Phosphatidylcholine-Specific Phospholipase C Activation in Epithelial Ovarian Cancer Cells

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Abstract

Elucidation of the mechanisms responsible for aberrant phosphatidylcholine (PC) metabolism in cancer cells may allow identification of novel biomarkers of tumor progression and design of new targeted anticancer therapies. We recently reported up-regulation of PC-specific phospholipases in epithelial ovarian cancer cells (EOC) compared with nontumoral (normal or immortalized) counterparts (EONT). In the present study, we focused, in the same cell systems, on levels, subcellular localization, and activity of PC-specific phospholipase C (PC-PLC), for which a key role in cell proliferation, differentiation, and apoptosis has been shown in several mammalian cells. A 66-kDa PC-PLC isoform, detected in nuclear and cytoplasmic compartments of both EOC and EONT cells, accumulated on the external plasma membrane of cancer cells only, where it colocalized with β1 integrin, in nonraft membrane domains. PC-PLC activity was 3-fold higher in total cell lysates and 5-fold higher in membrane-enriched fractions of EOC compared with EONT cells. Serum deprivation induced in EOC, but not in EONT, cells a 3-fold decrease in PC-PLC activity, associated with a 40% drop in S-phase fraction. The recovery of both variables to their original levels in serum-restimulated (or lysophosphatidic acid–restimulated) EOC cells was strongly delayed, for at least 24 h, in the presence of the PC-PLC inhibitor tricyclo-decan-9-yl-potassium xanthate (D609). The S-phase of serum-restimulated EONT cells was not sensitive to D609. These findings warrant further investigations on the role of PC-PLC and on the effects of its inhibition on the pathways responsible for constitutive EOC cell stimulation and cell proliferation.

Introduction

Epithelial ovarian cancer (EOC), the most common cause of death from gynecologic malignancy in the Western world, still presents a high case-fatality ratio with an overall 5-year survival lower than 45%, mainly due to asymptomatic disease progression at early stage, high recurrence rate, and frequent onset of chemoresistance after first line treatment (1, 2). The existing gap in the knowledge of molecular mechanisms responsible for EOC progression leads to current limitations of therapy regimens, still mainly restricted to surgery followed by chemotherapy with cytotoxic drugs. Only limited attention has been, thus far, devoted to alterations of metabolic fluxes and enzyme activities associated in these tumors with gene-driven deregulation of cell signaling pathways responsible for uncontrolled cell proliferation, cell invasiveness, and escape from default apoptotic programs.

An aberrant phosphatidylcholine (PC) metabolism is reported in cancer cells, in which substantial changes in the steady-state levels of PC cycle derivatives are commonly detected by in vitro and in vivo nuclear magnetic resonance signals (3–5). Among these, an increase in phosphocholine (PChO) content is a common feature of different cancer cells, beyond their otherwise wide phenotypic variability (5). Elucidation of the underlying molecular mechanisms may characterize novel biomarkers of tumor progression (6–8) and allow identification of metabolic targets for newly designed selective antitumor therapies (9–11).

We recently reported (8) a 3-fold to 8-fold increase in the PChO content in EOC cell lines compared with epithelial ovarian nontumoral (normal or immortalized) cells (EONT). Enzymatic assays showed that PChO accumulation in EOC cells was associated with activation of choline kinase, a biosynthetic enzyme well known for its implication in human carcinogenesis (12–14), and PC-specific phospholipases of the C and D type (PC-PLC and PC-PLD). These findings prompted our interest on investigating the role of PC-PLC in ovary tumor progression, also in view of our previous reports on ectopic localization and/or activation of this enzyme in oncogene-transformed and mitogen-stimulated fibroblasts (15, 16).

A growing body of evidence has pointed, since the 1990s, to the implication of PC-PLC in proliferation, differentiation, and apoptosis of mammalian cells. In fact, this enzyme has been implicated in cellular processes crucial for cell signaling, such as (a) mitogen-driven and oncogene-driven extracellular signal-regulated kinase phosphorylation and activation of gene transcription factors in fibroblasts (16, 17), (b) agonist stimulation of cells transfected with receptor proteins (18), (c) neuronal and endothelial cell differentiation (19–21), (d) programmed cell death (22, 23), and (e) activation of cells of the immune system (24–30).

Currently, the mammalian PC-PLC has not been cloned and its sequence is unknown. Only scanty knowledge has been accumulated on expression, subcellular distribution, and activity of PC-PLC in cancer cells. Nevertheless, indirect evidence on increased PC-PLC activity was reported in breast tumor cells (31). Moreover, the present unavailability of specific monoclonal antibodies (mAb) can be partly compensated by the use of rabbit polyclonal antibodies raised against bacterial (Bacillus cereus) PC-PLC, possessing proved...
selective cross-reactivity against mammalian PC-PLC (32). This approach allowed identification of PC-PLC isoforms in various normal and tumor cells of different origin (15, 16, 25–27, 32, 33).

This article reports evidence on subcellular localization and activity of PC-PLC in EOC cell lines compared with epithelial ovarian normal cells or immortalized cell variants. The possible role of this enzyme in cell proliferation was investigated by measuring PC-PLC activity in relation to alterations in the S-phase fraction induced in EOC cells by serum deprivation and subsequent restimulation by growth factors, in the presence or absence of a PC-PLC inhibitor.

Materials and Methods

Antibodies and reagents. Rabbit polyclonal anti–PC-PLC antibody raised against bacterial (B. cereus) PC-PLC and selectively cross-reacting with mammalian PC-PLC was obtained according to the procedure described by Clark and colleagues (32) and modified as reported in ref. 15. Immunochemical characterization and specificity of these antibodies for proteins with PC-PLC activity have already been described (15, 16, 25). In particular, specific binding inhibition tests showed that this antibody was directed against the catalytic site of a mammalian 66-kDa PC-PLC isoform (16). Anti–β-actin mAb was supplied by Sigma-Aldrich. Anti–folate receptor (FR) MO18V and MO19V (34), available through Alexis Life Sciences, Inc., and anti–α1 integrin MAB4 (35) mAbs were produced at the Fondazione IRCCS Istituto Nazionale dei Tumori, Milan. Rabbit anti–caveolin-1 (Cav-1) antibody was from Santa Cruz Biotechnology. Alexa Fluor-488 and Alexa Fluor-594 (Fab), fragment of goat anti-rabbit IgG (H + L) and Alexa Fluor-594 (F(ab)), fragment of goat anti-mouse IgG (H + L), purchased from Molecular Probes, Inc., were used as secondary antibodies.

Epidermal growth factor (EGF) was supplied by Calbiochem. Lysophosphatidic acid (LPA) was purchased from Avanti Polar Lipids, Inc. Triticum X-100, propidium iodide, tricyclodecan-9-yl-potassium xanthate (D609), 5-bromo-2′-deoxyuridine (BrdUrd), and all other chemicals and biochemicals were from Sigma-Aldrich.

Normal ovarian epithelial cells, immortalized cell variants, and cancer cells. Normal ovarian surface epithelial (OSE) cells (six independent preparations), stably immortalized OSE cell variants obtained by OSE transfection with SV40 large T antigen (IOSE) and by further IOSE transfection with cDNA of the catalytic subunit of human telomerase reverse transcriptase (hTERT), and EOC cell lines (IGROV1, OVCA3, SKOV3, and CABA 1) were obtained and cultured as reported (8). Ascitic fluids were collected during surgical procedures from four advanced stage ovarian cancer patients undergoing debulking surgery at the Fondazione IRCCS Istituto Nazionale dei Tumori at the first diagnosis and not previously treated with chemotherapeutic regimens. Histopathologic diagnosis for all patients was poorly differentiated serous papillary ovarian cancer. All clinical specimens used for this study were obtained with Institutional Review Board approval from patients who gave informed consent to use leftover biological material for investigative purposes. Tumor cells were isolated by centrifugation on a discontinuous 75% to 100% Ficoll gradient followed by separation from adherent cells, essentially as described (36).

Confocal laser scanning microscopy and flow cytometry analyses. PC-PLC subcellular distribution in either unfixed or fixed and permeabilized cells was characterized by indirect immunofluorescence and confocal laser scanning microscopy (CLSM) analysis, as previously described (16, 25–27).

CLSM observations were performed on a Leica TCS SP2 AOPS apparatus, using excitation spectral laser lines at 405, 488, and 594 nm and using the Leica Confocal Software (Leica Laserotechnik GmbH) and Adobe Photoshop software programs (Adobe Systems).

Signals from different fluorescent probes were taken in parallel, and colocalization was detected in yellow. Different fields of view (>200 cells) were analyzed on the microscope for each labeling condition, and representative results are shown.

Flow cytometry analyses were performed on cells suspended in 0.2% PBS-EDTA and stained as described (16).

Western blot analyses. Total cell lysates were obtained suspending cell pellets in ice-cold lysis buffer [150 mmol/L NaCl, 100 mmol/L Tris-HCl (pH 8), 1% Triton X-100, 1 mmol/L MgCl2, and protease inhibitor cocktail] for 20 min. Lysates were cleared of detergent insoluble materials by 10 min centrifugation at 20,000 × g.

Membrane fractions were prepared, and their purification was controlled as described (37).

Protein concentration was determined by Bio-Rad protein assay according to the manufacturer’s protocol (Bio-Rad Laboratories, Inc.) and total cell lysates or purified membranes (30 μg proteins) were analyzed by Western blotting, as previously described (27). Densitometry analyses of protein bands were performed with a Bio-Rad apparatus (Bio-Rad Laboratories Srl) using the Quantity One software.

In vitro PC-PLC and PLD activity analyses. Relative changes of PC-PLC and PLD activities were determined in whole-cell lysates and enriched membrane fractions using the Amplex Red assay kit (Molecular Probe, Inc.), as described by the manufacturer and adapted by Spadaro and colleagues (26).

Separation of lipid rafts by sucrose gradient. Cells grown in 175 flasks were washed in cold PBS, lysed in 1% Triton X-100 containing buffer [25 mmol/L MES (pH 6.5), and 150 mmol/L NaCl], and the lysate was mixed 1:1 with 80% sucrose solution. Gradient fractionation (5–30% sucrose) was carried out as previously described (38, 39). After ultracentrifugation at 300,000 × g for 17 h in TH61 Sorvall rotor, twelve 1-ml fractions were collected from the top of the gradient, and the same volume (90 μL) was taken from each fraction for analysis in SDS-PAGE in parallel with that of total cell lysate (35 μg total protein). To concentrate raft membranes, top fractions 3 to 6 were diluted 1:2 with 25 mmol/L MES (pH 6.5) and 150 mmol/L NaCl, and raft membranes were pelleted by centrifugation at 13,000 rpm for 30 min. Pellet was resuspended in loading buffer for SDS-PAGE analysis. Distribution of relevant proteins separated by 10% SDS-PAGE was analyzed by Western blotting.

Glycosyl-phosphatidylinositol–anchored FR and Cav-1 were used as markers of lipid rafts, whereas α1 integrin was considered as a nonraft marker. To analyze FR and α1 integrin reactivity, samples were analyzed under nonreducing conditions.

Immunohistochemical detection of PC-PLC in EOC tissues. Analyses were performed on a commercial tissue array slide constructed with paraffin sections from 50 ovarian cancer specimens of different histotypes (SuperBioChips Laboratories). Antigen retrieval was performed in 10 mmol/L citrate buffer (pH 6), at 120°C for 2 min; endogenous peroxidase was quenched with 3% H2O2 for 10 min, and slides were saturated in blocking solution (30 min) and then incubated with rabbit anti–PC-PLC antibodies (1 h at room temperature) and revealed with biotin-conjugated antirabbit secondary antibody followed by avidin-biotin complex (Vectastain Universal Elite ABC kit, Vector Laboratories, Inc.). Peroxidase reaction was developed with 3,3-diaminobenzidine, and sections were counterstained with hematoxylin. Slides incubated with the secondary antibodies alone provided negative controls; a control with hematoxylin/eosin was also prepared. Staining was evaluated considering both staining intensity and the proportion of cells showing positive reaction.

Statistical analysis. Data were analyzed using GraphPad software version 3.03. Statistical significance of differences was determined by one-way ANOVA or by Student’s t test (as specified). Differences were considered significant at P < 0.05.

Results

Increased levels of PC-PLC localization and activity on the membrane of EOC compared with EONT cells. CLSM analyses on unfixed cells showed massive (although different) levels of PC-PLC localization on the outer membrane surface of a number of EOC cell lines, as well as on that of cancer cells isolated from ascitic exudates (examples in Fig. 1A). This characteristic PC-PLC distribution was much less evident in hTERT and only barely detectable in OSE and IOSE cells. Flow cytometry analyses on unfixed cells showed a 5.0 ± 0.9 (SD)–fold increase (ANOVA


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P < 0.0001) in the median value of fluorescence intensity of OVCAR3 and IGROV1 compared with OSE and IOSE cells (Fig. 1B). No significant difference was found between hTERT and the other EONT cells (P = 0.09). Moreover, it is worth noting that, whereas only 25.0 ± 3.8% (SD) of unfixed OSE and IOSE cells were positive to PC-PLC immunostaining, this fraction increased to 53.7 ± 9.5% in hTERT (P = 0.003) and reached values as high as 84.6 ± 5.7% in EOC cell preparations (P < 0.0001).

Both EOC and EONT cells exhibited, however, similar PC-PLC intracellular distribution patterns, as shown by CLSM analyses on fixed and permeabilized cell preparations (examples in Supplementary Fig. S1). PC-PLC–positive granules were clearly detected in both cytoplasmic and nuclear regions, as well as in the filamentous structures protruding from cell periphery.

Immunohistochemical examinations of a commercial tissue array slide of ovarian tumor tissues, including 31 epithelial ovarian cancer sections, revealed high levels of PC-PLC in 27 samples (87%) with variable cellular localization (examples in Supplementary Fig. S2). In the PC-PLC–positive epithelial tumors, staining was localized to the cytoplasm. Among these cases, six also showed staining at the membrane level and the other six at the nuclear level (Table 1). PC-PLC localization in tumor specimens was in general agreement with the results obtained in EOC cell lines by CLSM analysis. In fact, as in CLSM examinations on fixed cells (Supplementary Fig. S1), the majority of the staining was mainly localized to the cytoplasm also in formalin-fixed paraffin-embedded tissue sections (Supplementary Fig. S2).

Immunoblotting analyses allowed detection of a distinct PC-PLC isoform with apparent molecular weight M<sub>r</sub> = 66 kDa in fresh, total cell lysates of all investigated EONT and EOC cells (Fig. 2A), in agreement with previous reports in other mammalian cells (15, 16, 25–27, 33). By densitometric analysis, the intensity of the corresponding protein band in most of the investigated cancer cell lines (OVCAR3, SKOV3, and IGROV1) was 1.9 ± 0.5 times higher than that observed in nontumoral cells. Moreover, the intensity of the PC-PLC band at 66 kDa was 2.6 ± 0.1–fold higher in membrane-enriched fractions of OVCAR3 compared with immortalized cells (Fig. 2B).

Amplex Red assays performed on total cell lysates showed no significant differences (P = 0.062) in the relative PC-PLC activity values of nontumoral immortalized cells (IOSE and hTERT); data...
were therefore pooled together, and their mean value was normalized to 1.0. In this arbitrary unit scale, the PC-PLC activity of EOC cell lines (Fig. 2C) in repeated experiments was 2.9 ± 0.7 (±SD)–fold higher than that of normal cells (ANOVA P < 0.0001). Assays on enriched membrane fractions (Fig. 2D) showed that the PC-PLC activity of OVCAR3 cells was 5-fold higher than that of EONT cells, and a similar result was obtained from ascitic cancer cells isolated from patient exudate.

These results altogether indicated that EOC differed from EONT cells in a substantial increase in the PC-PLC level on outer cell

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<th>Samples</th>
<th>No. sections</th>
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*Immunohistochemical score was obtained by adding the value of staining intensity (0, no staining; 1, faint staining; 2, moderate staining; 3, intense staining) to the estimated percentage of positive cells (0, no reactive cells; 1, 1–10% reacting cells; 2, 11–25% reacting cells; 3, 26–50% reacting cells; 4, over 50% reacting cells). Weak staining, score ≤ 3.

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**Table 1. Immunohistochemical detection of PC-PLC in an ovarian cancer tissue array slide**

**Figure 2.** Expression and activity of PC-PLC in cell lysates of nontumoral (OSE, IOSE, hTERT) and EOC cell lines at early confluence. Western blotting of total cell lysates (A) and membrane fractions (B). β-Actin and β1 integrin were used as loading control for densitometric analyses in A and B, respectively. Representative sets of experiments (of three (A) or two (B) performed) are reported. PC-PLC activity (C and D) was measured from the relative increase in Resorufin fluorescence in Amplex Red assay in cancer cells compared with IOSE and hTERT cells. C, relative increase in PC-PLC activity in whole-cell lysates (mean ± SD of five experiments; P < 0.0001 by ANOVA in EOC with respect to IOSE plus hTERT cell samples); D, relative increase in PC-PLC activity in PC-PLC activity in cell membrane preparations (mean ± SD of three experiments; P < 0.0002 by Student’s t test). The PC-PLC activity of ascitic cells from peritoneal exudates was measured in triplicate (variability ± 10%).
membrane, in a higher activity of this enzyme in total cell lysates, including membrane fragments and in an even higher activity in membrane-enriched fractions.

**PC-PLC localizes on EOC cells plasma membrane in nonraft domains.** The granular PC-PLC distribution pattern on plasma membrane of cancer cells suggested that the enzyme could localize in selected membrane domains. To investigate the nature of these domains, a sucrose gradient separation of lipid rafts was performed on OVCAR3 cell lysates (see Materials and Methods). The distribution of PC-PLC was monitored by Western blot and compared with that of nonraft (β1 integrin) and raft (FR and Cav-1) markers. Raft fractions were 18-fold concentrated to increase the sensitivity of PC-PLC detection. B–D, CLSM analyses (central optical sections) of unfixed cells double-stained with anti–PC-PLC antibodies (red) and mAb against β1 integrin (green). B, OVCAR3 cells; right, background labeling in absence of primary antibodies. C, ascitic cancer cells (from patient 4). D, hTERT cells. Colocalization in yellow. Nuclear staining by 4’,6-diamidino-2-phenylindole (blue). Scale bar, 20 μm.

**Figure 3.** PC-PLC localization in nonraft membrane domains and its colocalization with β1 integrin in epithelial ovarian cancer and immortalized cells. A, sucrose gradient separation of Triton X-100 lysate from OVCAR3 cells (see Materials and Methods). The distribution of PC-PLC in the recovered fractions (3–6 for rafts and 9–12 for nonrafts) and in total cell lysate (7) was monitored by Western blot analysis and compared with that of nonraft (β1 integrin) and raft (FR and Cav-1) markers. Raft fractions were 18-fold concentrated to increase the sensitivity of PC-PLC detection. B–D, CLSM analyses (central optical sections) of unfixed cells double-stained with anti–PC-PLC antibodies (red) and mAb against β1 integrin (green). B, OVCAR3 cells; right, background labeling in absence of primary antibodies. C, ascitic cancer cells (from patient 4). D, hTERT cells. Colocalization in yellow. Nuclear staining by 4’,6-diamidino-2-phenylindole (blue). Scale bar, 20 μm.

Overall, these results showed that PC-PLC localization on the plasma membrane of EOC cells occurs in discrete nonraft domains and suggest a spatial (and perhaps functional) relationship between this phospholipase and the β1 integrin adhesion protein.

**Changes of PC-PLC activity in OVCAR3 cells after serum deprivation and cell restimulation in the presence or absence of D609.** Amplex Red analyses showed that the mean value of PC-PLC activity decreased ~3-fold in OVCAR3 cells cultured for 48 h in FCS-deprived medium compared with control cells grown to subconfluence in complete medium (Fig. 4A). Under these conditions, the PC-PLC activity of EOC cells became comparable with that of EONT cells grown in complete medium. When serum-deprived cells were restimulated by FCS, the PC-PLC activity returned almost to the level measured in control cells within 1 h. Similar results were obtained when cell restimulation was induced by LPA alone [20 μmol/L in unsupplemented RPMI], a potent mitogen for EOCs (40). In fact, 1-hour LPA stimulation induced a 3.4 ± 1.1-fold increase in PC-PLC activity of serum-deprived cells.
However, when cell restimulation occurred in the presence of the PC-PLC inhibitor D609 (50 μg/mL), the PC-PLC activity in FCS-stimulated cells maintained, for at least 24 h, values comparable with those of serum-deprived cells (Fig. 4A). Cell incubation with D609 similarly prevented the recovery of PC-PLC activity in LPA-stimulated cells. Parallel Amplex Red PLD assays showed that the activity of this enzyme was not significantly altered by D609 in OVCAR3 cells under any of the adopted conditions of serum deprivation or serum resupplementation (Fig. 4B). It is also worth noting that the percentage of apoptotic cells, measured by flow cytometry on propidium iodide-stained EOC cells, was below 3% both in the presence and absence of D609.

Experiments performed on hTERT cells showed that PC-PLC activity remained practically unaltered (within ±10%) in these cells after 48 hours of serum deprivation and during subsequent FCS restimulation, irrespectively of cell exposure to D609 (data not shown). These results suggest the existence of a fine regulation mechanism controlling PC-PLC activity in ovarian cancer (but not in nontumoral, immortalized epithelial ovarian cells) in response to exogenous growth factors.

**Effect of D609 on the S-phase fraction of OVCAR3 and hTERT cells.** To investigate possible relationships between PC-PLC activity and cell proliferation in EOC and EONT cells, dual flow cytometry analyses were performed on OVCAR3 and hTERT cells stained with BrdUrd and propidium iodide at the end of the serum deprivation period and after cell restimulation by serum (FCS, for 8 or 24 h) either in the presence or absence of D609. Cell cycle measurements in these samples were compared with those of control cells cultured in the presence of complete medium while maintained in the presence of D609.

The cell cycle distribution of hTERT cells showed that PC-PLC activity was equally inhibited by D609 for at least 24 hours (data not shown). However, different from the nontumoral, immortalized cell lines studied, the S-phase fraction of these nontumoral, immortalized epithelial ovarian cells was even higher than that of a highly aggressive carcinoma cell line, such as OVCAR3. With respect to the latter, serum deprivation of hTERT cells induced a smaller decrease in the S-phase fraction (by 24% at 56 h and 36% at 72 h). Similar results were obtained when cell restimulation was exerted by LPA alone, instead of FCS. In particular, the recovery of the S-phase fraction in either FCS-restimulated or LPA-restimulated cells was equally inhibited by D609 for at least 24 hours (data not shown).

No apoptosis was induced by D609 under any of the experimental conditions.

Cell cycle analyses performed under the same conditions on hTERT cells gave a G0-G1/S/G2M percentage distribution of 36 ± 4%: 45 ± 10%; 13 ± 4% in subconfluent control cells. These values indicated that the basal S-phase fraction of these nontumoral, immortalized cells was even higher than that of a highly aggressive carcinoma cell line, such as OVCAR3. With respect to the latter, serum deprivation of hTERT cells induced a smaller decrease in the S-phase fraction (by 24% at 56 h and 36% at 72 h). Similar to OVCAR3 cells, FCS restimulation restored in 8 h the original S-phase fraction in hTERT cells. However, different from the carcinoma cell line, D609 did not prevent, at any incubation time, in these nontumoral cells the FCS-induced process of S-phase recovery (Fig. 5, bottom).

These results altogether indicate that cell exposure to the PC-PLC inhibitor results in a long-lasting inhibition of serum growth factor–induced proliferation of cancer cells, but does not inhibit proliferation of nontumoral immortalized cells.

**Discussion**

In this study, we report accumulation of PC-PLC on the outer surface of the plasma membrane in EOC cell lines, as well as in EOC cells isolated from patient ascites compared with in vitro
cultured EONT cells. Cellular PC-PLC redistribution in EOC cells lines was consistent with immunohistochemical detection of PC-PLC in a relevant subset of archival EOC tissue samples. The ectopic PC-PLC localization in EOC cells was associated with highly significant enzyme activation with respect to EONT cells, corresponding to a PC-PLC activity increase of 3-fold and 5-fold in total EOC cell lysates and membrane-enriched fractions, respectively. The lower increase in enzyme activity measured in total cell lysates suggests that the enzyme undergoes a much lower (if any) activation in nonmembranous cytoplasmic compartments. Furthermore, by exposing cells to either FCS or LPA in the presence of the PC-PLC inhibitor D609, we provided the first direct indication that PC-PLC activity in EOC cells is likely involved in the control of cell proliferation, mainly affecting the S-phase fraction.

**PC-PLC externalization on the plasma membrane of EOC cells.** The massive localization of the enzyme on the membrane surface of EOC (but not EONT) cells is in general agreement with similar findings reported on ras-transformed and mitogen-stimulated fibroblasts (15, 16), as well as on cytokine-activated natural killer (NK) cells (25–27). Among signaling-activated PC-specific phospholipases (41, 42) which are also known to translocate to the plasma membrane, PC-PLC presents the peculiar feature of accumulating on the outer, instead of the inner, membrane surface of mammalian cells. Furthermore, different from PC-PLD, whose localization is mainly restricted to caveolin-enriched microdomains (43), our study shows that, in EOC cells, PC-PLC is localized in nonraft domains in close proximity with β1 integrin.

Further investigations are needed to elucidate whether protein phosphorylation and/or interaction with specific cytoskeleton components are involved in PC-PLC activation and accumulation on the plasma membrane under conditions of constitutive or agonist-mediated cell stimulation of EOC cells. Indirect evidence on PC-PLC phosphorylation is inferred from a previous study (19), in which nerve growth factor–induced increase of an antiphosphotyrosine immunoreactive 66-kDa band in PC12 cells has been reported to be inhibited by cell exposure to D609. Regarding the possible involvement of cytoskeleton components in PC-PLC translocation to the membrane, we previously reported a kinesin-supported PC-PLC transport from the microtubule organizing center to the membrane and PC-PLC interaction with filamentous actin in interleukin 2–activated, but not in resting NK cells (25).

![Figure 5. S-phase fraction in OVCAR3 and hTERT cells after serum deprivation and subsequent restimulation in complete medium in the presence or absence of D609.](image-url)
Regarding functional aspects, externalization of PC-PLC on the cell membrane may confer to this activated enzyme the capability of participating in membrane receptor-mediated cell signaling, by producing diacyl glycerol (DG) second messengers from hydrolysis of PC, the major phospholipid component of the outer layer of eukaryotic cells. In fact, a persistent production of PC-derived DGs is reputed to contribute to long-lasting (PKC-mediated or PKC-independent) signaling events ending with gene transcription and DNA synthesis (43, 44). In this context, it is interesting to note that even exogenously added bacterial PC-PLC has been shown to elicit a potent mitogenic response in fibroblasts by promoting DNA synthesis and cell proliferation (45).

Furthermore, the ectopic localization of activated PC-PLC on cancer cell membrane may allow this enzyme to modulate the expression of some membrane receptors. In fact, we recently reported evidence on the crucial role of ectopically localized activated PC-PLC in selectively regulating the expression of CD16, the FcγR receptor on the membrane of human NK cells, with striking effects on CD16-mediated cytotoxicity (27). The changes reported herein of PC-PLC and β1 integrin colocalization of the membrane of serum-deprived and EGF-restimulated OVCAR3 cells (Supplementary Fig. S3) may point to a similar role of PC-PLC in regulating the expression and signaling of this adhesion protein in EOC cells. A previous report on the mutual relationships between PC-PLC and β1 integrin in regulating cell-substratum adhesion in vascular endothelial cells (46) seems to reinforce this hypothesis. Studies are in progress to further investigate and quantify possible interactions of PC-PLC with this adhesion molecule under different experimental conditions.

Relationships between PC-PLC activity and S-phase fraction in EOC cells. Serum deprivation caused a 3-fold decrease in the PC-PLC activity of OVCAR3 cells, inducing a drop in the activity of this enzyme down to the level steadily measured in EONT cells. PC-PLC down-regulation was associated in EOC cells with a 40% decrease in the S-phase fraction of serum-deprived OVCAR3 cells. PC-PLC reactivation in FCS-stimulated or LPA-stimulated OVCAR3 cells preceded a full recovery of the S-phase fraction to the value measured before serum deprivation. Similarly, although quantitatively different, changes in PC-PLC activity (5-fold decrease) and S-phase fraction (~100% drop) were reported in serum-deprived fibroblasts, where subsequent cell restimulation by platelet-derived growth factor (PDGF) promptly restored PC-PLC activity and the original proliferative status (16). The higher residual percentage of PC-PLC activity maintained in serum-deprived EOC cells compared with the residual PC-PLC activity reported in serum-deprived normal fibroblasts may be attributed to the well-known reduced dependence of cancer cells upon exogenous growth factors for their survival and proliferation (47). The long-lasting (for at least 24 h) inhibition on the recovery of PC-PLC activity induced by D609 in serum-restimulated or LPA-restimulated OVCAR3 cells is in general agreement with a similar effect observed in PDGF-stimulated fibroblasts (16), suggesting that altered PC-PLC activity and changes in the S-phase fraction may both be linked to common mitogen-dependent pathways. Further investigations are required to elucidate the nature of the underlying molecular mechanisms.

The present study also shows that the suggested relationships between PC-PLC activity and S-phase fraction in OVCAR3 do not hold true for the nontumoral immortalized hTERT cells, in which subcellular PC-PLC localization was mainly confined to inner cellular compartments. In spite of their higher S-phase fraction at subconfluence (probably due to telomerase transfection), these cells maintained a 3-fold lower PC-PLC activity compared with OVCAR3 cells and no substantial change was observed in PC-PLC activity in serum-deprived and FCS-stimulated cells, either in the presence or in the absence of D609. Noteworthy, only scanty alterations were observed in the S-phase fraction of hTERT cells exposed to D609.

Further studies are needed to elucidate whether other enzymes, besides PC-PLC, may also be directly or indirectly involved in D609-induced effects on EOC cell proliferation. In fact, it has been shown that PC-PLC inhibition by D609 may also interfere with cell signaling, by blocking, in some cell systems, mitogen-induced protein kinase activation (48, 49) or by inhibiting other enzymes, such as sphingomyelin synthase (50).

In overall, the results reported in the present work support the view that PC-PLC is directly involved in the complex machinery of pathways responsible for EOC cell proliferation. Further evidence on these mechanisms is expected from PC-PLC sequencing and cloning, which would allow a better elucidation of the role of this enzyme in the multiple pathways controlling the development of aberrant phenotypes typical of these (and probably other) malignant cells, together with the possible identification of novel targets for selective antitumor therapies. At the present stage, the different involvement of PC-PLC in EONT and EOC cell proliferation suggests that the use of a PC-PLC inhibitor (possibly one even more effective and specific than D609) might provide a powerful method to selectively interfere, likely in combination with antitumor drugs, with some of the aberrant pathways responsible for EOC proliferation and invasiveness.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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