Phospholipase Cγ1 Is Required for Metastasis Development and Progression

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Abstract

Cell motility and invasion play an essential role in the development of metastasis. Evidence suggests that the enzyme phospholipase Cγ1 (PLCγ1) may be involved in tumor progression and possibly development of metastasis. In this study, we show that down-regulation of PLCγ1 expression severely impairs activation of the small GTP-binding protein Rac and cell invasion in breast cancer cell lines and U87 *in vitro*. Experimental metastasis assays in nude mice show that inducible knockdown of PLCγ1 strongly inhibits development of MDA-MB-231–derived lung metastasis and reverts metastasis formation. In addition, analysis of 60 breast cancer patients' tissues revealed an increase of PLCγ1 expression in metastasis compared with the primary tumor in 50% of tissues analyzed. These data show a critical role of PLCγ1 in the metastatic potential of cancer cells, and they further indicate that PLCγ1 inhibition has a therapeutic potential in the treatment of metastasis dissemination. [Cancer Res 2008;68(24):10187–96]

Introduction

Metastasis, the ability of cancer cells to spread from a primary site and form tumors at distant sites, is the main cause of death associated with cancer. Several steps regulate the development of metastasis (local invasion, intravasation, survival, extravasation, initiation, and maintenance of micrometastases at distant sites and vascularization of the resulting tumors). Cell motility and invasion plays an essential role in such a process, and identification of molecules and characterization of the mechanisms regulating cell motility is critical to understand metastasis development (1–4). Directed cell migration is regulated by chemokines and growth factors that bind and activate specific receptors, such as receptor tyrosine kinases. Receptors convert these outside signals to activate a complex network of intracellular pathways that regulate cell cytoskeleton rearrangement and motility. In this regard, activation of the enzyme phospholipase Cγ1 (PLCγ1) is thought to play a critical role in both cytoskeletal changes and migration associated with the metastatic process (5, 6). Activation of PLCγ1 can occur in response to either growth factors or integrin receptors–dependent pathways and induces hydrolysis of phosphatidylinositol-4,5-bisphosphate (PtdIns-4,5-P_2) to form the second messengers diacylglycerol and inositol-1,4,5-trisphosphate, which in turn activate a number of signaling molecules (7, 8). PLCγ1 has been shown to play a critical role in cell migration, invasion, and spreading (9–12). Indeed, PLCγ1 is highly expressed in several tumors, such as breast carcinomas, and in highly metastatic colorectal tumor cell lines (13, 14). In particular, we showed previously that phosphoinositide 3-kinase (PI3K)–mediated PLCγ1 activation is required for epidermal growth factor (EGF)–induced migration of breast cancer cells (15) but the mechanism of PLCγ1-dependent cell migration and metastasis was not clearly defined. Although previous studies provide evidences that PLCγ could be involved in carcinoma invasion, it should be noted that this observation was based on the use of generic PLC chemical inhibitor (11) or dominant-negative PLCγ (16). Here, we investigate the role of PLCγ1 in cell invasion and metastasis using different approaches to modulate PLCγ1 expression in highly invasive cancer cell lines. Our results show that PLCγ1 is required for breast cancer cell invasion and Rac1 activation. These data reveal a functional link between PLCγ1 and Rac1 that provides insight into processes regulating cell invasion. Furthermore, we show that down-regulation of PLCγ1 expression inhibits the human breast cancer cell line MDA-MB-231–derived lung metastasis development in *in vivo* mice model. According to this, immunohistochemical analysis of tissues derived from breast cancer patients shows an increase of PLCγ1 expression in metastasis compared with the primary tumor in around 50% of tissues analyzed. These data show that PLCγ1 is necessary for breast cancer–derived metastasis development. Moreover, we show that inducible down-regulation of PLCγ1 expression after 14 days of injection of MDA-MB-231 in nude mice is able to revert metastasis formation, indicating that blockade of PLCγ1 has a therapeutic potential to counteract metastasis dissemination.

Materials and Methods

Materials and constructs. Sequence targeting the human PLCγ1 mRNA was subcloned into pSuper and pSuperior vectors (ref. 17; Supplementary Fig. S1*B*). Control vectors (pSuper 3Mut and pSuperior 3Mut) contain a three-point mutated sequence unable to target the human PLCγ1 mRNA (Supplementary Fig. S1*B*). Palm-HA-PLCγ1 was kindly provided by Dr. E. Bonvini (MacroGenics, Inc.). Antibodies were as follows: phosphorylated PLCγ1 (Tyr^{783}), PLCγ1, phosphorylated epidermal growth factor receptor (EGFR; Tyr^{992}), EGFR, phosphorylated AKT (Ser^{473}), AKT, phosphorylated

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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extracellular signal-regulated kinases (ERK), and ERKs from Cell Signaling; phosphorylated focal adhesion kinase (FAK; Tyr^{397}) from BioSource; Rac1, FAK, paxillin, and glyceraldehyde-3-phosphate dehydrogenase from Santa Cruz Biotechnology; phosphorylated Tyr (4G10) from UBI; and CD44 from Novocastra Laboratories Ltd.

Cell culture, transfection, and RNA interference. MDA-MB-231 and U87 cells were grown in DMEM; MDA-MB-435 and TSA were grown in RPMI. Media were supplemented with 10% fetal bovine serum (FBS), sodium pyruvate, penicillin, and streptomycin and incubated in 5% CO_2 at 37°C. TSA is an aggressive and poorly immunogenic cell line established from a moderately differentiated mammary adenocarcinoma that arose spontaneously in a multiparous BALB/c mouse.

Down-regulation of PLCγ1 was obtained by using pSuper retro and pSuperior retro–based vectors to express stable or inducible short hairpin RNA (shRNA), respectively. Retroviral stocks were generated as described (18). For the inducible system, down-regulation of PLCγ1 expression was obtained by culturing cells in medium containing 2 μg/mL doxycycline (Sigma) or 10 μg/mL tetracycline (Sigma). MDA-MB-231 stable cell lines expressing the membrane-associated, palmitoylated HA-tagged PLCγ1 were transfected with 5 μg of palmitoylated HA-tagged PLCγ1 (Palm-PLCγ-1-HA) using Lipofectamine 2000 (Invitrogen) and then selected with 500 μg/mL of G418.

Rac1 activation assay. For EGF stimulation, 2×10^6 cells plated in 10-cm dishes were serum starved for 24 h and then stimulated with 20 ng/mL EGF (Sigma) for 5 min. For serum growing condition, 1×10^6 cells were plated in 10-cm dishes and allowed to grow for 3 d in the absence or presence of doxycycline to down-regulate PLCγ1 expression. Cells were then lysed on ice in lysis buffer [50 mmol/L Tris (pH 7.2), 100 mmol/L NaCl, 5 mmol/L $MgCl_2$, 1 mmol/L DTT, 10% glycerol, 1% NP40] containing protease inhibitors, and Rac GTP pull-down assays were then performed using a GST-PAK-CRIB as described (19).

Cell migration and invasion assay. Cell migration was performed in Transwell chambers (tissue culture treated, 10-mm diameter, 8-μm pores, Nunc) as described (20). Where indicated, inserts were coated with 10 μg/mL fibronectin. Invasion assay in TSA and MDA-MB-435 cells was performed in Matrigel-coated Transwell inserts (Matrigel diluted 1:8). Cells (100,000 per transwell) were resuspended in RPMI containing 1% FBS and plated on top of the inserts, whereas RPMI containing 20% FBS was plated on the bottom. Cells were allowed to migrate for 48 h.

Three-dimensional collagen I invasion assay. Collagen I plugs (2 mg/mL) were prepared from fibrillar bovine collagen I (3.1 mg/mL; PureCol) by dilution in DMEM in accordance with the manufacturer's protocol (Nutacon). A collagen solution (2.5 mL) was placed in a 24-mm diameter, 8-μm pore transwell insert (Corning) and allowed to polymerize for 60 min at 37°C. Cells (2.5×10^4) were plated in 1.5 mL of serum-free DMEM on top of the plug. To establish a chemoattractant gradient, 2.5 mL of DMEM containing 20% FCS were added to the lower chamber and replaced every 10 to 12 h to maintain the gradient. For experiment with inducible PLCγ1 knockdown, cells were treated with doxycycline 48 h before plating. Cells were allowed to invade for 5 d, then fixed in 4% paraformaldehyde, washed thrice in PBS, permeabilized with 0.2% Triton X-100, and stained with Sytox green (Invitrogen, 1:5,000). Confocal imaging was performed using a BIO-RAD Radiance 2100 laser and an upright Nikon Eclipse E1000 microscope (10 × LWD objective) running LaserSharp 2000 software. Analysis was performed by taking sections at 6-μm intervals through 400 to 900 μm of the collagen plug. Cells that had invaded >100 μm were considered as invading cells. The percentage of invading cells was determined by dividing the sum of the fluorescence in invading sections by the total fluorescence of all sections. Levels of fluorescence were determined by Image J. Three-dimensional reconstructions were performed using LSM Browser (Zeiss) imaging software.

Cell proliferation assay. 3Mut and shPLCγ1 MDA-MB-231 cells (2×10^4) were seeded on six-well plates in DMEM containing 10% FBS. Proliferation was assessed at different days by using the cell counter CDA-500 (Sysmex).

Confocal microscopy and ruffling assay. Cells were treated as indicated, then fixed in 4% paraformaldehyde for 15 min at room temperature and blocked with 0.1% bovine serum albumin for 30 min at room temperature. Coverslips were then incubated for 2 h at room temperature with the appropriate primary antibody (antipaxillin, anti-FAK antibodies). Rhodamine-labeled phalloidin was used to visualize actin cytoskeleton. Confocal imaging was performed using a BIO-RAD Radiance 2100 laser and an upright Nikon Eclipse E1000 microscope running LaserSharp 2000 software. Ruffles formation was assessed as described (21).

Animals. Female nude athymic CD-1 nu/nu mice (7 wk old) and BALB/c mice were purchased from Charles River Laboratories and maintained under specific pathogen-free conditions with food and water provided ad libitum. The general health status of the animals was monitored daily. Procedure involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national and international laws and policies.

Experimental lung metastasis assay. Control (3Mut) and inducible/stable PLCγ1 knockdown MDA-MB-231 cells were injected into the tail vein of recipient mice (2×10^6 per mouse). Five weeks after cell implant/injection, lung tissues (five lungs per group) were excised and immediately fixed in 10% neutral buffered formalin. Lungs were then embedded in paraffin, and thick sections (10 μm; two sections per lung tissue) were cut and stained with H&E or with an anti-CD44 monoclonal antibody from Santa Cruz Biotechnology.

***In vivo* tumor growth.** pSuperior 3Mut and shPLCγ1 MDA-MB-231 cells were injected s.c. into the left and right flanks of each mouse, respectively (6×10^6 per mouse). When tumors reached a size of ~50 mm^3, mice were randomized in two groups ($n = 5$), with one group receiving tetracycline (2 mg/mL; Sigma) in drinking water. Tetracycline was replaced every 2 d. The diameter of s.c. growing tumors was measured with a caliper twice a week, and the experiments ended at day 35. The volume of s.c. growing tumors was calculated by the formula: tumor volume (mm^3) = (length × $width^2$) / 2. Differences in s.c. tumor growth, in the presence or in the absence of tetracycline, were evaluated with a one-way ANOVA followed by Fisher's test using the StatView statistical package (SAS Institute, Inc.). Differences were considered statistically significant at a level of $P < 0.05$. All data are expressed as mean ± SE.

Mammary fat pad was performed in groups of eight mice receiving two mammary fat pad inoculations consisting of 1×10^5 TSA 3Mut and shPLCγ1 cells resuspended in 50 μL media and mixed with equal volume of Matrigel. Cells were implanted orthotopically into the left and right intact fourth inguinal mammary fat pad of 6-wk-old to 8-wk-old female BALB/c mice. Tumor growth was measured daily with calipers.

Western blot of mice tissues. Tumor samples were collected, snap frozen, and analyzed by Western blot. Frozen specimens of tumor tissue were homogenized with a Polytron homogenizer in a lysis buffer (ratio, 1:1 w/v) containing 50 mmol/L Tris-HCl (pH 7.4), 5 mmol/L EDTA, 0.1% NP40, 250 mmol/L NaCl, 50 mmol/L NaF in the presence of leupeptine, pepstatine, aprotinin, and phenyl-methyl-sulfonyl-fluoride as proteases inhibitors. The homogenates were then centrifuged at 13,000 rpm for 10 min at 4°C, and the protein concentration was determined using a Bio-Rad assay kit (Bio-Rad). Eighty micrograms of proteins were separated on SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore). The membrane was probed with the indicated primary antibodies, washed with TBS-T (TBS 1× + 0.05% Tween 20), and then incubated for 1 h with horseradish peroxidase–conjugated secondary antibody in TBB buffer (TBS-T + 5% nonfat milk). Proteins were detected by enhanced chemiluminescence (Amersham).

Immunohistochemistry analysis. Expression of PLCγ1 was analyzed in formalin-fixed, paraffin-embedded human breast tumors and matching metastasis (n, 60 pairs), using an anti-PLCγ1 monoclonal antibody from Santa Cruz Biotechnology. Briefly, antigen retrieval was performed by heating for 15 min in 10 mmol/L sodium citrate buffer (pH 6.0; DAKO, S2031) in a microwave oven. The slides were incubated overnight at 4°C with a 1:150 dilution of the primary antibody, and the reaction was evidenced with Dako EnVision kit. Diaminobenzidine was used as the chromogen, and the slides were counterstained with Mayer hematoxylin. In control sections, the specific primary antibody was omitted or replaced with nonimmune serum or isotype-matched immunoglobulins (Dako). The

staining of at least 500 cells in primary tumors and metastatic lesions was evaluated by two independent pathologists (MP, RL) using a modified histochemical score (H-score; ref. 22) The H-score includes an assessment of both the intensity of staining and the percentage of stained cells. For the intensity, a score index of 0, 1, 2, and 3 corresponding to negative, weak, moderate, and strong staining intensity, respectively, was used, and the percentage of positive cells at each intensity was estimated. Disagreement between the pathologists was noted for <8% of samples. In these cases, consensus was reached by a joint reevaluation of the slide. A final score of 0 to 300 is the product of both the intensity and the percentage. An H-score difference of at least 25% between breast tumors and matching metastasis was considered indicative of a different PLCγ1 expression.

Results

Modulation of PLCγ1 levels in breast cancer cell lines. To study the role of PLCγ1 in cell metastasis, we analyzed the effect of both stable and inducible down-regulation of PLCγ1 expression obtained by RNA interference (RNAi) strategy. As a complementary approach, we tested the effect of overexpression of Palm-PLCγ1-HA (23).

Specific shRNA targeting PLCγ1 (shPLCγ1) were subcloned into a pSuper retro or pSuperior retro–based vectors to obtain a stable or inducible down-regulation of PLCγ1 expression, respectively (Supplementary Fig. S1). As a control, we generated cells expressing shRNA PLCγ1 carrying a three-point mutation (3Mut) or a 7-point mutation (7Mut), which prevent the shRNA targeting of PLCγ1 mRNA (Supplementary Fig. S1*B*). As both mutated shRNA PLCγ1 failed to down-regulate PLCγ1 expression (Supplementary Fig. S1*A*), we elected the 3Mut sequence as the best appropriate control for all further experiments. PLCγ1 knockdown (ranging from 90% to 100% of control) had no effect on EGFR phosphorylation (Supplementary Fig. S1*C*) and it was isoform-specific because PLCγ2 expression was unaffected (Supplementary Fig. S1*D*). For the inducible PLCγ1 knockdown, one selected tetracycline-inducible MDA-MB-231 clone was infected with the pSuperior shPLCγ1 and pSuperior 3Mut retro vectors. Addition of tetracycline-induced PLCγ1 knockdown in pSuperior shPLCγ1 but not in pSuperior 3Mut MDA-MB-231 cells (Supplementary Fig. S1*E*).

PLCγ1 regulates cytoskeletal rearrangements and Rac1 activation. Several lines of evidence indicate that the PLCγ1 substrate PtdIns-4,5-P_2 (24) and the enzyme itself (25) are involved in cytoskeletal rearrangement through modulation of actin-binding proteins. Therefore, we analyzed the actin cytoskeleton in control (3Mut) and PLCγ1 knockdown MDA-MB-231 cells in the presence of serum. We observed that both stable (data not shown) and inducible (pSuperior; Fig. 1*A*) PLCγ1 down-regulation induced a marked decrease in the number of membrane ruffles. To further investigate the effect of PLCγ1 knockdown in a distinct breast cancer cell line, we infected the murine mammary adenocarcinoma cells TSA with 3Mut and PLCγ1 shRNA (PLCγ1 down-regulation is

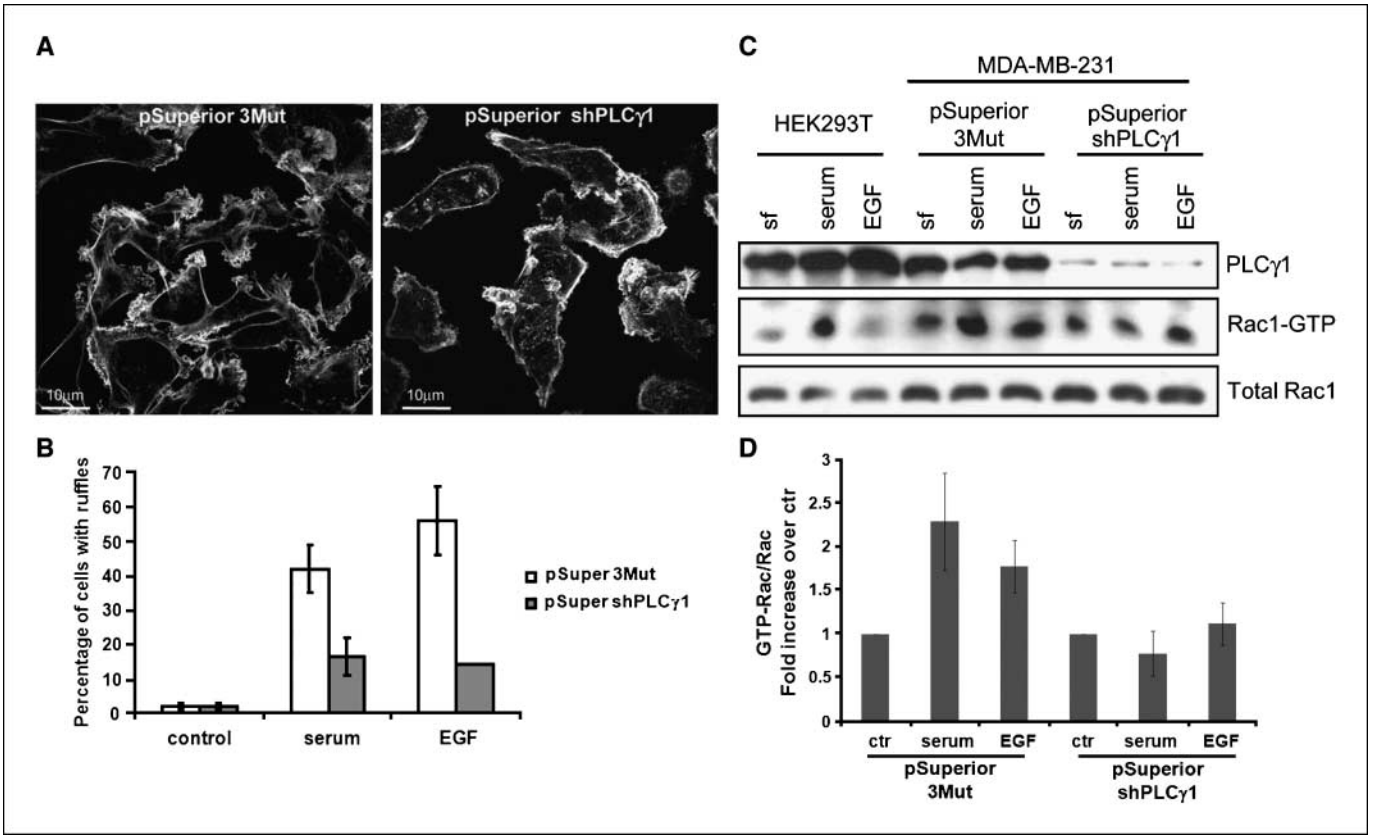

Figure 1. PLCγ1 regulates membrane ruffles formation and Rac activity in MDA-MB-231 cells. *A,* pSuperior 3Mut and shPLCγ1 MDA-MB-231 cells were kept in serum, treated with doxycycline for 48 h, and then fixed. Membrane ruffles formation was analyzed by confocal microscopy using a Rhodamine-labeled phalloidin antibody to visualize actin cytoskeleton. *Bar,* 10 μm. *B,* serum-starved TSAs were stimulated with serum or EGF for 6 min, fixed, and then analyzed by confocal microscopy. The number of cells showing membrane ruffles was assessed, as described (21). Data are mean ± SE of three independent experiments. *C,* the indicated cells were serum starved for 24 h and then unstimulated (*sf*) or stimulated with 20 ng/mL EGF (*EGF*) for 5 min. Alternatively, cells were kept in serum for 48 h (*serum*). Pull-down assay to detect GTP-bound Rac was performed, as described in Materials and Methods. Down-regulation of PLCγ1 levels was assessed by using a specific antibody. *D,* densitometry analysis of Rac activation. *Columns,* mean of three independent experiments; *bars,* SE.

shown below). Serum-starved TSA were stimulated with serum or EGF, and the percentage of cells showing ruffles was determined. Data revealed that both serum-induced and EGF-induced membrane ruffles formation was clearly reduced in PLCγ1 knockdown cells (Fig. 1*B*).

Because it is well established that the small GTP binding protein Rac is directly involved in membrane ruffles formation (26), we then analyzed the activation status of Rac in MDA-MB-231 cells. As shown in Fig. 1*C* and *D*, both serum and EGF were able to activate Rac1 in control 3Mut MDA-MB-231 cells. Similarly, serum was able to activate Rac1 in HEK293T cells used as control (Fig. 1*C*). On the contrary, inducible down-regulation of PLCγ1 significantly impaired both the EGF-induced and serum-induced Rac1 activation (Fig. 1*C* and *D*). Similar data were obtained in stable knockdown cells (data not shown). The EGF-induced activation of Akt was unaffected by PLCγ1 down-regulation, whereas a reduction in the late phase of ERK activation was detected (Supplementary Fig. S2).

To further assess the role of PLCγ1 in this process, we analyzed the effect of overexpression of a palmitoylated PLCγ1 mutant which is constitutively associated to the plasma membrane (Palm-PLCγ1-HA) and has been shown to act as a constitutively active PLCγ1 mutant (23). Overexpression of Palm-PLCγ1-HA induced a marked increase in membrane ruffles formation in serum

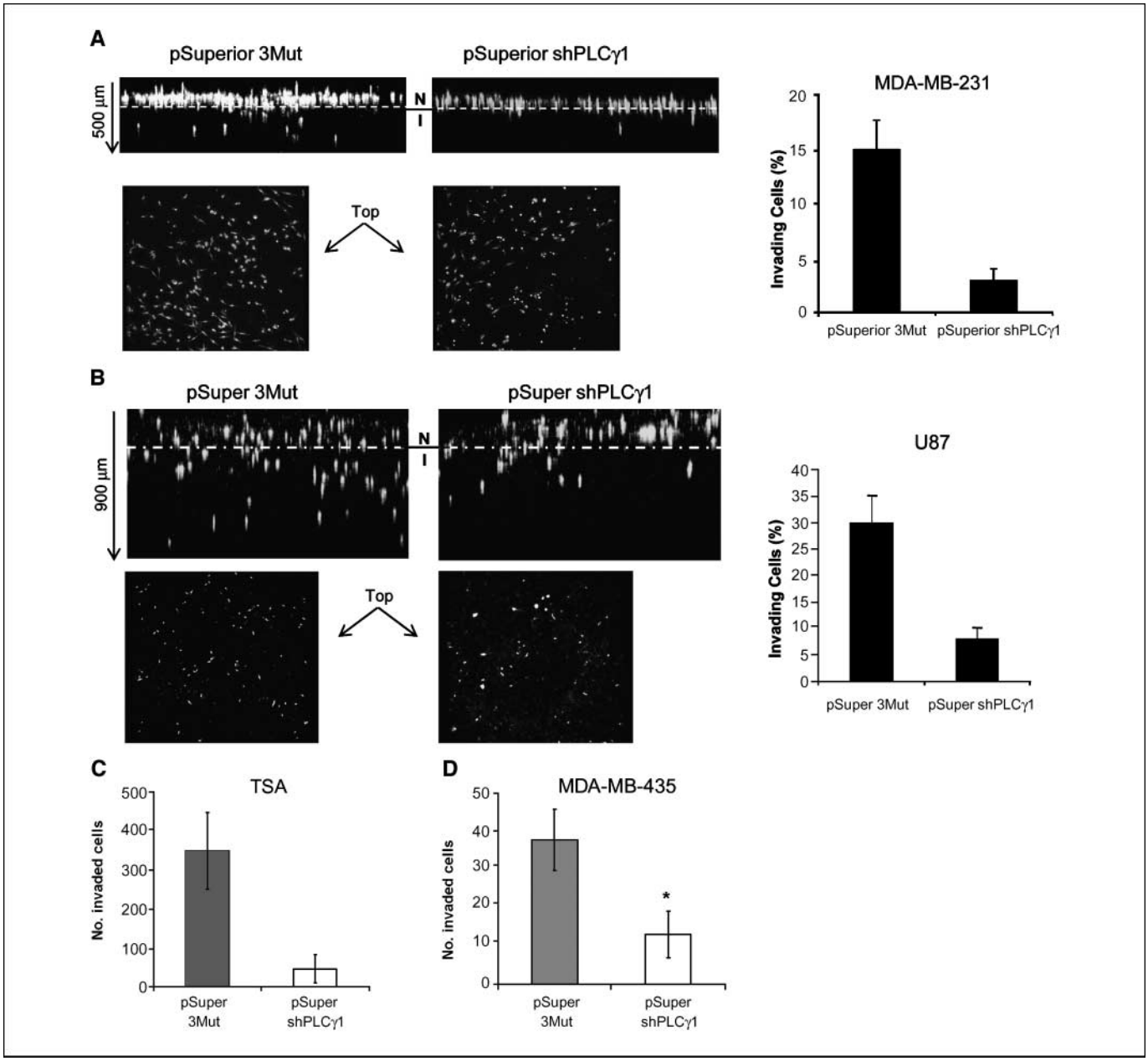

Figure 2. Down-regulation of PLCγ1 expression impairs cell invasion. Collagen assay in *A* and *B* was performed, as described in Materials and Methods. *A* and *B,* representative images of MDA-MB-231 (*A*) and U87 (*B*) within and on top of the collagen plug. Cells were seeded at the top of the filter and allowed to migrate for 5 d toward the chemoattractant (*bottom*). N and I indicate noninvading and invading cells. Quantitative analysis of invasion are also shown. *Columns,* mean of three independent experiments; *bars,* SD. *C* and *D,* serum-induced invasion of pSuper 3Mut and shPLCγ1 TSA (*C*) and MDA-MB-435 (*D*) on Matrigel-coated Transwell inserts. *Columns,* mean of three (*C*) and four (*D*) independent experiments; *bars,* SE. *, $P < 0.05$.

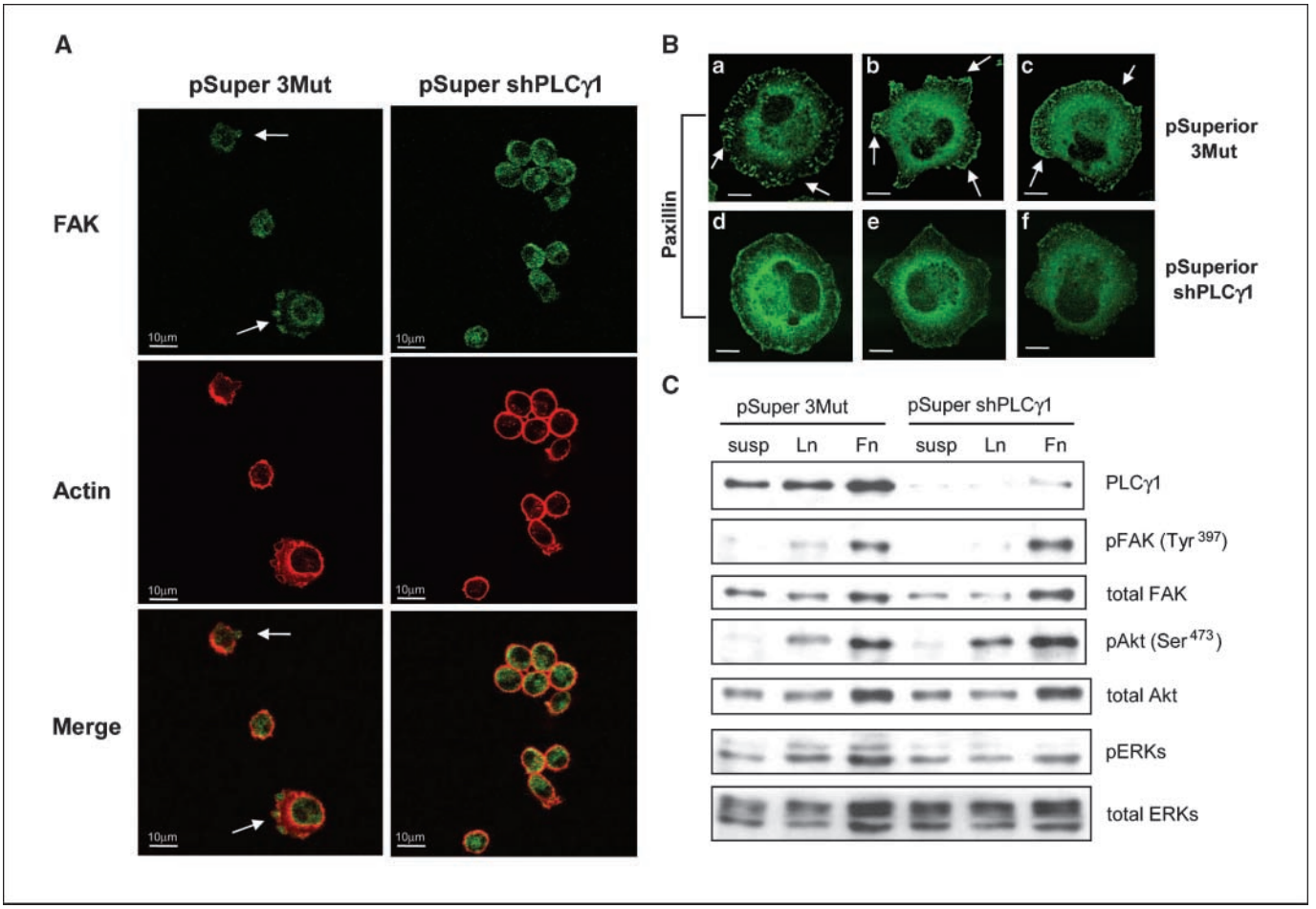

Figure 3. PLCγ1 expression is required for focal adhesion formation. *A,* pSuper 3Mut and shPLCγ1 MDA-MB-231 were seeded on glass coverslips coated with 20 μg/mL laminin and allowed to adhere for 20 min before fixing for confocal microscopy analysis. Focal adhesions were visualized by using an anti-FAK antibody. Actin was visualized by using rhodamine phalloidin. *B,* pSuperior 3Mut (*a, b, c*) and shPLCγ1 (*d, e, f*) cells were grown in the presence of 2 μg/mL doxycycline for 48 h, seeded on glass coverslips coated with 10 μg/mL fibronectin, and allowed to adhere for 60 min before fixing for confocal microscopy analysis. Arrows indicate focal adhesions visualized by using an anti-paxillin antibody. *C,* pSuper 3Mut and shPLCγ1 MDA-MB-231 were plated on laminin or fibronectin-coated well. After 20 min, cells were lysed and analyzed by Western blotting as indicated. Cells left in suspension (*susp*) were used as control.

(Supplementary Fig. S3*A*) and an increase in Rac activation (Supplementary Fig. S3*B*) in both serum-stimulated and EGF-stimulated MDA-MB-231 compared with cells expressing the empty vector pcDNA3. PLCγ1 overexpression is shown in Supplementary Fig. S3*C*. Taken together, these data indicate that PLCγ1 is required for Rac1 activation and membrane ruffles formation.

RNAi-mediated inhibition of PLCγ1 expression impairs cell migration, invasion, and cell spreading *in vitro*. The observation that PLCγ1 was able to modulate both Rac1 activation and cytoskeletal rearrangements strongly suggested that this enzyme might have a role in cell migration. This hypothesis was further supported by our previous work reporting a role for a PI3K/PLCγ1 pathway in migration of MDA-MB-231 cells (15). Migration assays in control and PLCγ1 knockdown MDA-MB-231 cells revealed that PLCγ1 down-regulation clearly reduced EGF-induced cell migration (Supplementary Fig. S4*A*) and basal cell migration on fibronectin (Supplementary Fig. S4*B*). Moreover tridimensional invasion assays showed that inducible (Fig. 2*A*) or stable (data not shown) PLCγ1 knockdown resulted in a complete impairment of cell invasion. Similar data were obtained by down-regulating PLCγ1 expression in U87 (Fig. 2*B*), a glioblastoma cell line whose cell migration has been shown to be PLC-dependent (27). Furthermore, a clear inhibition of invasion on Matrigel-coated transwell inserts was observed in TSA (Fig. 2*C*) and in human breast cancer cell line MDA-MB-435 upon PLCγ1 down-regulation (Fig. 2*D*). Efficiency of PLCγ1 knockdown in MDA-MB-435 and U87 cells is shown in Supplementary Fig. S5*A* and *B*, respectively. Taken together, these data show that PLCγ1 is necessary for cell migration and invasion *in vitro*.

A random motility assay in MDA-MB-231 cells overexpressing Palm-PLCγ1-HA revealed a defect in migration (data not shown). Indeed, these cells showed intense formation of protrusions all around the cells, possibly due to the PLCγ1 localization and subsequent Rac1 activation all around the perimeter of the cells. This may affect cell polarization, ultimately resulting in the observed impairment in cell migration.

To gain further insight into the mechanisms of the PLCγ1-dependent cell migration and invasion, we performed spreading assays on cells plated on laminin and fibronectin. Both stable (Fig. 3*A*) and inducible (Fig. 3*B*) PLCγ1 knockdown cells seem round and less spread than control (3Mut) cells. In addition, staining with antibodies to focal adhesion kinase (FAK; Fig. 3*A*) and paxillin (Fig. 3*B*) clearly revealed a decrease in focal adhesion number in PLCγ1 knockdown cells. Parallel biochemical analysis

showed that neither FAK nor Akt activation (assessed by monitoring phosphorylation of their residues Tyr^{397} and Ser^{473}, respectively) were impaired in these experimental conditions (Fig. 3*C*). On the contrary, a clear decrease of ERK phosphorylation was observed in PLCγ1 knockdown MDA-MB-231 cells plated on laminin and fibronectin (Fig. 3*B*). These data indicate that PLCγ1 down-regulation impairs spreading on laminin and fibronectin of MDA-MB-231 possibly through impairment of ERK activation.

PLCγ1 is necessary for breast cancer cell–derived lung metastasis *in vivo*. Data, thus far, revealed that PLCγ1 is required for cell migration and invasion. Because these processes are key steps in metastasis development, we decided to investigate the effect of PLCγ1 knockdown in an *in vivo* experimental metastasis assay. As shown in Fig. 4, control MDA-MB-231 (3Mut) cells injected in mice tail vein induced formation of lung metastases in 4 to 5 weeks. Strikingly, when we injected stable (Fig. 4*A*) or inducible (Fig. 4*B*) PLCγ1 knockdown MDA-MB-231 cells, metastasis formation was almost completely abrogated. In fact, four of five lung tissues in both stable and inducible PLCγ1 knockdown were completely metastasis-free and only one sample showed few metastases. Lung sections (stable; Fig. 4*A*) or external lung pictures (inducible; Fig. 4*B*) from this single mouse bearing few metastases are shown.

Because PLCγ1 may be involved in different steps involved in metastasis formation, we decided to investigate whether it was possible to block and possibly revert metastasis by down-regulating PLCγ1 expression after metastasis had already developed. To this purpose, we injected pSuperior shPLCγ1 cells and kept the injected mice in the absence of tetracycline. In this experimental condition, MDA-MB-231 cells were still expressing PLCγ1 and, indeed, were able to develop lung metastases after 14 days from injection (Fig. 5*A*). After 14 days, animals were divided in two groups, one untreated and one treated with tetracycline, to down-regulate PLCγ1 levels. Interestingly, we observed that reduction of PLCγ1 expression was able to induce regression of metastasis formation. Specifically, at the end point of the experiment (60 days), we found that four of five mice kept in the absence of tetracycline (control) showed lung metastases (Fig. 5*B*). On the contrary, after day 14, five of six mice treated with tetracycline showed completely metastasis-free lungs, whereas only the remaining single mouse presented few, barely detectable metastases (Fig. 5*B*). These data indicate that not only PLCγ1 has a key role in metastasis formation but also that blockade of PLCγ1 reverts metastasis formation and, therefore, could have therapeutic potential.

PLCγ1 expression is increased in lymph node metastasis compared with primary tumors in breast cancer patients. Data, thus far, strongly indicate that PLCγ1 has a role in breast cancer–derived lung metastasis development. To further assess the involvement of this enzyme in such a process, we performed immunohistochemical analysis of samples from 60 breast cancer patients to compare the expression levels of PLCγ1 in lymph node metastasis to primary tumors. Two of 60 primary tumors did not express PLCγ1, whereas all metastases showed specific PLCγ1 staining. Representative tissues are shown in Fig. 5*C*. We found that PLCγ1 expression is significantly increased in lymph node metastasis compared with primary tumors (Wilcoxon matched-pairs signed ranks test, W+ = 412.50, W− = 913.50, n = 51,

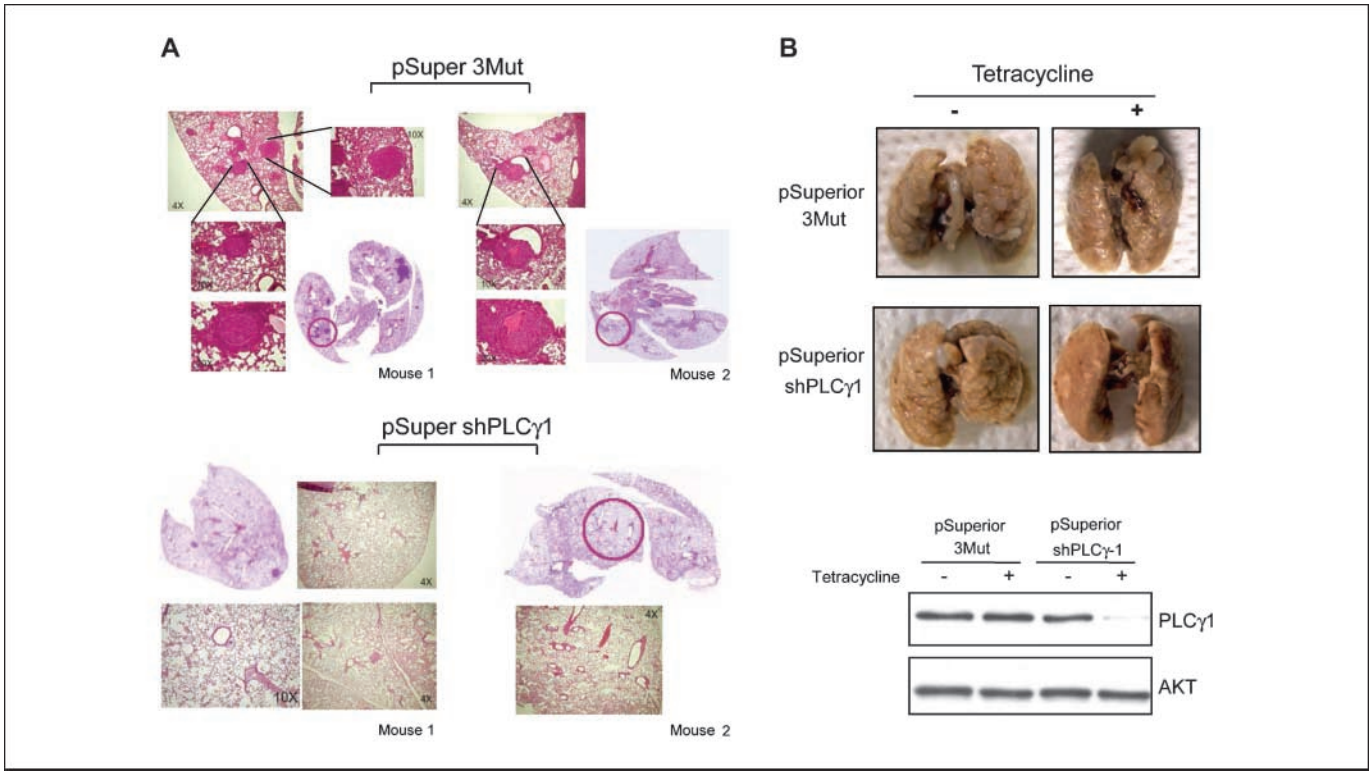

Figure 4. PLCγ1 is required for MDA-MB-231 cell–derived lung metastasis *in vivo*. *A,* control (*3Mut*) and stable shPLCγ1 cells were injected in the tail vein of nude mice. Experimental metastasis assay was performed, as described in Materials and Methods. Two representative images of lung sections stained with hematoxilin and eosin are shown. *B,* control (*3Mut*) and inducible shPLCγ1 cells were injected in the tail vein of nude mice. External lung pictures are shown. *Bottom,* Western blotting analysis of lysates from pSuperior 3Mut and shPLCγ1 cells after 48 h of treatment with tetracycline and before the injection.

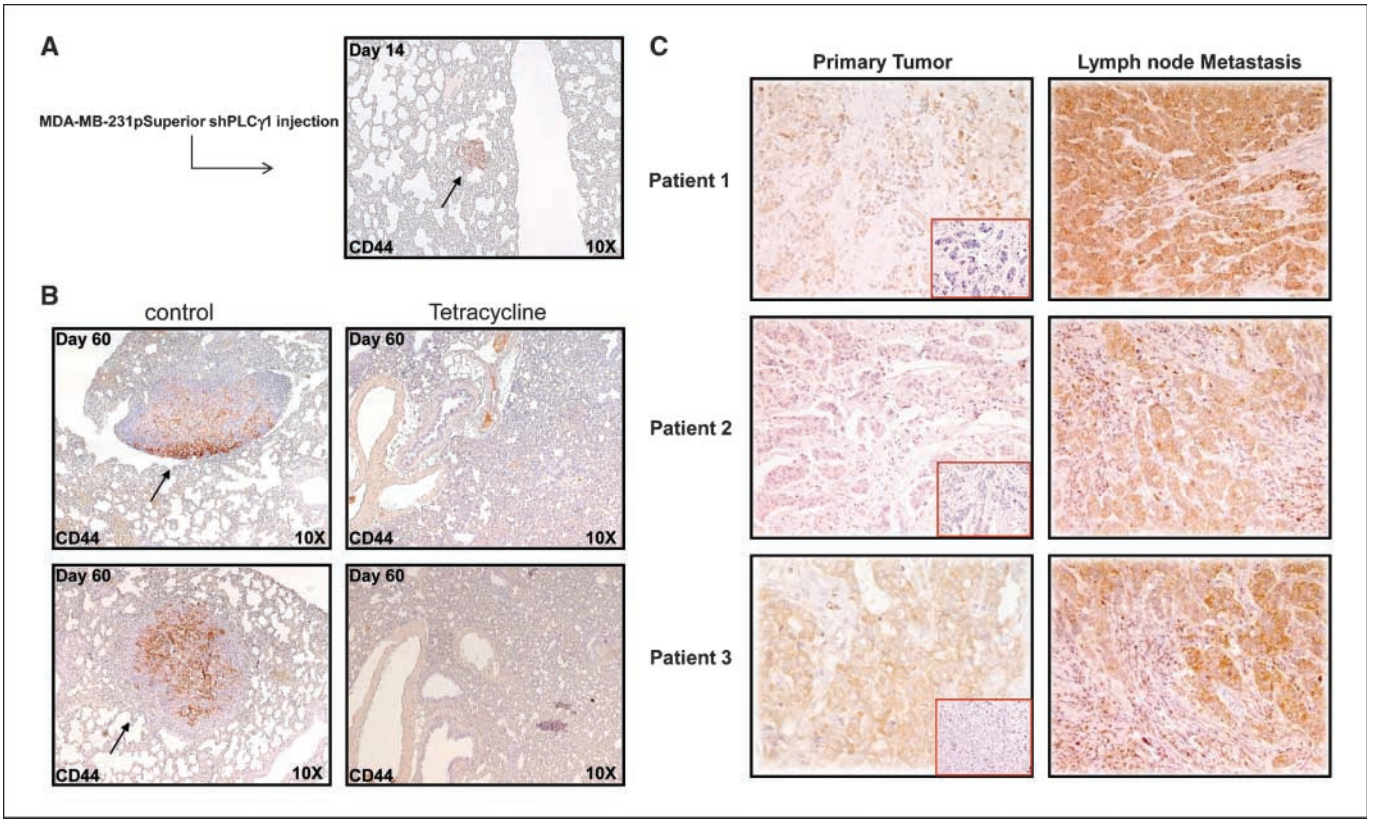

Figure 5. PLCγ1 is important for metastasis progression. *A* and *B*, pSuperior shPLCγ1 MDA-MB-231 cells (1×10^6) were injected in nude mice. Mice were kept in the absence of tetracycline to maintain PLCγ1 expression. After 14 d, three animals were sacrificed and the presence of micrometastasis in lung sections was analyzed by CD44 staining (*A*). The remaining mice were divided in two groups and treated ($n = 6$) or not ($n = 6$) with tetracycline to down-regulate PLCγ1 expression. Mice were sacrificed after 46 d (60 d from implant). Representative lung sections from untreated mice or mice treated with tetracycline are shown. Arrows indicate micrometastasis. *C,* expression of PLCγ1 in three primary breast tumors and matching lymph node metastases assessed by immunohistochemistry. Corresponding control images are shown in insets.

$P < 0.01911$). Data revealed that, in 48% of breast cancer patients, PLCγ1 expression was markedly increased in lymph node metastases compared with the corresponding primary tumors, further supporting the role for this enzyme in metastases development.

Effects of PLCγ1 down-regulation on primary tumor growth. To test the effect of PLCγ1 down-regulation on primary tumor growth, we studied *in vitro* and *in vivo* growth of pSuperior shPLCγ1 MDA-MB-231 cells. No difference in *in vitro* growth, assessed by cell counting, was observed in inducible PLCγ1 knockdown MDA-MB-231 cells compared with the control (3Mut) cells upon 5 days of culture (Fig. 6*A*). We then implanted inducible PLCγ1 knockdown MDA-MB-231 cells s.c. in nude mice and tested their growth *in vivo* in the presence or absence of tetracycline. A slight, although not significant, difference in primary tumor growth was observed in mice implanted with pSuperior shPLCγ1 MDA-MB-231 compared with control (3Mut)-injected mice (Fig. 6*B*). *In vivo* tumor growth of pSuperior 3Mut was unaffected by tetracycline treatment (data not shown). Efficiency of PLCγ1 down-regulation was verified in cells tested in *in vitro* growth (Fig. 6*A*) and in excised tumors (Fig. 6*B*). To better analyze the effect of PLCγ1 down-regulation on primary tumor growth, we implanted TSA cells in the mammary fat pad. Data revealed that tumor growth was slightly, but significantly, reduced in mice implanted with PLCγ1 knockdown cells compared with controls (Fig. 6*C*).

Discussion

Here, we show that PLCγ1 is a critical enzyme in the development and maintenance of tumor metastasis. Our data provide evidence that dissemination of tumor cells in models of breast cancer, as well as in the human disease, correlates with PLCγ1 expression. In addition our data indicate that loss of PLCγ1 affects actin polymerization and focal adhesion formation through modulation of the Rho family GTPase Rac. Interestingly, our data not only reveal that PLCγ1 is necessary for metastasis formation but they also show that down-regulation of the enzyme expression results in metastasis regression.

Taken together, our findings suggest that metastatic dissemination can be controlled by targeting PLCγ1 and its downstream signaling pathways.

Tumor metastasis is a complex process involving different sequential steps (1, 3, 4, 28), of which cell motility and invasion are critical events. Several chemoattractants can activate cell migration and invasion through activation of their specific receptors and downstream intracellular signaling pathways, ultimately resulting in reorganization of the actin cytoskeleton. Several components of such signaling pathways have been identified and found overexpressed in invasive tumor cells. Among these Rho/Rac GTPases are well-known regulators of cytoskeletal organization (2, 6). In particular Rac activation induces membrane ruffles, focal adhesion complexes, and lamellipodia formation (2, 26). The latter promotes cells to extend protrusions requested for cell polarity and forward

movement. The intracellular mechanism regulating Rac1 activation in cell migration is not completely understood. PI3Ks together with Rac seem to be key regulators of both polarization and cell migration (26), and several evidences suggest the existence of a mutual regulation of PI3K and Rac activity although its precise nature is, at present, unclear. Here, we show that PLCγ1 regulates Rac1 activation in breast cancer cells. Because we have shown that lipid product of PI3K contribute to PLCγ1 activation (29) and induces translocation of PLCγ1 to the leading edge of MDA-MB-231 migrating cells (15), our present data suggest that PLCγ1 may be the intracellular link between PI3K and Rac activity.

Given its essential role in cell motility Rac has been implicated in cancer cell invasion and metastasis. A direct correlation between Rac activation and metastatic potential of a panel of cell variants derived from MDA-MB-435 breast cancer cell line has been reported (30). In addition RNAi-mediated knockdown of Rac

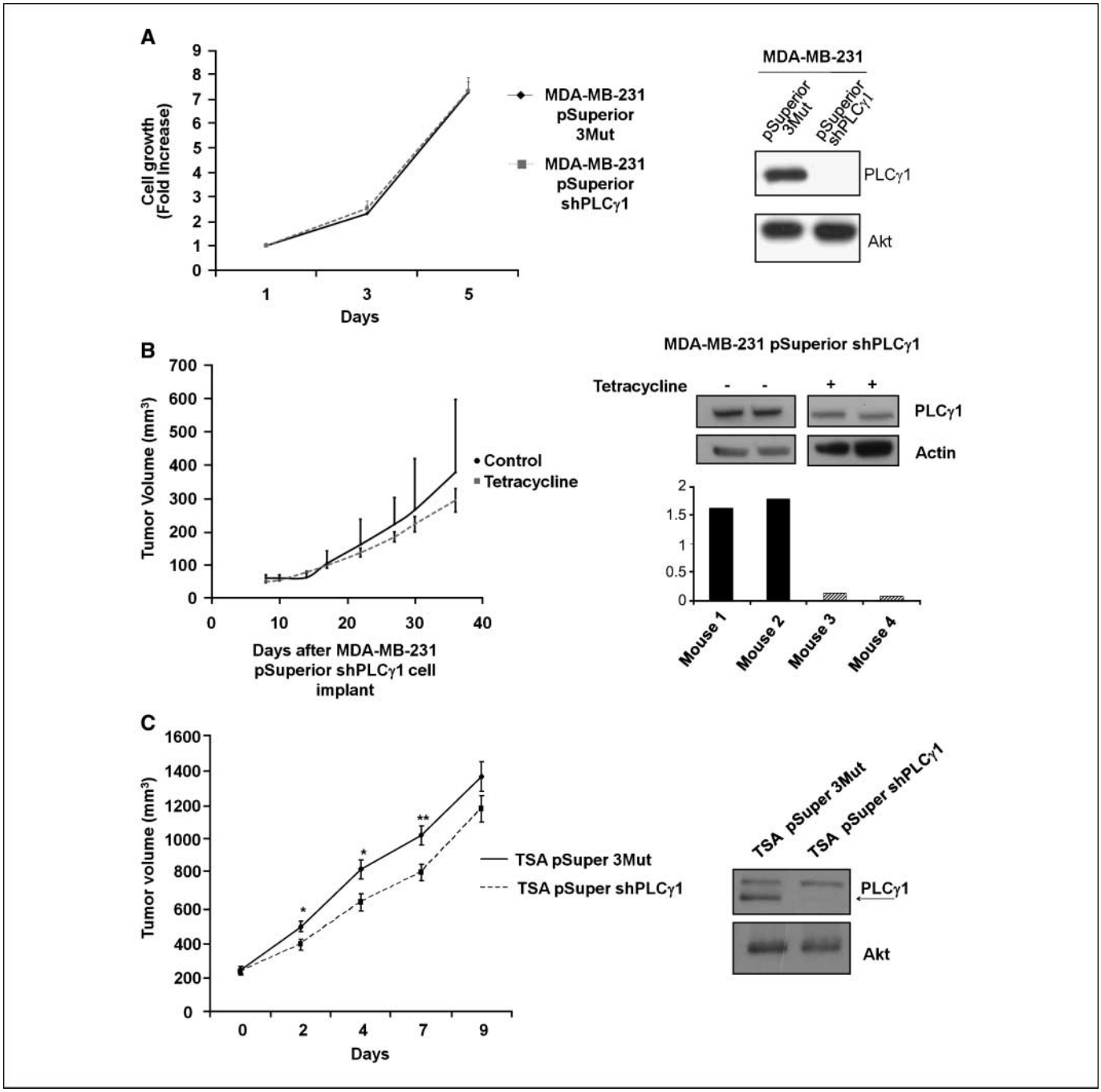

Figure 6. *In vitro* and *in vivo* tumor growth. *A,* pSuperior 3Mut and shPLCγ1 MDA-MB-231 cells treated with 2 μg/mL doxycycline were seeded and cultured in the presence of serum. At the indicated times, cells were trypsinized and counted. PLCγ1 knockdown in these cells is also shown. *B,* exponentially growing pSuperior shPLCγ1 MDA-MB-231 cells, treated or not with tetracycline, were injected into the right flank and the left flank, respectively, of the recipient mice. Tumor growth was assessed, as described in Materials and Methods. Western blotting analysis and corresponding densitometry analysis (*bottom*) of homogenates from tumors excised at the end of the experiments. *C,* 3Mut and shPLCγ1 TSA cells were implanted in the mammary fat pad, and tumor growth was assessed as indicated. *, $P < 0.05$; **, $P < 0.01$. PLCγ1 levels in pSuper 3Mut and shPLCγ1 TSA cells are also shown. Equal loading was confirmed by using an anti-Akt antibody.

strongly inhibits lamellipodia formation and cell migration of SNB19 glioblastoma cell line and BT549 breast cancer cell line (31).

Among the different Rac downstream effectors, Wiskott-Aldrich syndrome protein (WASP) family proteins seems to have an important role in cell migration and metastasis (32). Therefore, PLCγ1 may regulate actin cytoskeleton reorganization through Rac and WASP family proteins. However, we cannot rule out the possibility that PLCγ1 can regulate cell migration through downstream effectors other than Rac. In this respect, it is noteworthy that PLCγ1 directly activates cofilin (33). Increasing evidences indicate that cofilin is an essential regulator of cancer motility and invasion and that its activity is regulated by a PLC (33, 34). A role for PLCγ1 in cofilin activation is supported by our observations that PLCγ1 down-regulation induces changes in GFP-cofilin intracellular localization in knockdown MDA-MB-231 cells compared with their respective control.[4] Another intriguing possibility is that PLCγ1 can regulate cell migration and invasion through modulation of ERK activation. Indeed several lines of evidence indicate that ERK is involved in cell migration, invasion, and metastasis development (28). These data, together with our observation that down-regulation of PLCγ1 expression impairs a late-stage EGF-induced ERK activation (Supplementary Fig. S2) and ERK activation in cells plated on laminin or fibronectin (Fig. 3B), strongly suggest that ERK may be another downstream effector of PLCγ1 in regulating those processes.

Our data clearly indicate that PLCγ1 plays a key role in metastasis formation. Indeed both stable and inducible PLCγ1 down-regulation results in an almost complete inhibition of breast cancer–derived lung metastasis formation. Previous work using a dominant-negative PLCγ1 had shown a partial inhibition of metastasis formation in a model of spontaneous prostate carcinoma (16), although it should be noticed that the use of this construct may induce unspecific effects. On the contrary, our strategy was based on modulating PLCγ1 levels by both stable and inducible RNAi-dependent down-regulation. We further show that down-regulation of PLCγ1 expression is able to induce metastasis regression.

These data suggest that PLCγ1 is important not only in the early steps of the metastatic cascade, such as cell migration and invasion, but also at a late stage in metastasis development. Previous work has already shown that inhibition of PLCγ1 signaling correlated with a decreased breast cancer cell proliferation rate (35). Our data suggest that PLCγ1 regulates both cell migration and, possibly, metastasis proliferation. Interestingly, this effect seems to be metastasis-specific because the primary tumor growth is only slightly affected by PLCγ1 down-regulation. Moreover, this effect is significant only when cells are injected in the mammary fat pad. One explanation would be that PLCγ1 signaling might have a key role on the cell proliferation of a subcellular population of cancer cells critical for metastasis development or that PLCγ1 controls a signaling pathway that is more important for metastatic growth than for primary tumor growth. This is further supported by our data obtained in breast cancer patients and the recent data indicating that breast cancer metastases are molecularly distinct from their primary tumors (36).

Alternatively, because our data indicate that PLCγ1 is involved in focal adhesion formation and cell interaction with microenvironment, a lack of such interactions in the absence of PLCγ1 may explain the observed regression of micrometastasis after inducible knockdown of PLCγ1. This hypothesis is supported by recent data showing that small interfering RNA knockdown of PLCγ1 in human prostate carcinoma cell line (PC3LN3) induces defect in cell adhesion (37).

In conclusion, our data strongly suggest that PLCγ1 inhibitors may have potential therapeutic applications for the clinical treatment of tumor metastasis. At present, no specific PLCγ1 inhibitors are available, and therefore, there is a stringent need to develop novel-specific PLCγ1 inhibitors.

[4] G. Sala and M. Falasca, unpublished observation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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