Lysophosphatidic Acid Acyltransferase-β Is a Prognostic Marker and Therapeutic Target in Gynecologic Malignancies

Gregory M. Springett,1,2 Lynn Bonham,6 Amanda Hummer,3 Irina Linkov,5 Dipika Misra,2 Chia Ma,2 Gabriella Pezzoni,4 Stefano Di Giovinle,7 Jack Singer,6 Hiroaki Kawasaki,1 David Spriggs,2 Robert Soslow,4 and Jakob Dupont2

1Drug Discovery Program, Department of Interdisciplinary Oncology, H. Lee Moffitt Cancer Center, Tampa, Florida; Developmental Chemotherapy Laboratory, Departments of Medicine, Biostatistics, Pathology and Immunohistochemistry Core Laboratory, Memorial Sloan-Kettering Cancer Center, New York, New York; 2Cell Therapeutics Incorporated, Seattle, Washington; and 3Department of Neuropsychiatry, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan.

Abstract

Lysophosphatidic acid, the substrate for lysophosphatidic acid acyltransferase β (LPAAT-β), is a well-studied autocrine/paracrine signaling molecule that is secreted by ovarian cancer cells and is found at elevated levels in the blood and ascites fluid of women with ovarian cancer. LPAAT-β converts lysophosphatidic acid to phosphatic acid, which functions as a cofactor in Akt/mTOR and Ras/Raf/Erk pathways. We report that elevated expression of LPAAT-β was associated with reduced survival in ovarian cancer and earlier progression of disease in ovarian and endometrial cancer. Inhibition of LPAAT-β using small interfering RNA or selective inhibitors, CT32521 and CT32228, two small-molecule noncompetitive antagonists representing two different classes of chemical nanomolar concentrations. Inhibition of LPAAT-β also enhanced the survival of mice bearing ovarian tumor xenografts. Cytotoxicity was modulated by diacylglycerol effectors including protein kinase C and Ca2+/CaM-GEF1. LPAAT-β was localized to the endoplasmic reticulum and overexpression was associated with redistribution of protein kinase C. These findings identify LPAAT-β as a potential prognostic and therapeutic target in ovarian and endometrial cancer.

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Introduction

The diacylglycerol lipid, phosphatidic acid, is a versatile lipid second-messenger that functions as a cofactor in several critical signaling pathways that are operant in cancer cells. For example, the full activation of c-Raf-1 and B-raf is manifested only when phosphatidic acid physically interacts with a polybasic amino acid segment in these kinases (1). This interaction is required for the translocation of Raf kinases to the plasma membrane where they phosphorylate and activate their targets. Similarly, binding of phosphatidic acid to a polybasic domain in mTOR is essential for its activation (2). Hence, the role of phosphatidic acid is central to the regulation of proteins in both proliferative and survival pathways in tumor cells. Phosphatidic acid can be produced by the enzymatic conversion of phosphatidylcholine to phosphatidic acid and choline by phospholipase D (3). Alternatively, diacylglycerol can be phosphorylated by diacylglycerol kinase to produce phosphatidic acid. Finally, lysophosphatidic acid (LPA) can be acylated at the sn-2 position by lysophosphatidic acid acyltransferase (LPAAT) to generate phosphatidic acid.

Six human LPAAT isoforms have been identified and designated LPAAT-α, -β, -δ, -ε, -γ, and -ζ (4, 5). LPAAT-α (AGPAT1) and LPAAT-β (AGPAT2) have been studied in greatest detail thus far. LPAAT-α mRNA is ubiquitously expressed in humans, with highest levels in skeletal muscle (6). In contrast, the pattern of LPAAT-β expression is more tissue-specific, with highest levels in the liver and heart (4). The 278-amino acid sequence of LPAAT-β is 48% homologous to LPAAT-α (7). Inherited loss of function of LPAAT-β causes congenital generalized lipodystrophy 1, which is also known as Bardrdinelli-Seip syndrome (8, 9). This autosomal recessive disorder is characterized by an almost complete lack of body fat and severe insulin resistance, and this phenotype indicates that the LPAAT isoforms are not physiologically redundant.

In contrast, increased expression of LPAAT-β is associated with the malignant state. LPAAT-β expression is differentially up-regulated in tumor cells compared with normal tissues in a wide variety of tumor cell types, including lung, breast, colon, prostate, and gliomas (10). Expression has also been identified in tumor stroma and in tumor-associated vascular endothelium. In comparison, the expression of LPAAT-α is uniform in normal and tumor tissues (4). Ectopic overexpression of LPAAT-β stimulates the proliferation of LNCaP cells in low serum in vitro (10). Enhancement of proliferative capabilities has also been noted in human breast cancer cells (MCF-7) engineered to overexpress LPAAT-β (11). Conversely, knockdown of LPAAT-β expression in DU145 prostate cancer cells with small interfering RNA (siRNA) inhibits proliferation (10). With respect to its role in signaling, LPAAT-β mRNA is able to cooperate with Ras and Raf to enhance Erk activation when injected into oocytes in the Xenopus meiotic maturation assay. Indeed, inhibition of LPAAT-β expression with siRNA in mammalian cells suppresses basal Erk phosphorylation. Inhibition of LPAAT-β with small-molecule antagonists prevents the translocation of Raf to the plasma membrane and subsequent Erk phosphorylation. These inhibitors also suppress the activation of proteins in the phosphoinositide-3-kinase (PI3K)/Akt pathway, including Akt, mTOR, and S6 kinase (12).

LPA, the substrate for LPAAT-β, is a well-studied autocrine/paracrine signaling molecule which is secreted by ovarian cancer cells and is found at elevated levels in the ascites fluid of women with ovarian cancer (13, 14). LPA signaling may contribute to the accumulation of ascites by inducing the synthesis and release of...
vascular endothelial growth factor (15). LPA also regulates the proliferation, survival, adhesion, and migration of ovarian cancer cells in vitro, and its presence at high levels in serum may represent an early biomarker for ovarian cancer detection. These effects of LPA are a manifestation of its first-messenger role as an extracellular ligand for the Edg (endothelial differentiation gene) family of G protein–coupled receptors (16, 17). The signal transduction functions of intracellular pools of LPA and the phosphatidic acid that can be generated from it by LPAAT-β are poorly understood. In view of the biological activities of LPA and phosphatidic acid in ovarian cancer, we sought to investigate the role of LPAAT-β, an enzyme that can convert LPA to phosphatidic acid. In this report, we examine the expression of LPAAT-β in tissue microarrays of ovarian and endometrial cancers of various histologies. We found that LPAAT-β was expressed in 70% of serous ovarian cancers and that high expression was predictive of a poorer prognosis in ovarian and endometrial cancer. We then examined the effects of pharmacologic inhibition of LPAAT-β by a family of isoform-selective small-molecule inhibitors. These molecules exhibited cytotoxic activity at nanomolar concentrations in vitro and were effective in prolonging survival in a murine model of peritoneal carcinomatosis with a human ovarian tumor xenograft. Finally, we examined the subcellular localization of LPAAT-β in ovarian cancer cells and investigated the influence of LPAAT-β expression on protein kinase C (PKC) signaling. We present a case for the development of LPAAT-β as a new therapeutic target and prognostic marker in gynecologic cancers.

Materials and Methods

Cell lines and constructs. The following cell lines were obtained from the American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS): SK-OV-3, OVCAR3, SK-UT-1, SK-UT-1B, HeLa, and SK-LMS-1. MHO59, AN3CA, and HEC-1-A cells were kindly provided by Jeff Boyd (Department of Medicine, Clinical Genetics Service and Department of Surgery, Gynecology Service, Memorial Sloan-Kettering Cancer Center, New York, NY) and propagated in RPMI 1640 with 10% FBS. IGROV1 was cultured as previously described (18). OVCA 420 and OVCA 433 were kindly provided by Robert Bast (Translational Research, M.D. Anderson Cancer Center, Houston, TX). SKOV3-C1 was generated by infection of SK-OV-3 with a retrovector (MSCVneo-CalDAG-GEF1) engineered to express mouse CalDAG-GEF1 using methods essentially as described (19). Infected cells were selected in G418 500 μg/mL (Sigma, Inc., St. Louis, MO) for 2 weeks. MSCVneo-CalDAG-GEF1 was generated by inserting the full-length mouse CalDAG-GEF1 cDNA into the XhoI and EcoRI sites of retroviral vector MSCVneo (Clontech, Inc., Palo Alto, CA). The sequence of the complete insert was confirmed in both directions, and expression was confirmed by Western blotting with rabbit polyclonal anti-CalDAG-GEF antibodies (data not shown).

SKOV3-PKCα was generated by stable transfection of SK-OV-3 with 5 μg of pCMV-PKCα–enhanced green fluorescent protein (EGFP) DNA (Clontech), which contains a COOH-terminal fusion of PKC-α to EGFP. Transfections were done with the N-[1-(2,3-dioleoyloxy)propyll]-N,N-trimethylammoniummethylsulfate (DOTAP) liposomal reagent (Roche, Inc., Nutley, NJ) according to the suppliers’ instructions. Transfected cells were selected for 2 weeks in G418 (500 μg/mL). Fusions of the fluorescent protein DSRed to the COOH terminus and NH2 terminus of full-length human LPAAT-β were generated by ligation to pDsRed-N1 and pDsRed-C1 (Clontech), respectively. A third fusion of EGFP to the COOH terminus of LPAAT-β was also generated by ligation to pEGFP-N1 (Clontech). The resulting constructs (pDsRed-LPAAT-β, pLPAAT-β-DSRed, and pLPAAT-β-EGFP) were transiently transfected into SK-OV-3 or SKOV3-PKCα using the DOTAP method with 5 μg of DNA. SKOV3-AKT-Myr was generated by stable infection of SK-OV-3 with retroviral vector SRS-AKT-Myr (kindly provided by Alfonso Bellacosa, Fox Chase Cancer Center, Department of Microbiology and Immunology, Philadelphia, PA), which expresses a constitutively active myristoylated AKT mutant. SKOV3-vector control was generated by infection with empty SRS vector (20).

Western blot analysis. An LPAAT-β–specific monoclonal antibody, MoAb 4B12 (Covance Research Products, Denver, PA), raised against amino acids 155 to 166 of human LPAAT-β was used in immunoblotting experiments (10). Total protein extracts from cell lines in culture were prepared in lysis buffer [150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 7.5), 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 5 mmol/L MgCl2, protease inhibitor cocktail (Roche)] and 35 to 50 μg was run on 12% SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membranes and blocked with 5% bovine serum albumin. Membranes were incubated with MoAb 4B12 at a dilution of 1:2,500 in PBS with 1% Tween 20 for 1 hour at room temperature. Blots were then probed with horseradish peroxidase–conjugated anti-mouse secondary antibody (Amersham, Inc., Piscataway, NJ) for 1 hour. Bound antibody was detected by enhanced chemiluminescence (Perkin-Elmer, Inc., Boston, MA). Western blots were sequentially probed with antibodies to actin (sc-1616) and mouse anti-goat IgG–horseradish peroxidase (sc-2354) from Santa Cruz, Inc., Santa Cruz, CA.

Tumor microarrays and immunohistochemistry. The generation of ovarian and endometrial tumor tissue microarrays is described in detail elsewhere (21, 22). Briefly, patients undergoing surgery for gynecologic malignancies at the Memorial Sloan-Kettering Cancer Center had their tumor specimens banked under an Institutional Review Board–approved tissue acquisition protocol after signing informed consent. For the ovarian tumor microarray, all cases of Federation Internationale des Gynaecologistes et Obstetristes (FIGO) stage 1 or II epithelial ovarian cancer surgically staged at the Memorial Sloan-Kettering Cancer Center over a 20-year period (January 1980-December 2000) were identified. Patients with BRCA 1 mutations or a strong family history of breast or ovarian cancer were excluded. All remaining cases for which archival tissues were available were included. For the endometrial tumor array, all cases of patients undergoing surgery for endometrial cancer over a 10-year period (January 1991-December 2001) were selected. All samples were from tumor primary sites and were obtained before chemotherapy or radiation. Tumor microarrays were constructed using core needle biopsies of formalin-fixed paraffin-embedded tissue specimens from patients. Circular sections 0.6 mm in diameter were arrayed using a Beecher Instruments arrayer. The microarrays contained 129 endometrial cancer specimens and 87 ovarian tumor specimens. The clinical data were retrieved from a review of the medical records. For comparison, 14 normal ovaries were examined. These specimens were obtained from patients undergoing prophylactic oophorectomy (n = 3), resection of mucinous cystadenomas (n = 4), oophorectomy with benign epithelial inclusion cysts (n = 3), normal ovaries from patients with cervical cancer (n = 3), and normal ovary from a patient with endometrial cancer (n = 1). Specimens were processed using heat-induced epitope retrieval with citric acid (pH 6.0). Slides were stained with anti-LPAAT-β monoclonal antibody 4B12 at a 1:10,000 dilution followed by biotinylated horse anti-mouse IgG (1:500) from VectorLabs Inc. (Burlingame, CA). Finally, horseradish peroxidase–conjugated streptavidin (1:500) was added, and the slides were developed with 3,3′-diaminobenzidine chromogen solution (DAKO, Inc., Carpinteria, CA). Tumor specimens were scored as LPAAT-β–positive when displaying intense staining compared with adjacent normal tissue.

Confocal microscopy. Live SK-OV-3 and SKOV3-PKCα cells were plated in 0.17-mm-thick glass delta-T cover dishes (Biotechnics, Inc., Butler, PA) in complete medium and allowed to adhere for 24 hours. Cells were subsequently treated with phosphor 12-myristate 13-acetate (PMA; 200 nmol/L) or transfected with pDsRed1-C1, pDsRed-LPAAT-β, pLPAAT-β-DSRed, or pLPAAT-β-EGFP. At indicated times posttreatment, the cells were imaged with a 63× water immersion objective on a Leica (DM IRE2) laser scanning dual photon inverted confocal microscope. Digitally captured images were assembled using Paint Shop Pro (Jasc Software, Inc., Ottawa, Ontario, Canada). As indicated, selected images were processed by deconvolution with AutoDeblur (AutoQuant Imaging, Inc., Troy, NY).

Compounds and drugs. The identification of LPAAT-β inhibitors has been previously described (10, 12). Compounds were dissolved in DMSO at
indicated concentrations. For in vitro experiments, cisplatin was obtained from Sigma and dissolved in PBS at a concentration of 1 mM/L. Stock solutions were stored at −70°C for 2 weeks. For in vivo experiments, Platamime was obtained from Pharmacia and dissolved in saline at 1 mg/mL.

The c-fjun-NH2-kinase (JNK) selective inhibitor, SP600125 (Biomol, Inc., Plymouth Meeting, PA), was dissolved in DMSO and applied to cultures in complete medium at a final concentration of 20 μmol/L. PMA (Sigma) was added to complete medium (200 nmol/L in DMSO) as indicated.

Alamar blue viability assay. Cell viability was determined using the Alamar blue reagent (BioSource International, Inc., Camarillo, CA; ref. 18). Cells were plated in 96-well plates at 1 × 10^5 cells/well in 200 μL of complete medium with either vehicle (DMSO) or compounds at varying concentrations. Cells were incubated with compounds for 72 hours, and 25 μL of Alamar blue was then added. After an additional 6 hours of incubation, fluorescence intensity at 530 nm (excitation) and 590 nm (emission) was determined on a CytoFluor Series 4000 multi-well plate reader (PerSeptive Biosystems, Inc.). The percentage of growth inhibition was calculated as the ratio of the mean fluorescence of the treated cultures to control vehicle–treated cultures. Each point on the resulting viability curve is the mean of 16 replicates (n = 16). The Alamar blue assay is based on the reduction of the dye by the metabolic activity of living cells, which produces a colorimetric and fluorometric change in the dye.

Lysophosphatidic acid acyltransferase-β small interfering RNA treatment of ovarian tumor cells. Several siRNAs targeting LPAAT-β were synthesized by Dharmacon Research (Lafayette, CO). The sense strands for each siRNA duplex were as follows. LPAAT-β-specific siRNA beginning at nucleotide 604, 5′-GUGUGUGUCCUCUCUCUCdTdT-3′. LPAAT-β 3′ untranslated region beginning at nucleotide 953, 5′-UCCGGGCUUCCAAGCGGAdTdT-3′, and EGFP control siRNA, 5′-GCAGCAGCGACUCCCCAGdTdT-3′. SK-OV-3 and IGROV1 cells were plated on 96-well plates and transfected with 25 nmol/L siRNAs in serum-free Opti-MEM I (Invitrogen, Carlsbad, CA) using OligofectAMINE transfection reagent according to the manufacturer's protocol (Invitrogen). Following 12 hours of incubation at 37°C, M5 medium with 30% FBS was added, and 3 days later, Alamar blue viability assay was done. As mock transfection, cells were exposed to OligofectAMINE alone in the absence of oligonucleotides. For LPAAT-β Western blot analysis, 1 × 10^5 SK-OV-3 and IGROV1 cells were plated on six-well plates and transfection was done with 200 nmol/L siRNA or the controls of OligofectAMINE or medium alone.

Animal procedures. Immunodeficient female CD1 nude mice were obtained from Charles River Laboratories. IGROV1 cells (2.5 × 10^5 cells/mouse) were suspended in PBS were injected into the peritoneum of 6- to 8-week-old mice (n = 15) on day 0. LPAAT-β inhibitor CT-32521 dissolved in 6% DMSO was added to complete medium at a final concentration of 20 μg/mL. PMA (Sigma) was added to complete medium (200 nmol/L in DMSO) as indicated.

Survival as a function of time was estimated using the Kaplan-Meier methodology. S-Plus Software (Insightful Corporation, Seattle, PA), was dissolved in DMSO and applied to cultures in complete medium at a final concentration of 20 μg/mL. PMA (Sigma) was added to complete medium (200 nmol/L in DMSO) as indicated.

Statistical analysis. Survival as a function of time was estimated using Kaplan-Meier methodology. S-Plus Software (Insightful Corporation, Seattle, WA) was used for analysis of survival and the histologic marker, LPAAT-β expression and histology were assessed with Fisher's exact test. In murine experiments, compound- and vehicle-treated groups were compared using the log-rank test. Differences in LPAAT-β expression and histology were assessed with Fisher's exact test. In murine experiments, compound- and vehicle-treated groups were compared using the log-rank test. Differences in LPAAT-β expression and histology were assessed with Fisher's exact test. In murine experiments, compound- and vehicle-treated groups were compared using the log-rank test. Differences in LPAAT-β expression and histology were assessed with Fisher's exact test. In murine experiments, compound- and vehicle-treated groups were compared using the log-rank test. Differences in LPAAT-β expression and histology were assessed with Fisher's exact test. In murine experiments, compound- and vehicle-treated groups were compared using the log-rank test. Differences in LPAAT-β expression and histology were assessed with Fisher's exact test. In murine experiments, compound- and vehicle-treated groups were compared using the log-rank test. Differences in LPAAT-β expression and histology were assessed with Fisher's exact test. In murine experiments, compound- and vehicle-treated groups were compared using the log-rank test. Differences in LPAAT-β expression and histology were assessed with Fisher's exact test. In murine experiments, compound- and vehicle-treated groups were compared using the log-rank test. Differences in LPAAT-β expression and histology were assessed with Fisher's exact test. In murine experiments, compound- and vehicle-treated groups were compared using the log-rank test. Differences in LPAAT-β expression and histology were assessed with Fisher's exact test. In murine experiments, compound- and vehicle-treated groups were compared using the log-rank test. Differences in LPAAT-β expression and histology were assessed with Fisher's exact test. In murine experiments, compound- and vehicle-treated groups were compared using the log-rank test. Differences in LPAAT-β expression and histology were assessed with Fisher's exact test. In murine experiments, compound- and vehicle-treated groups were compared using the log-rank test. Differences in LPAAT-β expression and histology were assessed with Fisher's exact test. In murine experiments, compound- and vehicle-treated groups were compared using the log-rank test. Differences in LPAAT-β expression and histology were assessed with Fisher's exact test. In murine experiments, compound- and vehicle-treated groups were compared using the log-rank test. Differences in LPAAT-β expression and histology were assessed with Fisher's exact test. In murine experiments, compound- and vehicle-treated groups were compared using the log-rank test. Differences in LPAAT-β expression and histology were assessed with Fisher's exact test. In murine experiments, compound- and vehicle-treated groups were compared using the log-rank test. Differences in LPAAT-β expression and histology were assessed with Fisher's exact test. In murine experiments, compound- and vehicle-treated groups were compared using the log-rank test. Differences in LPAAT-β expression and histology were assessed with Fisher's exact test.

Results

Lysophosphatidic acid acyltransferase-β is up-regulated in gynecologic malignancies. In view of the ability of LPAAT-β to promote cell proliferation and the transformed phenotype, we examined the expression of this protein in human gynecologic tumors and normal ovarian epithelium using an LPAAT-β monoclonal antibody. We employed an ovarian tumor microarray comprised of 87 early-stage cancers and an endometrial cancer microarray of 129 tumors. The arrays were generated from the archival specimens of all patients who underwent surgery for ovarian and endometrial cancer over a 20- and 10-year period, respectively. These arrays contained carcinomas of various histologies as well as borderline neoplasms of low malignant potential. Nonmalignant ovarian epithelium was examined in 14 specimens from patients undergoing oophorectomy for prophylaxis, resection of benign cysts, or cervical cancer. In normal tissues, the ovarian epithelium was negative for LPAAT-β expression (Fig. 1A). This was the case for the surface epithelium, tubal epithelium, and the epithelium of inclusion and paratubal cysts. Focally weak staining was seen in the ovarian stromal cells and in endothelial cells. In contrast, ovarian carcinomas displayed intense cytoplasmic staining primarily within the epithelial component (Fig. 1B and C). Expression was detected in the tumor stroma at lower levels. Overall, 38 of 87 (44%) ovarian tumors expressed LPAAT-β (Table 1). The majority of aggressive papillary serous carcinomas (12 of 18; 67%), clear cell carcinomas (10 of 15; 67%), and endometrioid carcinomas (7 of 13; 54%) were LPAAT-β positive. In contrast, only 6 of 17 (35%) borderline and 1 of 17 (6%)
Table 1. Expression of LPAAT-β in ovarian and endometrial tumors

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>No. of cases</th>
<th>LPAAT-β+</th>
<th>LPAAT-β−</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovarian</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All ovarian tumors</td>
<td>87 (100%)</td>
<td>38 (44%)</td>
<td>49 (56%)</td>
<td></td>
</tr>
<tr>
<td>All carcinomas</td>
<td>70 (100%)</td>
<td>32 (46%)</td>
<td>8 (54%)</td>
<td></td>
</tr>
<tr>
<td>Serous</td>
<td>18 (100%)</td>
<td>12 (67%)</td>
<td>6 (33%)</td>
<td></td>
</tr>
<tr>
<td>Clear cell</td>
<td>15 (100%)</td>
<td>10 (67%)</td>
<td>5 (33%)</td>
<td></td>
</tr>
<tr>
<td>Endometrioid</td>
<td>13 (100%)</td>
<td>7 (54%)</td>
<td>6 (46%)</td>
<td></td>
</tr>
<tr>
<td>Mucinous</td>
<td>17 (100%)</td>
<td>1 (6%)</td>
<td>16 (94%)</td>
<td></td>
</tr>
<tr>
<td>Mixed histology</td>
<td>7 (100%)</td>
<td>2 (29%)</td>
<td>5 (71%)</td>
<td></td>
</tr>
</tbody>
</table>
| Borderline (low malignant potential) | 17 (100%) | 6 (35%)  | 11 (65%) | <0.001
| Normal ovarian                |              | 0 (0%)   | 14 (100%)|    |
| Epithelium                    | 14 (100%)    |          |          |    |
| Endometrioid                  | 54 (100%)    | 7 (13%)  | 47 (87%) |    |
| Serous                        | 16 (100%)    | 8 (50%)  | 8 (50%)  | 0.0036 |
| Clear cell                    | 11 (100%)    | 5 (45%)  | 6 (55%)  | 0.0235 |
| Malignant mixed Mullerian tumors | 11 (100%) | 8 (73%)  | 3 (27%)  | 0.0001 |
| FIGO I and II                 | 26 (100%)    | 3 (11%)  | 23 (89%) |    |
| FIGO III                      | 70 (100%)    | 26 (37%) | 44 (63%) | 0.0229 |

*Fisher’s exact test.
†Comparing all carcinomas to borderline low malignant potential tumors.
‡Comparing to endometrioid.
§Compared to FIGO I and II.

mucinous neoplasms were positive. When all ovarian carcinomas were compared with borderline tumors, LPAAT-β overexpression was significantly (P < 0.001) associated with the presence of carcinoma. The expression pattern of LPAAT-β in endometrial tumors (Fig. 1D) and malignant mixed Mullerian tumors (Fig. 1E) was similar to that in ovarian tumors with intense staining of the epithelial component. Although only 15% (7 of 47) of endometrioid carcinomas of the endometrium were positive for LPAAT-β, 50% of serous (P = 0.0036), 45% of clear cell (P = 0.0235), and 73% of malignant mixed Mullerian tumors (P = 0.0001) of the endometrium were positive (Table 1). In malignant mixed Mullerian tumors, the sarcomatous component also displayed strong staining. All human ovarian carcinoma cell lines tested had detectable LPAAT-β expression by Western blot at varying levels (Fig. 1F, lanes 1-8). This was also true of MHOIC-39 (lane 5), a primary culture of ovarian carcinoma from ascites fluid. There was no relationship between the sensitivity of the cell lines to platinum and the expression of LPAAT-β (compare Fig. 1F, lanes 1 and 2 or 3 and 4). Endometrial and cervical carcinoma cell lines were also positive, whereas uterine leiomyosarcoma cell lines were very low or negative.

Expression of lysophosphatidic acid acyltransferase-β correlates with prognosis. Because the expression of LPAAT-β correlated with tumor histology, we asked whether the presence of this protein influenced clinical outcome. The medical records of patients with tumors included in the tissue microarrays were reviewed for disease status and survival data. Median follow-up for ovarian and endometrial cancer cases was 59 and 37 months, respectively. Analysis by Kaplan-Meier methods revealed that LPAAT-β expression was associated with a significantly reduced progression-free survival (P = 0.009; Fig. 2A) and overall survival (death hazard ratio, 4.7; 95% confidence interval, 1.8-17.3; P = 0.01) in ovarian cancer (Fig. 2B). At 10 years, the overall survival of patients with LPAAT-β-positive ovarian carcinoma was 58% compared with 93% for LPAAT-β-negative tumors. It should be noted that the ovarian tumor microarray is comprised entirely of early-stage tumors (stage IA-IIIC). Nevertheless, LPAAT-β expression was able to differentiate between tumors with a 10-year overall survival of 90%, which is typical of early-stage disease and tumors with a substantially worse prognosis (58% overall survival) despite early stage. Similarly, progression-free survival was worse in LPAAT-β-positive (P = 0.01) endometrial cancer (Fig. 2C). No statistically significant difference in overall survival was noted in endometrial cancer (Fig. 2D).

Small-molecule lysophosphatidic acid acyltransferase-β inhibitors are cytotoxic to ovarian and endometrial carcinoma cell lines in vitro. The finding of differential up-regulation of LPAAT-β expression in ovarian and endometrial tumors and the correlation of this expression with clinical outcome led us to test a translational research hypothesis—that selective inhibition of LPAAT-β in gynecologic cancers would be an effective antitumor therapeutic strategy. To this end, we tested the activity of a family of isoform-selective small-molecule inhibitors of LPAAT-β. These compounds were identified in a high-throughput chemical library screen for molecules that could inhibit LPAAT-β in a cell-free biochemical assay (10). They are aryldiaminotriazines that display uncompetitive inhibition of sn-1-18:1 LPA binding to LPAAT-β at nanomolar concentrations. They display no activity against LPAAT-α at concentrations of up to 40 μmol/L (12). The inhibitors do not inhibit phospholipase D1 hydrolysis, phospholipase D2 activity, or PI3K activity (12), thus, suggesting relative specificity for LPAAT-β. We tested five of these first- (CT32521, CT32648) and second-generation (CT32228, CT32176, CT32615) LPAAT-β inhibitors and one control compound (CT32212) with a similar structure but weak LPAAT-β inhibitory activity in cell proliferation assays.
in vitro. Kill curves were generated using the fluorescence-based Alamar blue assay, which allows generation of kill curves without washing steps or manual counting of cells (23). Representative cytotoxicity curves are shown in Fig. 3A-D, where each point is the ratio of the mean fluorescence of a compound-treated culture to a vehicle (DMSO)-treated culture (n = 16 independent determinations within an experiment, two experiments). CT32228 is representative of this family of compounds with an IC50 (50% growth inhibitory concentration) of 50 and 104 nmol/L on human ovarian carcinoma cell lines SK-OV-3 and MHOC59, respectively (Fig. 3A and B). A similar range of activity was seen with human endometrial carcinoma cell lines AN3CA (IC50 = 37 nmol/L) and HEC-1A (IC50 = 100 nmol/L; Fig. 3C and D). A control compound without LPAAT-β inhibitory activity, CT32212, was cytotoxic only at concentrations that were one to two orders of magnitude greater on these cell lines (IC50 ≥1,500-10,000 nmol/L). CT32228 exhibited little activity against the uterine leiomyosarcoma cell line, SK-LMS (Fig. 3E), which has little or no LPAAT-β expression detectable by Western blot (Fig. 1F, lane 11). The relative potencies of the series of compounds used in this study were similar in all four cell lines tested (Fig. 3A-D). This allowed us to do a structure-function analysis to ascertain the critical determinants of antiproliferative activity against gynecologic cancer cell lines. The presence of substituents in the R3-NH-para-phenyl position (Fig. 3F) seems to be essential for activity because the positioning of the groups in the R4 ortho-phenyl position as in CT32212 is associated with loss of activity. Aliphatic groups (CH2OH) at R3 were more potent than halogenated groups (Cl > Br > CF3). Similarly, aliphatic substitution at R1 (CH2CH2OH > CH3) was associated with greater activity. In our series, CT32615 with aliphatic substitutions at both R1 and R3 was the most active compound, with an IC50 as low as 25 nmol/L against MHOC59 ovarian carcinoma cells.

Inhibition of lysophosphatidic acid acyltransferase-β protein expression with small interfering RNA reduces ovarian cancer cell line viability. In order to confirm the biological effect of LPAAT-β abrogation in SK-OV-3 and IGROV1 ovarian cancer cells, we used siRNAs directed against LPAAT-β mRNA to inhibit protein expression. In these experiments, SK-OV-3 and IGROV1 cells transfected with LPAAT-β siRNA1 (L1) had decreased expression of LPAAT-β protein detectable by Western blot analysis (Fig. 4A). SK-OV-3 and IGROV1 cell lines transfected with the LPAAT-β siRNAs (L1) also had diminished survival in the Alamar blue viability assay compared with cells transfected with control EGFP siRNA (P < 0.0001; Fig. 4B). No growth-inhibitory effect was observed in mock-transfected cells (O). This result was confirmed with a second LPAAT-β siRNA2 (L2) directed against a distinct sequence. SK-OV-3 and IGROV1 cell survival was decreased by 48% and 50%, respectively, with these siRNAs (three experiments). Thus, both pharmacologic- and siRNA-mediated inhibition of LPAAT-β activity leads to growth inhibition of ovarian tumor cells in vitro.

Lysophosphatidic acid acyltransferase-β inhibitors are effective against ovarian tumor xenografts in vivo. Peritoneal carcinomatosis is responsible for much of the morbidity and mortality associated with progressive ovarian cancer. Therefore, to test the efficacy of LPAAT-β inhibition in vivo, we used a murine peritoneal carcinomatosis model (24). Immunodeficient nude mice were inoculated in the peritoneal cavity with 2.5 × 106 cells of the IGROV-1 human ovarian carcinoma xenograft. When injected i.p., these cells grow as rapidly progressive peritoneal carcinomatosis, which is typically fatal in 10 to 12 days. Inoculated mice (n = 8 per treatment group) were treated i.p. with either vehicle (DMSO) or LPAAT-β inhibitor CT32521 at 37 mg/kg/d on days 3 to 7. Because the platinum drug class is the most clinically effective group of agents currently available against ovarian carcinoma, for comparison, we used a control group of mice (n = 10) treated i.p. on days 3, 7, and 11 with 6 mg/kg/d of cisplatin or saline vehicle. Survival by treatment group was analyzed by Kaplan-Meier methods and the results from a representative experiment (n = 3) are plotted in Fig. 5A and summarized in Fig. 5B. Mice treated with vehicle expired in 9 to 10 days, and mice treated with cisplatin experienced improved survival (median survival, 25 days; P < 0.0001). CT32521 treatment produced improved survival compared with vehicle (median survival, 19 days; P < 0.0001). CT32521 was not generally as effective as cisplatin in most experiments. Administering CT32521 at a lower dose (25 mg/kg/d) or on a 3-day schedule (days 3, 7, and 11) was not effective in comparison with vehicle (data not shown). CT32521 was well tolerated, with only one toxic death out of 30 mice treated at the 37 mg/kg/d dose level. Two toxic deaths
of 35 mice treated with platinum were observed. We conclude that pharmacologic inhibition of LPAAT-β activity is an effective therapeutic strategy in this preclinical animal model.

The cytotoxicity of lysophosphatidic acid acyltransferase-β inhibition is modulated by diacylglycerol/phorbol ester signaling. Because phosphatidic acid may contribute to P3K/AKT/mTOR signaling by facilitating activation of mTOR, we sought to determine whether LPAAT-β inhibitors could influence survival signals transmitted by mutational activation of AKT. We found that overexpression of constitutive active myristoylated AKT (AKT-Myr) in SK-OV-3 ovarian carcinoma cells failed to produce any shift in the cytotoxicity curve and IC50 of CT32288 when compared with control SK-OV-3 cells (Fig. 6A). This is consistent with blockade of the pathway at a point distal to AKT by CT32288. In addition, we were unable to identify statistically significant differences in clinical outcome based on AKT or PTEN expression in our ovarian and endometrial tumor tissue arrays (data not shown). These findings led us to evaluate the role of other signaling proteins that might influence the sensitivity of tumor cells to LPAAT-β inhibitors. Consistent with studies on myeloma cell lines, we found that treatment with the JNK-1, -2, -3–selective inhibitor SP600125 at 20 μmol/L slightly enhanced the resistance of SK-OV-3 cells to the cytotoxic effect of CT32228 (Fig. 6B; ref. 25). This effect did not achieve statistical significance, and there was no definitive correlation of the susceptibility to CT32228 and the levels of JNK-1 and -3 protein expression when examined by Western blotting in all cell lines used in Fig. 3 (data not shown).

We were led to examine diacylglycerol effector pathways because phosphatidic acid and diacylglycerol are interconvertible by lipid phosphate phosphatase and diacylglycerol kinase (Fig. 7; ref. 26). Diacylglycerol effectors, such as PKC, have a well-described role in modulating the sensitivity of ovarian cancer cells to cytotoxic

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**Figure 3.** Structure and activity of LPAAT-β inhibitors in ovarian and endometrial carcinoma cell lines in vitro. A–D, the antiproliferative effects of small-molecule LPAAT-β inhibitors were tested against a panel of two ovarian, two endometrial, and one uterine leiomyosarcoma cell lines. A, SK-OV-3. B, MHOC59. C, AN3CA. D, HEC-1A. E, SK-LMS1. Compounds used were: CT32212 (green circle), CT32648 (blue diamond), CT32521 (red square), CT32228 (yellow triangle), CT32176 (lavender triangle), and CT32615 (solid pink circle). F, the compound structures are shown with corresponding IC50 values for each cell line indicated below. Curves and IC50 values are from a representative experiment (n = 2, with each point being the mean of 16 independent replicates).
chemotherapy (27). In addition to PKC, nonkinase diacylglycerol effector proteins, such as the calcium- and diacylglycerol-regulated guanine nucleotide exchange factors (CalDAG-GEF), are able to regulate some aspects of malignant transformation (28–30). Accordingly, using transfection or retroviral transduction, we engineered derivatives of SK-OV-3, which constitutively overexpress either CalDAG-GEF1 (SKOV3-CD1; Fig. 6C) or PKC-α (SKOV3-PKCα; Fig. 6D). Both of these diacylglycerol effectors were able to enhance the resistance of SK-OV-3 cells to CT32228. This finding suggests that some portion of phosphatidic acid produced by LPAAT-β might be converted to diacylglycerol and used for second-messenger signaling to PKC and CalDAG-GEF effectors. Indeed, treatment of control SK-OV-3 with phorbol ester (PMA), which is an exogenous diacylglycerol-mimetic, reduced the cytotoxicity of CT32228 and increased the IC₅₀ from 50 to 60 to >100 nmol/L (Fig. 6C and D). This effect was more pronounced in SKOV3-CD1 (Fig. 6C) and SKOV3-PKCα (P < 0.0001; Fig. 6D). This implicates both kinase and nonkinase diacylglycerol effector systems in the proliferative/survival effects of LPAAT-β signaling. The possibility that manipulating LPAAT-β activity might influence PKC signaling is particularly intriguing because the expression of some PKC isoforms in ovarian tumors reportedly has prognostic implications (31). This might represent one mechanistic basis for the negative effect of LPAAT-β expression on clinical outcome observed in Fig. 2.

Consequently, we next sought to obtain more direct evidence of an effect of LPAAT-β expression on PKC function. Because the rapid translocation of conventional PKC isoforms in response to transient elevation of diacylglycerol or phorbol ester treatment can be observed in live cells using confocal microscopy with green fluorescent protein tags, we used EGFP-tagged PKCα as a diacylglycerol sensor (32). When treated with 200 nmol/L PMA for >5 minutes, SKOV3-PKCα cells displayed typical translocation of PKCα-EGFP primarily to the cell margins at the plasma membrane and to endoplasmic reticulum (Fig. 8A). With more prolonged exposure (>15 minutes), PKCα is known to be down-regulated in an ubiquitin-dependent process and the intensity of fluorescent signals accordingly decrease (data not shown; refs. 33, 34). SKOV3-PKCα cells transiently transfected with control constructs expressing DsRed protein over 12 to 24 hours acquired uniform cytoplasmic and nuclear red fluorescent signal (Fig. 8B, middle) with void areas consistent with absent expression in the endoplasmic reticulum. Within the cytoplasmic compartment, the distribution of PKCα-EGFP overlapped with DsRed (Fig. 8B, right) except in the endoplasmic reticulum where only the green signal of endoplasmic reticulum–localized PKCα was seen. Hence, transfection of DsRed alone did not affect the distribution of PKCα-EGFP under these conditions. When fusions of DsRed to either the COOH terminus (DsRed-LPAATβ) or NH₂ terminus (LPAATβ-DsRed) of LPAAT-β were expressed, punctate signals were observed throughout the cytoplasm in a pattern consistent with endoplasmic reticulum localization (Fig. 8C, middle). The patchy pattern is likely related to the propensity of the tetrameric DsRed protein to form aggregates when overexpressed as a fusion protein (35). Cells
expressing DsRed-tagged LPAAT-β displayed redistribution of PKCα-EGFP (Fig. 8C, left). Over 12 to 24 hours, PKCα-EGFP concentrated at the cell margins and at spots throughout the cytoplasm typical of the endoplasmic reticulum (Fig. 8C, left, top row). With COOH-terminus-tagged LPAAT-β at 48 to 72 hours, larger cytoplasmic aggregates were found in which PKCα-EGFP and LPAAT-β-DsRed colocalized (Fig. 8C, bottom row, right). Because of the tendency of DsRed fusions with LPAAT-β to aggregate, we sought to confirm the subcellular localization of LPAAT-β with a third fusion to EGFP, which, unlike DsRed, is monomeric. Using three-dimensional reconstruction of a stacked series of deconvoluted images of SK-OV-3 cells expressing LPAAT-β-EGFP revealed the reticular pattern of the endoplasmic reticulum (Fig. 8D). The most intense signal was in the perinuclear region consistent with the rough endoplasmic reticulum. A similar pattern has been noted in ECV-304 cells (10). Together, these findings are consistent with the notion that increased LPAAT-β expression and activity can influence the subcellular distribution of PKC. A proposed model for this signaling circuitry is presented in Fig. 7.

Figure 6. Diacylglycerol/phorbol ester signaling modulates the cytotoxicity of LPAAT-β inhibitors. A, cytotoxicity of CT32228 to SK-OV-3 cells expressing a constitutively active myristoylated AKT mutant (AKT-Myr) or empty vector control. B, cytotoxicity to SK-OV-3 cells of CT32228 alone or CT32228 combined with JNK-selective inhibitor, SP600125 (20 μmol/L). C, cytotoxicity of CT32228 to control SK-OV-3 cells or SK-OV-3 overexpressing CalDAG-GEF1 protein (SKOV3-CD1) in the presence or absence of PMA (200 nmol/L). D, cytotoxicity of CT32228 to control SK-OV-3 cells or SK-OV-3 overexpressing PKCα protein (SKOV3-PKCα) in the presence or absence of PMA (200 nmol/L). Comparing control SK-OV-3 to SKOV3-PKCα + PMA (P < 0.0001). Representative kill curves are shown. Each point is the mean of 16 determinations (n = 3 experiments).

Discussion

With the completion of the Human Genome Project, the task of selecting which of 30,000+ proteins are appropriate therapeutic targets and/or prognostic markers for clinical translation is formidable. Although there is no unanimity of opinion on what the criteria for target validation should be, certain requirements are clearly desirable (36). The candidate target must be produced in the specified tumor type. Ideally, the target should be differentially expressed in tumors compared with normal tissue. The function of the target should contribute to the initiation and/or maintenance of some aspect of the malignant phenotype. Finally, inhibition of the target’s function in a pharmacologically tenable manner should be cytotoxic or at least cytostatic to cancer cells. We have presented evidence that LPAAT-β is overexpressed in ovarian and endometrial carcinomas relative to normal tissue. Those patients expressing DsRed-tagged LPAAT-β displayed redistribution of PKCα-EGFP (Fig. 8C, left). Over 12 to 24 hours, PKCα-EGFP concentrated at the cell margins and at spots throughout the cytoplasm typical of the endoplasmic reticulum (Fig. 8C, left, top row). With COOH-terminus-tagged LPAAT-β at 48 to 72 hours, larger cytoplasmic aggregates were found in which PKCα-EGFP and LPAAT-β-DsRed colocalized (Fig. 8C, bottom row, right). Because of the tendency of DsRed fusions with LPAAT-β to aggregate, we sought to confirm the subcellular localization of LPAAT-β with a third fusion to EGFP, which, unlike DsRed, is monomeric. Using three-dimensional reconstruction of a stacked series of deconvoluted images of SK-OV-3 cells expressing LPAAT-β-EGFP revealed the reticular pattern of the endoplasmic reticulum (Fig. 8D). The most intense signal was in the perinuclear region consistent with the rough endoplasmic reticulum. A similar pattern has been noted in ECV-304 cells (10). Together, these findings are consistent with the notion that increased LPAAT-β expression and activity can influence the subcellular distribution of PKC. A proposed model for this signaling circuitry is presented in Fig. 7.

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Figure 7. A model for the function of LPAAT-β and phosphatidic acid in signaling. LPAAT-β in endoplasmic reticulum membranes generates phosphatidic acid by addition of a second acyl chain to the sn-2 position of LPA. Phosphatidic acid within membranes binds and contributes to the full activation of mTOR and Raf. A portion of phosphatidic acid is converted to diacylglycerol by lipid phosphate phosphatase-3, which is also an integral membrane protein found in part in the endoplasmic reticulum. If polyunsaturated-diacylglycerol is generated, it may function as a second-messenger to PKC and/or CalDAG-GEF (RasGRP) family members. Conventional and novel PKC isoforms translocate to membranes in response to diacylglycerol stimulation, and CalDAG-GEF1 stimulates GTP/GDP exchange on Rap1 resulting in activation of Rap1. Rap1-GTP activates B-Raf and the MEK/Erk cascade. PKC can also regulate this pathway through its action on Ras/c-Raf.
with LPAAT-β-positive tumors had a poor clinical outcome compared with patients whose tumors were negative for LPAAT-β expression. The ovarian tumors in our tissue microarray were from patients with early-stage disease. In an examination of LPAAT-β expression in a panel of stage III to IV patients, we have found that 68% are positive. Further study will be needed to determine whether the negative prognostic implications of LPAAT-β expression extend to patients with advanced-staged disease, which is the most common presentation. Nevertheless, the ability of LPAAT-β expression to differentiate between early-stage ovarian cancer patients who will do well and those who will be at higher risk for poor outcome would be clinically useful as those patients may benefit from adjuvant therapy despite having early-stage tumors. The discriminative capability of LPAAT-β expression in this population and the correlation with high-grade (papillary serous) histology also implies that this protein may influence critical biological features of ovarian cancer cells, such as adhesion, migration, invasion, and angiogenesis, which might contribute to early dissemination of disease. We further showed that inhibition of LPAAT-β enzymatic activity with small-molecule inhibitors, exemplified by CT32228, is cytotoxic to ovarian and endometrial cancer cells at nanomolar concentrations in vitro and can prolong the survival of mice inoculated with human ovarian tumor xenografts. That siRNA knockdown of LPAAT-β also inhibits the viability of ovarian cancer cells supports the conclusion that it is the inhibition of this target that is the mechanism by which CT32228 kills cancer cells. The relative resistance to CT32228 of SK-LMS, which has little detectable LPAAT-β protein expression, would suggest that this leiomyosarcoma cell line is not dependent on LPAAT-β activity for continued proliferation and survival. However, we have noted that the relative expression of LPAAT-β protein does not correlate closely with the relative sensitivity of the cell lines to these inhibitors (compare Fig. 1F, lanes 3 and 5 or 9 and 12 to Fig. 3A and B or C and D, respectively). Similarly, enforced overexpression of LPAAT-β in ovarian carcinoma cell line with a retroviral vector did not produce a significant change in sensitivity to several of these inhibitors. This suggests that it may be the extent of dependence of a particular tumor on LPAAT-β that controls the sensitivity to the inhibitors rather than the absolute expression level alone. This finding is not unprecedented, and in the era of targeted cancer therapeutics, it is a recurring theme. For example, the expression level of estrogen receptor correlates poorly with tamoxifen sensitivity in breast cancer cells that also express HER2/neu (37). Similarly, sensitivity to anti-Her2 monoclonal antibody trastuzumab in breast cancer cells does not correlate with tamoxifen expression (38). Lack of correlation between tumor target expression and drug sensitivity also exists for the proteosome inhibitor PS-341 (bortezomib; ref. 39), the topoiso- merase I inhibitor CPT-11 (irinotecan; ref. 40), and the epidermal growth factor receptor–targeting drugs C225 (erbitux) or ZD1839 (Iressa; ref. 41). Only recently has the beginning of a molecular understanding of epidermal growth factor receptor–targeting drugs been reported (42).

With regard to LPAAT-β, we have examined both expression and enzymatic activity of the tumor cells and its relationship to the IC$_{50}$ of the inhibitory compounds. As with all the instances above, the relationship is not simple (e.g., cells with twice the expression/activity are twice as resistant). Possible mechanistic reasons for the lack of correlation could be signaling interference, counter-regulatory mechanisms, relative differences in the dependence of a given cell line on the pathway or enhanced cellular drug efflux. Based on these findings, we conclude that LPAAT-β is a valid target for clinical translation in gynecologic malignancies.

### Figure 8
Localization of PKC-α in ovarian cancer cells expressing LPAAT-β.

A, SK-OV-3 cells constitutively expressing COOH-terminal EGFP-tagged PKC-α (SKOV3-PKCα) were treated with PMA 200 nmol/L for 0 minutes (left), 1 minute (middle), and 5 minutes (right). Arrows, sites of plasma membrane translocation (magnification, ×63). B, SKOV3-PKCα cells were transfected with control vector expressing DsRed protein. PKCα-EGFP displays cytoplasmic and endoplasmic reticulum localization (left). DsRed is also distributed in the cytoplasm (middle) and nucleus. The cytoplasmic distribution of PKCα-EGFP is not altered and overlaps with that of DsRed (right). C, SKOV3-PKCα cells transfected with NH2-terminal DsRed-tagged LPAAT-β (SKOV3-DsRed-LPAATβ) display redistribution of PKCα to the cell margins (arrows) and endoplasmic reticulum 12 hours posttransfection (left, top row). DsRed-LPAATβ is found in scattered aggregates (middle). Cells in the same field without strong red signals maintain a uniform cytoplasmic PKCα pattern (right, top row). SKOV3-PKCα cells transfected with COOH-terminal DsRed-tagged LPAAT-β (SKOV3-LPAATβ-DsRed) display large aggregates in a pattern consistent with endoplasmic reticulum distribution (left and middle, bottom row) in which PKCα and LPAAT-β colocalize (right, bottom row; magnification, ×100). D, SK-OV-3 cells expressing COOH-terminal EGFP-tagged LPAAT-β were imaged as a stacked series, deconvoluted and three-dimensional reconstructed. LPAAT-β is distributed throughout the rough endoplasmic reticulum in the perinuclear region and the smooth endoplasmic reticulum peripherally.

8 Unpublished data.
9 L. Bonham, unpublished data.
It has become increasingly clear that lipids like LPA and phosphatidic acid are much more than intermediates in membrane biosynthesis. Phosphatidic acid also functions as a cofactor in the activation of multiple signaling molecules including Raf, PKC-ζ, phosphoinositol 4-kinase, phospholipase C-γ, and mTOR (Fig. 7; refs. 1, 2, 43–45). Considering this dual role, the question arises as to whether there are distinct subcellular pools of phosphatidic acid that are used for different purposes and to what extent these pools intermingle. If phosphatidic acid pools used for signal transduction and membrane biosynthesis are in equilibrium, this potentially presents an opportunity to manipulate the quantity of phosphatidic acid available for signaling by targeting the metabolic enzymes that can directly or indirectly contribute to phosphatidic acid homeostasis and thereby influence cell growth and survival. This approach has been shown in principle with respect to other metabolites in the biosynthetic pathway, such as LPA and diacylglycerol. For example, overexpression of plasma membrane localized lipid phosphate phosphatase can dephosphorylate extracellular LPA and thereby attenuate its positive effect on ovarian cancer cell proliferation, migration, and invasiveness (46, 47). Similar observations have been made with respect to diacylglycerol where activation of PKC and CalDAG-GEF family members can be dampened by overexpression of diacylglycerol kinase, which converts available diacylglycerol to phosphatidic acid (48, 49). Alternatively, overexpression of diacylglycerol acyl transferase reduces total cellular diacylglycerol by converting it to triglyceride, which inhibits proliferation and anchorage-independen growth in transformed fibroblasts (50). Our findings suggest that by depriving ovarian cancer cells of a source of phosphatidic acid from LPAAT-α, the survival of these cells can be undermined. Until recently, it was not appreciated that the phosphatidic acid supplied by LPAAT-β could contribute to signaling. This role had been ascribed largely to phosphatidylincholine-phospholipase D, which can generate phosphatidic acid from phosphatidylincholine. The fatty-acyl chains in acyl-phosphatidic acid generated by phospholipase D are composed largely of saturated and monounsaturated fatty-acids. The fatty-acyl chains in acyl-phosphatidic acid generated by LPAAT-β would be mostly saturated and destined for use in membrane biosynthesis, and the effects of LPAAT-β on the distribution of PKCα observed here would be through an indirect mechanism. For example, phosphatidic acid from LPAAT-β may contribute to the activation of phospholipase C-γ, which then produces polyunsaturated-diacylglycerol for signaling. Further biochemical studies will test these models. A LPAAT-δ phosphatidic acid-PKC signaling axis would potentially be clinically relevant to a number of malignancies where PKC expression has prognostic importance. For example, PKC-α expression is positively correlated with ovarian tumor grade and median survival (31). In human ovarian carcinoma cell lines, changes in PKC-α expression are associated with sensitivity to chemotherapy (27). PKC-α expression is inversely correlated with estrogen receptor status in breast and endometrial cancers and may be associated with tamoxifen treatment failure in breast cancer (54, 55). Differential expression of PKC-α and -β has been noted in early prostate carcinoma compared with adjacent benign pseudoepithelium (56). It remains to be clarified which of the isofoms of PKC identified in ovarian and endometrial carcinomas are most relevant to the pathogenesis of these malignancies. In the studies reported here, PKC-α was selected as a representative member of the conventional class. Early phase I and II trials of PKC-α inhibitors in ovarian cancer have been disappointing and suggest that other isoforms may be more appropriate targets in this disease (57, 58). Determination of the influence of LPAAT-β signaling on these isofoms would be important in devising potential combination targeted therapies against both LPAAT-β and PKC.

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