerol-3-phosphate formed by a redox-coupled dehydrogenase reaction (3–8, 10, 33, 34).

Here we show that mLPAP can be used to selectively dephosphorylate LPA in lipid extracts from biological samples. This enzyme can therefore be used for measurement of LPA by coupling this selective dephosphorylation to a highly sensitive enzymatic assay for phosphate. Recombinant LPAP is relatively simple to generate, and the basic assay procedure uses commercially available reagents and common laboratory equipment. The lower sensitivity limit of the assay is ~2–5 pmol, which is more than adequate for detection of LPA production by cultured cells.

We have used this assay to make four novel observations about the mechanisms involved in the generation of LPA by cultured ovarian cancer cells. First, we show that these cells generate functionally significant levels of LPA. Serum-deprived SK-OV-3 cells release LPA into the culture medium in a time-dependent manner, reaching an approximate concentration of ~4 nM after 1 h. This rate of LPA production is somewhat higher than that reported for cultured adipocytes (33, 35). Although LPA concentrations were not determined, several ovarian cancer cell lines have been shown to release radiolabeled LPA into the culture medium constitutively and in response to LPA itself (16).

Our data substantiate and extend these findings and are consistent with the proposal that ovarian cancer cells themselves are the source of LPA in ovarian cancer ascites fluid. The levels of LPA generated by these cells are sufficient for biological activity, and they support the idea that autocrine actions of LPA may provide a relevant stimulus for growth and survival of ovarian cancer cells (14).

Our second observation is that nucleotide agonists promote LPA release by ovarian cancer cells. The most efficacious agonist tested, UTP, increased LPA production almost 4-fold over basal. Pharmacological studies and RNA analysis implicate a G-protein-coupled P2Y4 receptor in this process. Activation of phospholipase-C and consequent Ca2+ mobilization has been reported in response to purinergic agonists in two other ovarian cancer cell types (36, 37). While it is now clear if there are differences in purinergic responses between normal ovarian surface epithelium and ovarian cancer cells, our results suggest that purinergic agonists may play an unappreciated role in the development and progression of this disease. Unlike LPA, purinergic agonists are generally not capable of eliciting a mitogenic response, yet surprisingly, extracellular ATP was found to stimulate proliferation of an ovarian cancer cell line (37). Our finding that purinergic agonists stimulate production of LPA by SK-OV-3 cells raises the possibility that the mitogenic actions of nucleotides are indirect and mediated by autocrine actions of LPA produced in response to purinergic receptor activation.

Third, our work identifies a role for PLD in the signaling pathways initiated by nucleotide agonists in ovarian cancer cells. Members of the P2Y class of receptors couple to members of both the pertussis toxin-sensitive Gs family of heterotrimeric G-proteins and to the pertussis toxin-insensitive G12/13 families and possibly G12/13 families. Consequent responses to agonist activation of these receptors therefore typically include inhibition of adenylate cyclase and activation of inositol lipid-specific PLC with subsequent Ca2+ mobilization and increases in protein kinase-C activity (25). We found that purinergic agonists also produce a robust stimulation of PLD activity in SK-OV-3 cells. Overexpression studies using adenovirus vectors indicated that PLD2 was selectively responsive to purinergic receptor activation. To substantiate the results of these overexpression studies, we used catalytically inactive mutants of PLD1 and PLD2, which have been shown to exert inhibitory effects on endogenous PLD activities and presumpively PLD-regulated processes (31, 32). Although the mechanisms involved have not been defined, it is likely that the interfering actions of these mutants arise from competition with their endogenous counterparts for regulatory proteins. We found that adenovirus-mediated expression of an inactive mutant of PLD2 but not PLD1 produced a significant inhibition of purinergic-receptor-stimulated PLD activity in SK-OV-3 cells. Although PLD2 can clearly respond to extracellular signals, in contrast to PLD1, which is directly responsive to GTP binding proteins and protein kinase-C, little is known about the regulatory mechanisms involved (30).

Differential localization of PLD1 or PLD2 to specific intracellular membranes likely underlies some of the regulatory and functional differences between these two enzymes. Although attempts to localize endogenous PLD enzymes in SK-OV-3 cells using antibodies available to us were inconclusive, studies using transient overexpression of GFP-tagged variants of PLD1 and PLD2 indicated that both enzymes localized predominantly to intracellular membranes, possibly endosomes, and not to the plasma membrane (unpublished observations). While these findings do not readily explain the differential coupling of PLD2 to purinergic receptor activation, they suggest that the enzyme is being activated in an intracellular membrane compartment.

Finally, our work implicates activation of a specific PLD enzyme, PLD2, in the pathways by which purinergic agonists promote LPA production by ovarian cancer cells. These enzymes generate PA by hydrolysis of PC, and while a variety of genetic and cell biological evidence supports roles in membrane trafficking, their precise roles in cell regulation remain to be defined. Western blotting, RNA analysis, and in vitro assays conducted under conditions that can discriminate between the two enzymes reveal that SK-OV-3 cells express both PLD1 and PLD2. Adenovirus-mediated overexpression of PLD2 produced a modest increase in constitutive LPA production by SK-OV-3 cells and a more substantial increase in purinergic agonist-stimulated LPA production. The latter of these processes was significantly attenuated by infection of the cells with an adenovirus for expression of a catalytically inactive PLD2

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mutant. Together, these data suggest that PLD2 functions in pathways that control agonist-regulated and possibly constitutive-LPA production by ovarian cancer cells. How this is achieved is at present unclear. The simplest possibility is that PLD2 generates LPA directly by hydrolysis of lysophosphatidylcholine, but this does not seem to be the case, as we found that neither PLD1 nor PLD2 can hydrolyze lysophosphatidylcholine under conditions in which they are highly active against PC. However, our results demonstrate that lysates from SK-OV-3 cells as well as culture medium contained a Ca<sup>2+</sup>-independent PLA<sub>2</sub> activity that preferentially hydrolyzed PA to generate LPA. Enzymes with this type of activity have been described by several groups, but at present we do not know if the relevant genes are expressed in SK-OV-3 cells (38, 39).

Our results suggest a potential pathway for LPA synthesis by SK-OV-3 cells in which PLD-catalyzed formation of PA from cellular phospholipid substrates precedes formation of LPA by PLA<sub>2</sub> activity, and are consistent with the previous observation that primary alcohols inhibit LPA production by ovarian cancer cells (16). We cannot, however, exclude an indirect regulatory role for PLD in LPA synthesis and, given the widely demonstrated roles for these enzymes in control of membrane transport and exocytosis, and the likely roles of these processes in delivering enzymes and substrates for LPA production into the extracellular space, this possibility merits further investigation.

Ovarian cancer ascites fluid contains biologically active levels of LPA, which stimulate the growth, survival, motility, and resistance to chemotherapeutics of primary ovarian cancer cells and ovarian cancer cell lines but not of immortalized ovarian surface epithelium. This altered responsiveness to LPA may reflect changes in expression of LPA receptors in ovarian cancer cells or up-regulation of LPA signaling responses, most notably the PI3-kinase pathway (14). Overexpression of the LP3, which inactivates LPA in the extracellular space and thereby opposes the signaling actions of the lipid, was shown to result in decreased growth and tumorigenic potential and increased rates of apoptosis in several ovarian cancer cell lines, including SK-OV-3 cells (40). Our results suggest that the pathways responsible for LPA synthesis by ovarian cancer cells may provide a similar target for therapeutic intervention in ovarian cancer.

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