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Phospholipase C-β2 promotes mitosis and migration of human breast cancer-derived cells

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Like most human neoplasm, breast cancer has aberrations in signal transduction elements that can lead to increased proliferative potential, apoptosis inhibition, tissue invasion and metastasis. Due to the high heterogeneity of this tumor, currently, no markers are clearly associated with the insurgence of breast cancer, as well as with its progression from *in situ* lesion to invasive carcinoma. We have recently demonstrated an altered expression of the β2 isoform of the phosphoinositide-dependent phospholipase C (PLC) in invasive breast tumors with different histopathological features. In primary breast tumor cells, elevated amounts of this protein are closely correlated with a poor prognosis of patients with mammary carcinoma, suggesting that PLC-β2 may be involved in the development and worsening of the malignant phenotype. Here we demonstrate that PLC-β2 may improve some malignant characteristics of tumor cells, like motility and invasion capability, but it fails to induce tumorigenesis in non-transformed breast-derived cells. We also report that, compared with the G_0/G_1 phases of the cell cycle, the cells in $S/G_2/M$ phases show high PLC-β2 expressions that reach the greatest levels during the late mitotic stages. In addition, even if unable to modify the proliferation rate and the expression of cell cycle-related enzymes of malignant cells, PLC-β2 may promote the G_2/M progression, a critical event in cancer evolution. Since phosphoinositides, substrates of PLC, are involved in regulating cytoskeleton architecture, PLC-β2 in breast tumor cells may mediate the modification of cell shape that characterizes cell division, motility and invasion. On the basis of these data, PLC-β2 may constitute a molecular marker of breast tumor cells able to monitor the progression to invasive cancers and a target for novel therapeutic breast cancer strategies.

Introduction

Phosphoinositide-specific phospholipase C (PLC) is one of the key enzymes in the metabolism of inositol lipids. It plays a crucial role in multiple transmembrane signal transduction pathways that regulate numerous cell processes, including proliferation and motility (1). At present, 13 mammalian PLC isoforms have been reported and characterized, divided in the five subfamilies β, γ, δ, ϵ and ζ. They differ in structure and activation mechanism and show expression patterns that reflect the function identified for each isozyme in the diverse tissues (1–3).

The different PLC isozymes show discrete localization in both cytoplasm and nuclei of a number of cell types, in which they regulate events dependent on the inositol lipid cycle. In particular, the modifications of the phosphoinositide pool, as a consequence of PLC activity, have been reported to be involved in actin cytoskeleton reorganization, chromatin remodeling and gene transcription (4,5).

Altered expression and activity of specific PLC isoforms are commonly detected in a variety of different human cancers and are often associated with the appearance and progression of tumoral phenotypes. For instance, β isozymes are frequently associated to hematopoietic malignancies and neuroendocrine tumors (6–9). Members of the γ subfamily have been found to be up-regulated in neoplastic leukocytes (10) and in colon carcinoma, where they may play a role in carcinogenesis (11). In addition, PLC-δ isozymes have been found to be involved in the neoplastic evolution of gastric mucosa and hepatic cells (12,13).

While a role in promoting cell cycle progression by targeting cyclin–cyclin kinase complexes has been reported for PLC-β1 (14), a general role of PLC in regulating tumorigenic features of cancer-derived cells has been coupled to its capability to modulate the local levels of phosphoinositides. A central role in this context seems to be played by the preferred PLC substrate PtdIns $(4,5)P_2$ that, in addition to be a precursor of the second messengers inositol 1,4,5-trisphosphate and diacylglycerol, is a substrate for Phosphoinositide 3-kinase. PtdIns $(4,5)P_2$ may directly influence actin cytoskeleton organization, which is deregulated in a number of human tumors (15).

Evidence from different groups indicate that in breast cancer, like in most human neoplasms, some PLC isoforms show altered expressions, even if their involvement in neoplastic transformation is currently poorly understood. For instance, a number of data report that PLC-γ1 is over-expressed in breast cancer cells, in which it has a role in EGFR-driven tumor progression and cell migration (16–18). Also PLC-δ4 was found to be up-regulated in some breast tumors and its over-expression seems to increase the proliferation rate of low-tumorigenic breast cancer cells (19). Recently, we have demonstrated that PLC-β2 is over-expressed in a large majority of breast tumors and that its amount correlates with a poor clinical outcome, constituting a molecular marker of breast cancer severity (20).

On the basis of our previous data, the aim of this work was to establish if PLC-β2 is directly involved in the carcinogenic steps of promotion and progression of human mammary neoplasia. In this study, we demonstrate that, even though PLC-β2 fails to induce tumorigenesis in non-transformed breast-derived cells, it has a major role in promoting migration. We also provide evidence that PLC-β2 induces transition from G_0/G_1 to $S/G_2/M$ phases of the cell cycle, which appears to be a critical event in cancer progression, without affecting cell cycle-associated enzymes. PLC-β2, by modifying the phosphoinositide pool, may then be responsible for inositol lipid-related modifications of cytoskeleton architecture that occur during division, motility and invasion of tumor cells.

Materials and methods

Immunohistochemical analysis of breast tissues

Formalin-fixed and paraffin-embedded samples from normal breast and primary infiltrating breast cancers with different histological grade, estimated by using Elston and Ellis criteria (21), were obtained from the Department of Experimental and Diagnostic Medicine of University of Ferrara (Ferrara, Italy), after informed consent according to the Helsinki declaration of 1975. All patients were treated with surgery for primary breast cancer and none of them received neoadjuvant treatment or had distant metastasis at the time of primary surgery.

For analysis of PLC-β2 expression, tissue sections were deparaffinized by means of the clearing agent Bioclear (Bio-Optica, Milan, Italy), rehydrated in distilled water through graded alcohols (ethanol 100, 95 and 70%, respectively) and subjected to immunohistochemical analysis with an anti-PLC-β2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) using the Ultraystain Polyvalent-HRP immunostaining kit (YLEM, Rome, Italy). In particular, the

Abbreviations: MFI, mean fluorescent intensity; PBS, phosphate-buffered saline; PI, propidium iodide; PLC, phospholipase C.

slides were incubated in 3% (vol/vols) H_2O_2 and the endogenous peroxidase was blocked with Super Block reagent (YLEM). The samples were then incubated at room temperature for 90 min with anti-PLC-β2 rabbit polyclonal IgG (1:100 in 10 mM Tris–HCl, pH 8.0, 150 mM NaCl). The slides were further incubated for 10 min at room temperature with anti-polyvalent biotinylated antibody and, after rinsing in the same buffer, streptavidin–horseradish peroxidase was applied for 10 min. The antibody localization was detected by the addition of substrate/chromogen mix (AEC Cromogeno Kit, YLEM) and the sections were finally counterstained with Mayer's hemallume solution and rinsed for ∼10 min in tap water. Negative controls were obtained by omitting the primary antibody. Each tissue sample, after hybridization with the anti-PLC-β2 antibody, was analyzed using an optical microscope (Carl Zeiss Axiophot 100, Oberlochen, Germany).

Breast-derived cell lines

The breast cancer-derived cell lines MDA-MB-231 and MCF7 and the non-transformed MCF10A cells were purchased from the American Type Culture Collection (Rockville, MD). The low-tumorigenic BT-474 cell line was from ICLC (Genova, Italy). MDA-MB-231 and MCF7 were grown in Dulbecco's modified Eagle's medium (Gibco-BRL, Grand Island, NY) supplemented with 10% fetal calf serum (Gibco-BRL). MCF10A cells were cultured in Dulbecco's modified Eagle's medium and Ham's F-12 medium (1:1, vol/vols) containing 10 μg/ml bovine insulin, 100 ng/ml cholera toxin, 0.5 μg/ml hydrocortisone, 20 ng/ml recombinant human epidermal growth factor and 10% horse serum. BT-474 were maintained in RPMI 1640 growth medium (Gibco-BRL) supplemented with 10% fetal calf serum, 1 mM Na pyruvate and 0.01 mg/ml bovine insulin in 5% CO_2 atmosphere. Subconfluent cells were counted daily and cell morphology was evaluated using an inverted phase-contrast microscope (Nikon, Melville, NY).

Serum-starved cells were obtained by culturing the different cell lines for 48 h in the presence of 1% fetal calf serum.

Immunochemical analysis

Cells were harvested when 70–80% confluent, washed twice with cold phosphate-buffered saline (PBS) containing 1mM Na_3VO_4 and lysed with a buffer containing 50 mM Tris–HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM ethylenglycol-bis (β-aminoethylethen)-N, N′-tetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml each Aprotinin and Leupeptin and 1 mM Na_3VO_4 (all from Calbiochem, La Jolla, CA).

For immunoprecipitation experiments, after 1 h at 4°C, the insoluble materials were removed and supernatant (1 mg protein) was incubated with a polyclonal antibody against human PLC-β2, followed by precipitation with protein A-Sepharose, as previously reported (20).

Total lysates (50 μg protein) and immunoprecipitates were resuspended in Laemmli's sample buffer, separated on 7.5% polyacrylamide denaturing gels and blotted to nitrocellulose membranes (Amersham Life Science, Little Chalfont, UK). The membranes were then incubated with antibodies directed against PLC-β2 (Santa Cruz) and against cyclin A, cyclin D1, cyclin D3, CDK4, actin and β-tubulin (Sigma Chemicals, St Louis, MO). The final detection was performed using the Enhanced Chimiluminescence system (Perkin-Elmer, Boston, MA), according to the manufacturer's instructions and densitometric analysis of autoradiograms was performed on the ImageQuant TL (Amersham Bioscience, Little Chalfont, UK).

RNA interference assay

Specific siRNAs were designed by selecting four target sequences in the coding region of PLC-β2 mRNA (accession number NM 004573). Sense and antisense sequences were chemically synthesized by Qiagen S.p.A. (Milan, Italy). The lyophilized siRNA was dissolved in sterile suspension buffer (100 mM potassium acetate, 2 mM magnesium acetate, 30 mM Hepes-KOH, pH 7.4) to obtain a 20 μM solution, and then nucleotides were heated to 90°C for 1 min followed by 1 h at 37°C. As a control, non-silencing fluorescein-labeled duplex siRNA, also purchased from Qiagen, was used.

Transfection was conducted with Lipofectamine 2000 (Invitrogen, Milan, Italy) in six-well plates according to the manufacter's specification. In summary, the day before transfection, cells were trypsinized, counted and seeded at 5×10^5 cells/well into six-well plates, so that they were 40–45% confluence on the day of transfection. Lipofectamine 2000, diluted in Opti-MEM I medium (Gibco-BRL), was supplemented to the RNA mixture and 100 μl of the mixture were then added to each well. The transfected cells were incubated at 37°C in a CO_2 atmosphere for 24–48 h and then subjected to immunochemical analysis.

PLC-β2 over-expression

All cell lines were transfected with plasmids expressing an Enhanced Green Fluorescent Protein (EGFP)-tagged full-length human PLC-β2. DNA constructs were obtained by subcloning the specific fragments into expression vectors (Pharmacia Biotech, Uppsala, Sweden) in the correct reading frame, amplifying plasmidic DNA in competent bacteria DH5α (Invitrogen) and extracting DNA by means of a specific purification kit (Qiagen Plasmid Maxi Kit).

DNA was supplemented to Lipofectamine 2000 (Invitrogen), diluted in Opti-MEM I medium and the mixture was then added to each well of the six-well plate containing 9×10^5 cells/well (90% confluence). The transfected cells were incubated for 24–48 h at 37°C in a humidified 5% CO_2 atmosphere and then subjected to immunochemical analysis.

Proliferation assay

The analysis of cell proliferation was carried out by performing an MTT (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide) colorimetric assay (Roche, Penzberg, Germany), according to the manufacturer instructions. Breast-derived cells (2×10^3) were seeded into microplates (tissue culture grade, 96 wells, flat bottom), in a final volume of 100 μl culture medium per well, for overnight incubation at 37°C and 5% CO_2. Ten microliters of the MTT labeling reagent (final concentration 0.5 mg/ml) were then added to each well and the microplates were further incubated for 4 h. The MTT solution was than removed and 100 μl of solubilization solution were added to each well to dissolve formazan crystals. After an overnight incubation (37°C and 5% CO_2), the spectrophotometrical absorbance of the samples was read with an Anthos 2010 ELISA reader operating at 540 nm (Anthos Labtec Instruments, Wals Salzburg, Austria).

Chemomigration assay

Cell migration was determined using a 24-transwell migration chamber assay (Costar, Boddenheim, Germany) equipped with 6.5 mm diameter, 8 μm pore size polycarbonate inserts. Cell migration was quantified by the number of cells that were migrated directionally through collagen IV (40 μg/ml)-coated inserts toward serum as a chemoattractant. Cells (0.5×10^5) were resuspended in 100 μl of serum-free medium containing 0.1% bovine serum albumin and added to the transwell inserts. In all, 600 μl of medium were added to the lower compartments of the chambers. After 24 h of incubation, cells attached to the bottom surface of the membrane were fixed and stained with crystal violet. Non-migrated cells were then removed by wiping with a cotton swab and at least four random fields of vision were counted for quantification of cell migration using a light microscope (Carl Zeiss Axiophot 100).

In vitro cell invasion assay

Cell invasion assay was performed according to the protocol from Chemicon (Tamecula, CA). The Cell Invasion Assay Kit (ECM550) utilizes an Invasion Chamber, which consists of a 24-well tissue culture plate and 12 cell culture inserts. The inserts contain an 8 μm pore size polycarbonate membrane, over which a thin layer of ECMatrix™ solution is applied (ECM layer occludes the membrane pores, blocking non-invasive cells from migrating through; in contrast, invasive cells migrate through the ECM layer). Cells (0.5×10^5) in 300 μl of serum-free media were added to each insert and 500 μl of media containing 10% fetal bovine serum (chemoattractant) was added to the lower chamber. Chambers were then incubated in 5% CO_2 and at 37°C for 24–72 h. Non-invading cells as well as the ECM gel layer were removed using a cotton-tipped swab. On the contrary, the invasive cells on lower surface of the membrane were stained by dipping inserts in a staining solution for 20 min; then counted after observation through a light microscope.

Immunocytochemical analysis

The different breast-derived cell lines, grown onto glass slides, were fixed with freshly prepared 4% paraformaldehyde (10 min at room temperature), washed in PBS (5 min) and reacted with the polyclonal anti-PLC-β2 antibody in NET gel (150 mM NaCl, 5 mM ethylenediaminetetraacetic acid, 50 mM Tris–HCl, pH 7.4, 0.05% Nonidet P-40, 0.25% carragenin, 0.02% NaN_3), for 2 h at room temperature. Samples were then reacted with a secondary antibody (fluorescein isothiocyanate-conjugated anti-rabbit IgG; Sigma Chemicals) in NET gel for 45 min at room temperature. After two washes with NET gel and PBS, samples were incubated (from 30 s to 5 min) with 0.5 μg/ml 4′,6-diamidino-2-phenylindole, then washed in PBS, dried with ethanol, mounted in glycerol containing 1,4-diazabicyclo [2.2.2] octane to retard fading and analyzed with a fluorescence microscope.

The same experiment was performed in the presence of an anti-PLC-β2 blocking peptide (Santa Cruz) to verify the specificity of the anti-PLC-β2 antibody.

Cytofluorimetric analysis of PLC-β2 expression in the different phases of cell cycle

For detection of intracellular PLC-β2, 5×10^5 cells were fixed with 2% paraformaldehyde for 5 min and then reacted for 30 min with 2 μg/ml anti-PLC-β2 antibody in NET gel, followed by 30 min with 15 μg/ml fluorescein isothiocyanate-conjugated anti-rabbit IgG (Jackson Immunoresearch Laboratories, West Grove, PA) as secondary antibody.

Samples were then washed and incubated with 25 μg/ml propidium iodide (PI) and 0.1 mg/ml RNase (Sigma) in PBS for 30 min. Secondary antibody-matched controls were used to assess non-specific fluorescence, and after staining procedures, samples were analyzed with a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Analysis of PI fluorescence was performed with FL-2 detector in a linear mode. Finally, data were analyzed using CELLQuest 3.2.1.f1 software (Becton Dickinson). Mean fluorescence intensity (MFI) ratio was calculated as the ratio between the MFI of PLC-β2-stained and MFI of control-stained cells (secondary antibody alone). The results were evaluated by using analysis of variance with subsequent comparisons by Student's *t*-test and with the Mann–Whitney rank-sum test (22).

Cell cycle analysis

The number of cells in each phase of the cell cycle was evaluated by means of flow cytometry after PI staining of ethanol-fixed cells. After a wash in PBS, 5×10^5 cells were fixed in 500 μl of cold 70% ethanol at 4°C for at least 2 h. The cells were then centrifuged, washed once in PBS, resuspended in 500 μl PBS and incubated in the dark at room temperature for 30 min in the presence of 100 μg/ml RNAse and 20 μg/ml PI (Sigma Chemicals). The PI fluorescence of individual nuclei was measured using a FACScan (Becton-Dickinson, San Josè, CA). The proportions of cells in the G_0/G_1, S and G_2/M phases of the cell cycle were automatically calculated by the Lysis II analysis software (Becton-Dickinson).

Statistical analysis

The results were expressed as means ± standard deviations of three or more experiments performed in duplicate. Statistical analysis was performed by using the two-tailed Student's *t*-test for unpaired data.

Results

PLC-β2 expression positively correlates with malignant features of breast cells

We have previously demonstrated that PLC-β2 is weakly expressed in healthy mammary tissue and more abundant in tumor samples (20). In particular, the staining intensity increase in parallel with tumorigenic features, defined as final 'grade', of cancer samples, as summarized in the Table I.

Figure 1A, illustrating an immunohistochemical analysis performed with an anti-PLC-β2 antibody on normal breast tissues and breast tumors with different tumorigenic characteristics, demonstrates that grade 1 tumors, showing a partially differentiated phenotype, react poorly, whereas the highly undifferentiated grade 3 tumors express the largest amount of the enzyme.

Immunochemical analysis with an anti-PLC-β2 antibody was performed on the non-transformed breast-derived cell line (MCF10A) and on three breast cancer-derived cell lines (MCF7, BT-474 and MDA-MB-231) with different tumoral features. As reported in Figure 1B, a low amount of PLC-β2 is present in immortalized non-transformed, highly proliferating MCF10A cells. On the other hand, cancer-derived cells express this PLC isozyme at levels that positively correlate with their tumoral characteristics. In fact, small amounts of PLC-β2 were found in the low-tumorigenic MCF7 and BT-474 cell lines, which show modest proliferation rate and invasiveness, compared with the elevated amount of protein expressed by the highly tumorigenic MDA-MB-231 cells, characterized by a faster growth and an elevated invasive potential.

The correspondence between levels of PLC-β2 and tumorigenic potential in both tissues and breast-derived cells corroborates the use of the selected cell lines for this study.

Table I. Expression of PLC-β2 in breast tissue positively correlates with malignancy of invasive breast cancers

Features	Negative (%)	Weak staining (%)	Moderate staining (%)	Strong staining (%)	*P*
Normal tissues	20.0	80.0	0.0	0.0	
Breast tumors					<0.001
Final grade 1	18.2	54.5	9.1	18.2	
Final grade 2	0.0	16.1	71.0	12.9	
Final grade 3	0.0	0.0	17.4	82.6	

Tissue microarrays containing 10 samples from normal breast tissues and 77 from invasive breast cancers were subjected to immunohistochemical analysis with an anti-PLC-β2 antibody and the staining intensity was estimated on a four-step scale by means of Adobe Photoshop analysis of acquired images. Tumors were categorized according to arbitrarily defined criteria into four groups, including completely negative (no staining at all), weak (staining intensity ≤ 100 000 pixel), moderate (staining >100 000, ≤ 200 000 pixel) and strong (staining >200 000 pixel). The staining corresponding to protein levels was then correlated with final grade of the different tumors (20).

PLC-β2 promotes migration of breast cancer-derived cell lines

Since, as we have reported above, the amount of PLC-β2 in breast-derived cells correlates with their malignancy, we have investigated if this protein is involved in promoting and/or modifying some tumor-related events. For this purpose, cell proliferation and metastatic potential were investigated in all breast-derived cell lines in which PLC-β2 expression was negatively modulated by silencing its mRNA (Figure 2A) or increased by transient transfection with a plasmid containing the full-length cDNA of human PLC-β2 linked with the EGFP-cDNA (Figure 2B). As reported in Figure 2C, neither down-modulation nor over-expression of PLC-β2 significantly affected the proliferation rate of both non-transformed and tumoral cell lines. On the other hand, negative and positive modulation of PLC-β2 expression did not influence the very low motility of MCF10A and BT-474 cells, but induced significant modifications of the migration capability of cell lines displaying an intrinsic potential to migrate toward a chemoattractant (Figure 2D). In particular, the reduced expression of PLC-β2 resulted in a significant inhibition of MDA-MB-231 cell migration, whereas the over-expression of the protein increased the motility of both MCF7 and MDA-MB-231 cell lines, particularly of the highly tumorigenic cells (Figure 2D).

We have then evaluated if modulation of PLC-β2 expression may influence the invasion capability since cell movement is a prerequisite for cancer invasion and metastasis. As shown in Figure 2E, both down-modulation and over-expression of the protein did not modify the ability of the non-tumorigenic MCF10A and of the low-tumorigenic BT-474 to invade through Extracellular Matrix-coated porous filters in response to a chemochinetic stimulus. On the contrary, the over-expression of PLC-β2 did not induce changes of the invasive potential of MCF7 and MDA-MB-231 cells, whereas down-modulation of PLC-β2 induces a large decrease in the invasiveness of both malignant cell lines, particularly evident in the highly tumorigenic cells.

The amount of PLC-β2 increases during progression of cells along the S and G_2/M phases of the cell cycle

Immunocytochemical analysis with an anti-PLC-β2 antibody was performed on glass slide growing cells. The results, shown in Figure 3A, confirmed the immunochemical data, since cell staining intensity, indicating the protein amount, was progressively higher starting from the non-transformed MCF10A to the highly invasive MDA-MB-231 cells. In all cell lines, the highest levels of PLC-β2 were found in the late phases of the mitotic division. The specificity of the anti- PLC-β2 antibody was demonstrated by performing immunocytochemical analysis in the presence of the specific blocking peptide (Figure 3B).

To determine the amount of PLC-β2 in the different phases of the cell cycle, a bi-parametrical analysis of PLC-β2 expression in cells with different DNA content was performed. The results, reported in Figure 4A, show a significant increase of PLC-β2 expression during the progression of cells from G_0/G_1 to S and G_2/M phases of the cell cycle, particularly evident in the more tumorigenic MCF7 and MDA-MB-231 cells.

As reported in Figure 4B, the cytofluorimetric determination of PLC-β2 on the whole-cell population confirmed the immunochemical results, showing that the highly invasive MDA-MB-231 contains the largest amount of this protein. The same analysis performed on cells grown on a serum-deprived mediun confirms the correlation between PLC-β2 expression and specific phases of cell cycle. In particular, serum-starved cells that accumulate in the G_0/G_1 phases show

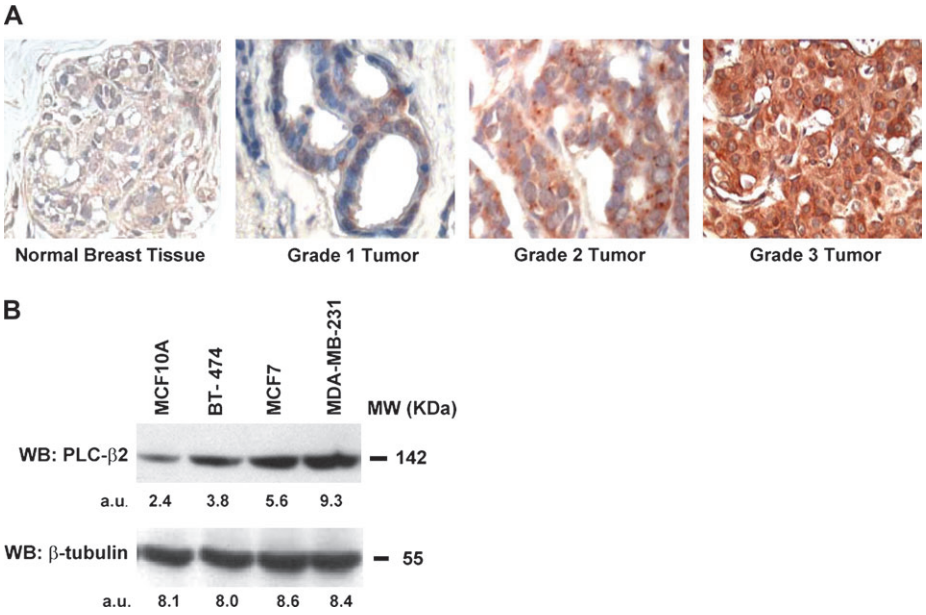


Fig. 1. PLC-β2 expression correlates with malignancy of breast cancer cells. (**A**) Immunohistochemical analysis performed with an anti-PLC-β2 antibody on deparaffinized tissue sections of human healthy and tumoral breast tissues with different histopatological final grade. Magnification: 40×. (**B**) Western Blot analysis performed with the indicated antibodies on total lysates from different breast-derived cell lines. Densitometric analysis of autoradiograms was performed and the results were reported as arbitrary units (a.u.). The data are representative of three separate experiments, performed in duplicate.

a significantly reduced expression of PLC-β2 in comparison with whole-serum growing cells (Figure 4B).

PLC-β2 induces accumulation of the cells in the G_2/M phases of the cell cycle without modifying cell cycle-related enzymes

On the basis of the above reported findings, suggesting that an increased expression of PLC-β2 takes place during DNA synthesis and cell mitosis, a further set of experiments was aimed at assessing whether the modulation of PLC-β2 expression is capable of inducing changes in cell cycle profile.

As shown in Figure 5A, no significant modifications of the number of the MCF10A, BT-474 and MCF7 cells in the different phases of cell cycle were observed when PLC-β2 expression was inhibited. On the contrary, the down-modulation of PLC-β2 induced a significant accumulation of the highly invasive MDA-MB-231 cells in the G_0/G_1 phases and a strong reduction of the same cell population in the G_2/M phases.

The over-expression of PLC-β2 induced, in MCF7 and MDA-MB-231, a significant decrease of the number of cells in G_0/G_1 and an increase of cells in G_2/M phases, whereas no effects on the number of cells located in the different phases of the cell cycle were observed in MCF10A and BT-474 cell lines over-expressing the enzyme (Figure 5B).

We have also evaluated the effects of a modulated expression of PLC-β2 on the number of apoptotic cells by means of cytofluorimetric analysis. As reported in Figure 5A and B, neither down-modulation nor over-expression of PLC-β2 significantly affected apoptosis, in all breast-derived cell lines.

To understand the role of PLC-β2 in regulating cell cycle machinery, we then analyzed the expression of proteins that, in breast tumor cells, are critical for the transition between the different phases of cell cycle. In particular, immunochemical analysis with antibodies directed against cyclin A, cyclin D1, cyclin D3 and CDK4 were performed on growing non-transformed and tumoral breast-derived cell lines in which PLC-β2 expression was modulated. As reported in Figure 5C, with the exception of the non-transformed MCF10A that does not express cyclin D1, all examined cell cycle-related proteins are expressed in breast tumor-derived cells, without significant modifications due to the over-expression or down-modulation of PLC-β2.

Since cytoskeleton is involved in both migration/invasiveness and progression along cell cycle, the association between PLC-β2 and actin was analyzed in tumor-derived cells. As reported in Figure 6, the presence of actin in the anti-PLC-β2 immunoprecipitates was particularly abundant in the more tumorigenic MCF7 and MDA-MB-231.

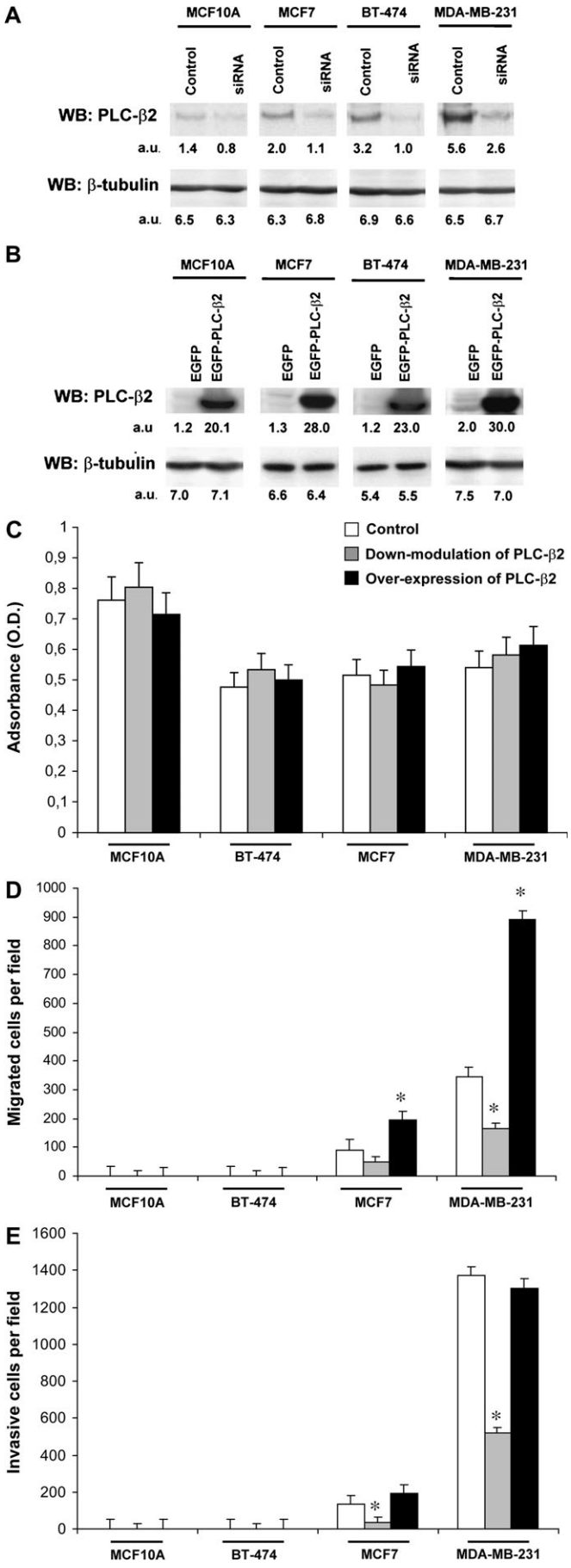
Discussion

Most human malignancies have aberrant expression of signal transduction elements that induce a deregulation of critical cellular processes like proliferation, differentiation, survival and motility (23). PLC, a rate-limiting enzyme in the generation of second messengers in response to a large variety of growth factors and mitogens, is involved in tumorigenic features of a wide variety of tissues (6–12).

In breast cancer, the role of the different PLC isozymes in neoplastic transformation is poorly understood. So far, high levels of PLC-γ1 has been found in breast tumor cells in comparison with normal mammary tissues, and the over-expression of this PLC isozyme has been related to the epidermal growth factor-driven tumor cell migration and invasiveness (18,24). Also PLC-δ4 has been found to be up-regulated in a relatively low percentage of breast tumors and its forced expression in low-tumorigenic breast cancer-derived cells has been observed to enhance their proliferation rate (19).

By means of immunohistochemical analysis on tissue microarrays composed of breast cancer specimens and normal epithelia, we have recently demonstrated that PLC-β2, poorly expressed in normal tissues, is up-regulated in tumor cells (20). We have found that the expression of PLC-β2 correlates strikingly with the aggressive behavior of primary tumors by analyzing the relationship between PLC-β2 levels and biological and clinicopathological factors. In particular, a shorter relapse-free interval time and an lower overall survival have been demonstrated in patients whose primary tumors expressed high levels of PLC-β2, compared with patients whose breast carcinoma expressed modest levels of the protein (20).

On the basis of the above-mentioned findings, indicating a strong correlation between PLC-β2 levels and worse prognosis of breast cancer, we have tried to establish whether this PLC isozyme plays a role in promoting and/or modifying the malignancy of breast cancer



cells. With this aim, we have positively and negatively modulated the expression of PLC-β2 in breast-derived cell lines, and the most reliable tumoral characteristic were analyzed. As experimental models, we have used a breast-derived non-transformed cell line expressing, as the normal mammary tissue, low levels of the enzyme and three different breast cancer-derived immortalized cells, in which this enzyme is expressed at levels that positively correlate, as in tumor tissues, with their tumor-related de-differentiated phenotype.

The disturbance in signal transduction pathways often results in failure of growth control. Since this may cause hyperproliferation of cells and ultimately cancer, we have first analyzed the role of PLC-β2 in modulating the proliferation rate of the different cell lines. Our results failed to show any effects of modulated PLC-β2 expression on proliferation of both non-transformed and tumoral cell lines. Accordingly, the basal levels of the enzyme expressed by the different cell lines do not correlate with their proliferation rate. In particular, the non-transformed highly proliferating MCF10A cells possess a very low amount of PLC-β2 if compared with the breast tumor-derived BT-474, proliferating very slowly. On the other hand, the weakly proliferating BT-474 cells express PLC-β2 at levels significantly higher than the more rapidly growing MCF7 cells. These data indicate that PLC-β2 in breast cancer, at variance with other PLC isoforms, like PLC-γ1 and PLC-δ4, is not able to accelerate the cell cycle.

Breast cancer starts as a local *in situ* carcinoma, but it can progress into an invasive tumor and culminate in metastasis to the lymph nodes and distant organs with a frequency/localization that depends on the type of tumor. The ability of cancers to invade and metastasize requires the acquisition of a specific phenotype that enable the cells to escape from the localized site and reach an ectopic location in which they may find a favorable growth environment (25). Unfortunately, a number of recent data have revealed that the portrait of breast tumors remains stable during progression and that no major changes appear to explain why a tumor may evolve to the metastatic stage, and at present, no markers are clearly associated with the progression of mammary tumors from *in situ* to invasive (26).

Deregulation of cell motility stimulated by various extracellular factors seems to be a critical step in tumor progression. This has focused the attention on initiators of signaling cascades that regulate tumor cell migration. In this effort, PLC has been shown to function as a key molecular switch since the members of this family (both γ1 and γ2 isoforms) are involved in several aspects of motility regulation (27–29). In breast cancer, inhibition of both PLC-γ1 expression and activity resulted in loss of growth factor-induced cell motility and invasiveness (16,18).

Here we have demonstrated that a forced expression of PLC-β2 is not able to induce a migration capability in non-migrating cells, like the non-transformed MCF10A and the low-tumorigenic BT-474, but it is sufficient to strongly increase the motility of the more tumorigenic MCF7 and MDA-MB-231 cells. Accordingly, a reduced expression of the enzyme decreases the motility of the most tumorigenic cell lines, which express high basal levels of PLC-β2. No effects of a reduced PLC-β2 expression were found on the non-transformed and on the low-tumorigenic cells regarding the ability of PLC-β2 to modify the invasive potential of breast-derived cells, whereas the invasive potential shown by the highly tumorigenic cell lines was largely reduced.

Fig. 2. PLC-β2 is involved in migration and invasiveness of breast cancer-derived cell lines. Non-transformed and tumoral breast-derived cells were subjected to RNA interference assay (siRNA) (**A**) or were transiently transfected with an EGFP-tagged construct containing the full-length cDNA of PLC-β2 (EGFP-PLC-β2) (**B**). Cell lysates were then subjected to Western Blot analysis to evaluate PLC-β2 expression. β-tubulin content constitutes an indicator of the loaded proteins. Autoradiograms were analyzed and the densitometric results were expressed as arbitrary units (a.u.). After down-modulation and over-expression of PLC-β2, cell proliferation (**C**), migration (**D**) and invasive potential (**E**) of the different breast-derived cells were evaluated. Reported results are the means ± standard deviation of three evaluations. The asterisks indicate statistically significant results.

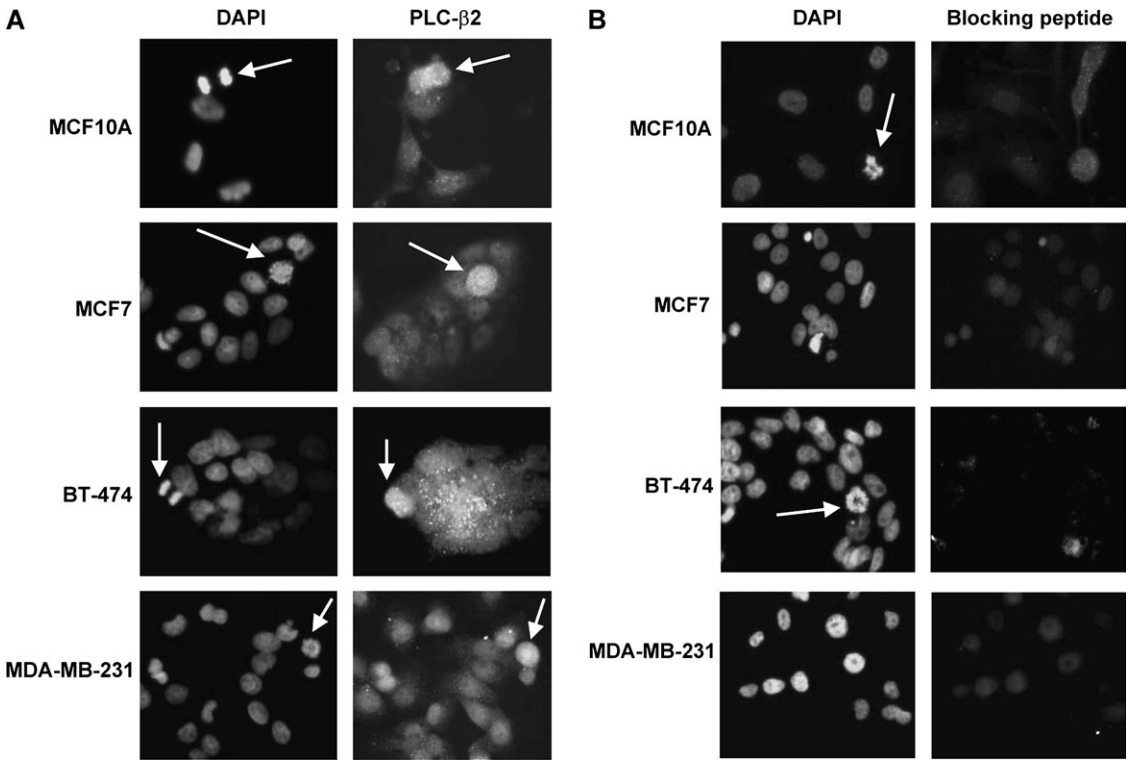


Fig. 3. PLC-β2 expression is highest in the late mitotic stages. Immunocytochemical analysis of PLC-β2 expression was performed on non-transformed and tumoral breast-derived cell lines (**A**). The specificity of the antibody was assayed by performing the same experiment in the presence of the blocking peptide (**B**). The nuclear morphology was detected by specific staining of DNA with 4′,6-diamidino-2-phenylindole (magnification 100×). The arrows indicate cells that are in the late mitotic stages.

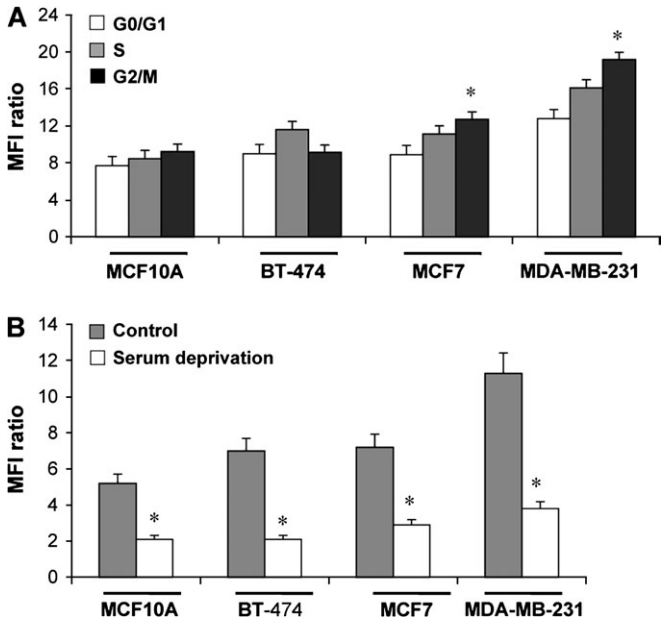


Fig. 4. The amount of PLC-β2 increases during progression of cells along the S and G_2/M phases of the cell cycle. Cytofluorimetric analysis of PLC-β2 expression was performed in cells that are in the different phases of cell cycle (**A**) and in breast-derived cell lines growing in control conditions or in a serum-deprived medium (**B**). The data, representative of three separate experiments performed in duplicate, are reported as MFI ratio ± standard deviation. The asterisks indicate statistically significant results.

On the contrary, an increased expression of PLC-β2 did not induce invasiveness in non- and low-tumorigenic cells and did not modify the already elevated invasive ability of the highly tumorigenic cells.

All these data indicate that PLC-β2 does not possess the capability to induce the modification of breast-derived cells required to assume an invasive phenotype but, in tumor cells that display an intrinsic tumorigenic potential, it is part of intracellular mechanisms able to change their migration/invasiveness aptitude.

By means of immunohistochemical analysis, we have found that in primary breast tissues PLC-β2 expression correlates with the response to the monoclonal antibody MIB-1, which reacts with an antigen that is only present in the nucleus of proliferating cells (20,30). Also in breast-derived cell lines, PLC-β2 is particularly abundant in cells that actively proliferate. In particular, both immunocytochemical analysis and cytofluorimetrical evaluation of the intracellular amount of PLC-β2 demonstrated that this protein accumulates in cells that are in the S/G_2/M phases of the cell cycle. We have also found that a forced expression of PLC-β2 is able to induce an accumulation of tumoral breast-derived cells in the G_2/M phases of the cell cycle and that a reduced expression of PLC-β2 in cells that show high basal level of this protein induced an accumulation of cells in the G_0/G_1 phases.

Breast cancer, as other neoplasia, shows alterations in cellular proteins that regulate the transition through the different phases of the cell cycle. In this context, a particular significance is displayed by cyclin D1. In addition to playing a role as a critical modulator of the G_1/S transition, it seems to be important in the loss of proliferative control during mammary oncogenesis in a way not mediated through regulation of its cyclin-dependent kinase activity (31). Also cyclin D3 plays a critical role in mammary tumorigenesis and seems to activate an oncogenic pathway in mammary epithelial cells that is distinct from that induced by cyclin D1 (32). All D cyclins were shown to bind the cyclin-dependent kinase CDK4 that is over-expressed in a significant fraction of breast cancers and seems to be essential for breast tumorigenesis (33).

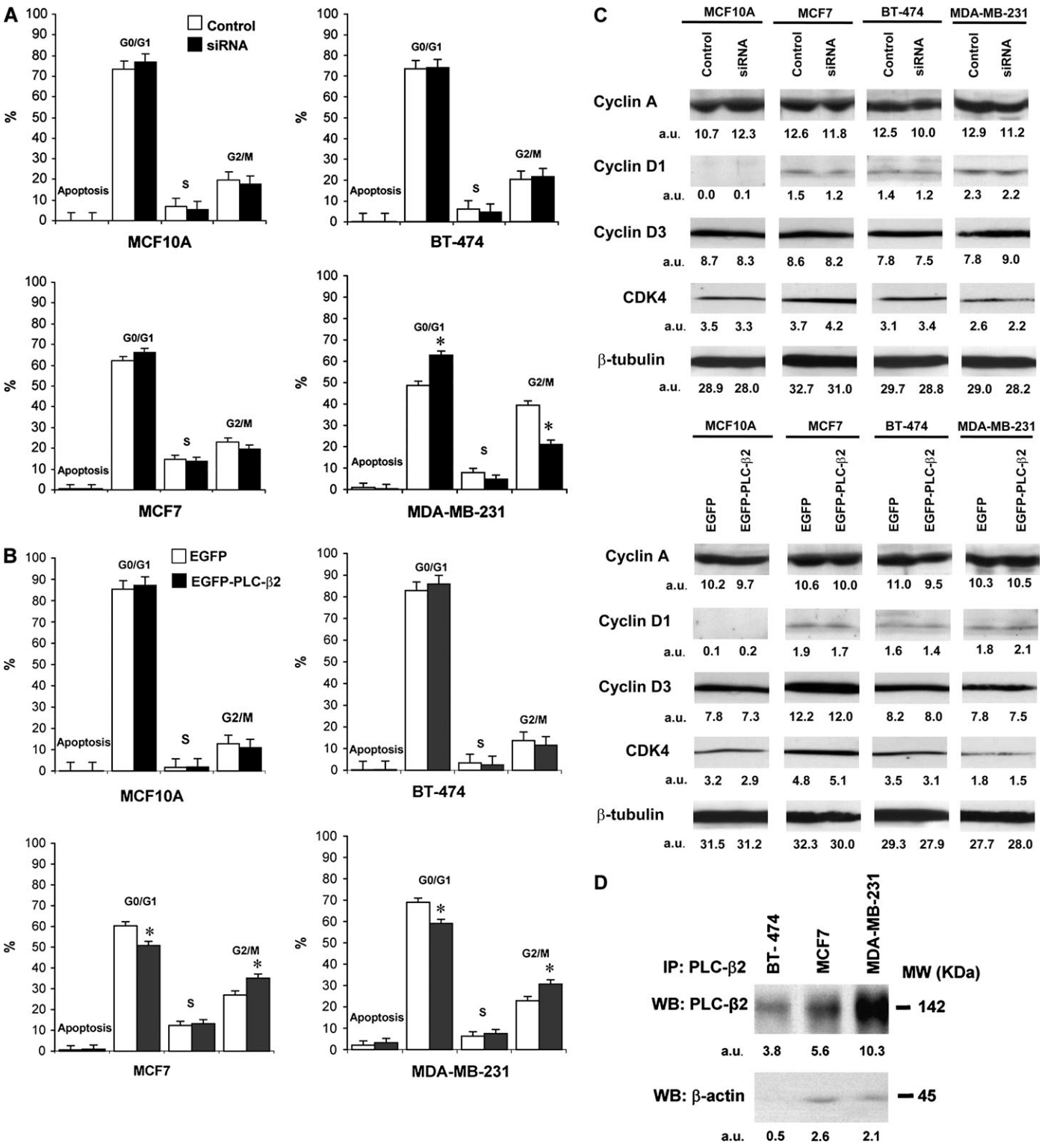


Fig. 5. PLC-β2 accumulates cells in G_2/M without modifying cell cycle-related enzymes. Breast-derived cell lines in which PLC-β2 was either silenced (**A**) or over-expressed (**B**) were subjected to analysis of cell cycle distribution and immunochemical analysis of the indicated cell cycle-related molecules (**C**). The results are representative of three independent experiments performed in duplicate. The asterisks indicate statistically significant results. In (**D**), immunochemical analysis with the indicated antibodies was performed on anti-PLC-β2 immunoprecipitates. Densitometric analysis of autoradiograms was performed and the results were reported as arbitrary units (a.u.). The data are representative of three separate experiments, performed in duplicate.

Here we demonstrate that in both non-transformed and tumoral breast-derived cells, modulated expression of PLC-β2 did not modify the expression of cyclin D1, cyclin D3 and of their partner CDK4, as well as of cyclin A, that regulates the transition along the S/G_2/M phases and whose transcription is dependent on the D cyclins activity (32). This suggests that a role in regulating cyclin-related enzymes in breast-derived cells may be excluded for PLC-β2. This in contrast to PLC-β1, which appears to regulate the cell cycle of murine

erythroleukemia cells by acting on the cyclin D3–CDK4 complex (14).

All the above reported data suggest that PLC-β2 plays a role in transduction mechanisms responsible for the modifications of cell morphology required for cell motility, DNA synthesis and mitosis of breast-derived tumor cells. Concerning the mechanism involved, it is well documented that phosphoinositides, and particularly PtdIns $(4,5)P_2$, the preferred substrate of PLC-β2, regulate the activity of actin-binding proteins during cytoskeleton changes leading to migration (34), according to the consolidated evidence that the amount of cytoskeleton-associated phosphoinositides may have a crucial role in the development of human diseases (15,35–37).

Here, we demonstrate the association between PLC-β2 and the actin component of cytoskeleton, particularly evident in the more invasive cell lines. These preliminary data suggest that the altered levels of PLC-β2 expressed in breast tumor cells may affect the amount of PtdIns$(4,5)P_2$, responsible, in turn, of deregulated control of actin cytoskeleton during cell motility and invasion. Similarly, the large amount of PLC-β2 found in dividing cells also suggests that PLC-β2 plays a role in regulating the phosphoinositide-related changes of cytoskeleton architecture that occur during mitosis.

Even though we have not been able to demonstrate a clear role of PLC-β2 in breast carcinogenesis, this work reports for the first time a role of this enzyme in promoting some malignant features that allow breast cancer progression. Since breast tumor phenotype does not appear to change extensively during tumor evolution from *in situ* to invasive carcinoma, molecularly based approaches targeted at specific factors, including components of the cell signaling mechanism, can play a crucial role in the early detection and in monitoring the evolution of breast cancer. In this context, PLC-β2 constitutes an indicator of the tumor stage, useful to predict its progression to invasiveness, metastasis and recurrence and a potential specific target for future therapeutic strategies of mammary carcinomas.

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