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Effects of Active and Inactive Phospholipase D2 on Signal Transduction, Adhesion, Migration, Invasion, and Metastasis in EL4 Lymphoma Cells

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ABSTRACT

The phosphatidylcholine-using phospholipase D (PLD) isoform PLD2 is widely expressed in mammalian cells and is activated in response to a variety of promitogenic agonists. In this study, active and inactive hemagglutinin-tagged human PLD2 (HA-PLD2) constructs were stably expressed in an EL4 cell line lacking detectable endogenous PLD1 or PLD2. The overall goal of the study was to examine the roles of PLD2 in cellular signal transduction and cell phenotype. HA-PLD2 confers PLD activity that is activated by phorbol ester, ionomycin, and okadaic acid. Proliferation and Erk activation are unchanged in cells transfected with active PLD2; proliferation rate is decreased in cells expressing inactive PLD2. Basal tyrosine phosphorylation of focal adhesion kinase (FAK) is increased in cells expressing active PLD2, as is phosphorylation of Akt; inactive PLD2 has no effect. Expression of active PLD2 is associated with increased spreading and elongation of cells on tissue culture plastic, whereas inactive PLD2 inhibits cell spreading. Inactive PLD2 also inhibits cell adhesion, migration, and serum-induced invasion. Cells expressing active PLD2 form metastases in syngeneic mice, as do the parental cells; cells expressing inactive PLD2 form fewer metastases than parental cells. In summary, active PLD2 enhances FAK phosphorylation, Akt activation, and cell invasion in EL4 lymphoma cells, whereas inactive PLD2 exerts inhibitory effects on adhesion, migration, invasion, and tumor formation. Overall, expression of active PLD2 enhances processes favorable to lymphoma cell metastasis, whereas expression of inactive PLD2 inhibits metastasis.

Phospholipase D (PLD) enzymes catalyze the hydrolysis of membrane phospholipids, yielding phosphatidic acid and a polar head group. Two forms of mammalian PLD, PLD1 and PLD2, have been characterized. These enzymes are widely expressed in mammalian tissues; individual cells usually express one or both isoforms (Meier et al., 1999). T-lymphocytes, in which PLD activity and expression levels are very low (Kinsky et al., 1989; Meier et al., 1999), are an exception.

Phorbol esters have been shown to activate both PLD1 and PLD2 in intact mammalian cells (Gibbs and Meier, 2000). Our laboratory has established that EL4, a murine lymphoma cell line, lacks detectable basal or phorbol ester-induced PLD activity (Bradshaw et al., 1996) and does not express detectable levels of mRNA for either PLD1 or PLD2 (Meier et al., 1999; Gibbs and Meier, 2000). EL4 therefore provides a useful model in which to assess the roles of PLDs in signal transduction.

The roles of PLD-generated metabolites in signal transduction are still being delineated. However, it is clear that phosphatidic acid (PA) can bind to proteins and modulate their

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ABBREVIATIONS: PLD, phospholipase D; FBS, fetal bovine serum; HA, hemagglutinin; LPA, lysophosphatidic acid; PA, phosphatidic acid; PBt, phosphatidylbutanol; PEt, phosphatidylethanol; PMA, phorbol 12-myristate 13-acetate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; PKC, protein kinase C; FAK, focal adhesion kinase; PI3K, phosphatidylinositol-3 kinase; mTOR, mammalian target of rapamycin; Erk, extracellular signal-regulated kinase; LY294002, 2-(4-morpholinyl)-8-phenyl-1(4*H*)-benzopyran-4-one hydrochloride.

activities. For example, PA can reportedly promote the membrane association and activation of Raf-1, member of the Erk mitogen-activated protein kinase cascade (Ghosh et al., 1996). Other reported protein targets of PA are phosphatidylinositol 5-kinase (Divecha et al., 2000), NADPH oxidase (Palicz et al., 2001), mTOR (Fang et al., 2001), Pak1 (Symons, 2000), and sphingosine kinase 1 (Delon et al., 2004). In addition, PA is rapidly metabolized to other lipids with biological activity. Diglyceride, produced from PA by lipid phosphate phosphatases, can activate protein kinase C (PKC) isoforms and other receptors. PA may potentially be converted to lysophosphatidic acid (LPA) via the action of PA-using forms of LPA_1 or LPA_2. LPA binds to G-protein-coupled receptors to elicit a wide range of cellular responses.

The roles of individual PLD isoforms in receptor-mediated signal transduction remain to be fully delineated. PLD1 is dependent on small GTP-binding proteins (e.g., ARF and Rho) for activity, is localized to intracellular membranes, and participates in vesicle trafficking (Cockcroft, 2001). PLD2 was described originally as a constitutively active enzyme localized to the plasma membrane (Colley et al., 1997). Subsequent studies have shown that PLD2 can be activated in intact cells by agonists and can be regulated by small GTP-binding proteins (Hiroyama and Exton, 2005), heterotrimeric GTP-binding proteins (Preininger et al., 2006), and PKC isoforms (Chen et al., 2004). Overexpression of PLD2 can result in changes in cytoskeletal organization (Colley et al., 1997). The mechanistic basis for these changes has not yet been fully established. Independent of catalytic activity, PLD2 can participate in protein-protein interactions via its phox homology domains (Lee et al., 2006).

In the current study, we use EL4 to examine the role of PLD2 in signaling. PLD2 was expressed in EL4 cells and characterized. Effects on cell phenotype were investigated. The results show that active PLD2 promotes FAK and Akt phosphorylation and cell-substrate adhesion. Inactive PLD2 exerts inhibitory effects on proliferation, adhesion, migration, invasion, and tumor formation.

Materials and Methods

Cell Culture. Clonal EL4 cell lines were maintained as described previously (Ku and Meier, 2000) in RPMI 1640 medium with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Norcross, GA). All were grown on standard tissue culture plastic, except for WT2, which was grown on suspension culture plastic (Corning, Acton, MA). Stably transfected cell lines were maintained in the presence of 0.25 mg/ml G418 (Calbiochem, San Diego, CA). Du145 and OVCAR-3 cells were grown in RPMI 1640 medium with 10% FBS, PC3 cells in F12K with 10% FBS, and PC12K cells (on Primaria plates) in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS.

Vector Construction and Transfections. PLD2 was cloned from total RNA extracted from PC-3 cells (human prostate carcinoma) by reverse transcription-polymerase chain reaction. The resulting full-length construct was subcloned into a mammalian expression vector driven by the CMV promoter (PBSK+CMV). During subcloning, a 9-amino acid hemagglutinin (HA) tag was inserted between the second and third amino acids of PLD2. The HA-PLD2 construct was subsequently subjected to site-directed mutagenesis using a PCR-based QuikChange kit (Stratagene, La Jolla, CA) to create a substitution from lysine to arginine in the catalytic domain of PLD2 at amino acid 758, generating a catalytically inactive protein. After sequence confirmation, constructs were introduced into EL4 cells using electroporation. Before transfection, 100 μg of DNA for either catalytically active or inactive HA-PLD2 was added to a cell suspension with 10 μg of vector DNA encoding neomycin resistance, driven by the RSV promoter. The cell suspension (2×10^7 cells in 0.4 ml of PBS) was subjected to electroporation using a BTX Electro Cell Manipulator with parameters of 140 V, 720 Ω, and 3175 μF across a BTX cuvette with 0.2 cm electrode gap (Harvard Apparatus Inc., Holliston, MA). Pulse lengths varied between 25 and 28 ms. Cells were placed in fresh medium for 48 h at 37°C before 1 mg/ml G418 was added. Clonal cell lines were isolated after limiting dilution on 96-well tissue culture plates.

Immunoblotting. After treatment, clonal EL4 cells were collected by centrifugation at 1200*g*. Adherent cells were harvested by scraping before the centrifugation. Cells were lysed in a lysis buffer containing 20 mM Tris, pH 7.4, 1% Triton X-100, 150 mM NaCl, 1 mM EGTA, 30 mM sodium pyrophosphate, 100 μM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. Extracts were sedimented at 10,000*g* for 10 min at 4°C to remove insoluble material. For some experiments, this Triton-insoluble material was further solubilized using lysis buffer supplemented with 1% SDS and 0.5% deoxycholate. Samples equalized for protein (100 μg), as determined by Coomassie Protein Assay (Pierce, Rockford IL), were separated by SDS-PAGE on 12.5% Laemmli gels, transferred to polyvinylidene difluoride paper, incubated with primary antibodies, and developed using enhanced chemiluminescence (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) (Ku and Meier, 2000). Blots were imaged by densitometry and quantified using NIH Image software (http://rsb.info.nih.gov/nih-image/). Antibodies were obtained from the following suppliers: actin, BD Transduction Laboratories (Lexington, KY); phospho-Akt, Cell Signaling Technology Inc. (Danvers, MA); phospho-ERK, Promega (Madison, WI); Erk1, Santa Cruz Biotechnology (Santa Cruz, CA); FAK, Santa Cruz Biotechnology and BD Transduction Laboratories; phospho-FAK (Tyr397), BD Transduction Laboratories and Biosource International (Camarillo, CA); phospho-FAK (Tyr861), Biosource International; phosphotyrosine, Transduction Labs; and HA, BAbCO (Richmond, CA).

PLD Activity Assays. PLD activity was analyzed as described previously (Meier and Gibbs, 1999). In brief, for intact cell assays, cells were cultured for 18 h in 35-mm dishes in 2 ml of DMEM containing 5 μCi/ml [^{3}H]palmitic acid (PerkinElmer Life and Analytical Sciences, Waltham, MA). For the experiment shown in Fig. 1C, cells were incubated in flasks containing 10 μCi/ml [^{3}H]palmitic acid. Washed cells were incubated in fresh DMEM with 10 μM ionomycin, 1 μM okadaic acid, or 100 nM 12-myristate 13-acetate (PMA) for 30 or 60 min at 37°C in the presence of 0.5% ethanol. Phosphatidyl ethanol was extracted, separated by thin-layer chromatography, and quantified by liquid scintillation spectrometry. Data were normalized to the total counts per minute recovered from each sample and then analyzed using Prism and Instat software (GraphPad Software Inc., San Diego, CA). Membrane PLD activity was assessed using the fluorescent substrate BODIPY-phosphatidylcholine (Invitrogen, Carlsbad, CA). Products were separated by thin-layer chromatography and then imaged and quantified using a FluorImager (GE Healthcare).

Cell Proliferation, Spreading, Adhesion, Invasion, and Migration Assays. For proliferation assays, EL4 cell lines were seeded at a density of 1×10^5 cells/ml in triplicate 35-mm plates. At various times, cells were collected, and trypan blue was added. Live cells were counted using a hemacytometer. For assessment of cell spreading, cells were seeded on standard tissue culture plastic and incubated for 24 h. Cells were photographed using a Nikon Diaphot microscope equipped with interference contrast optics. Major/minor axis ratios were assessed using NIH Image software. For assessment of adhesion rates, cells were detached from tissue culture plates using trypsin and then allowed to reattach to fresh plates in fresh medium. At various times, unattached cells were removed by rinsing three times with PBS. The remaining attached cells were removed using trypsin and counted using a hemacytometer. Cell invasion was

assessed using a modified Boyden chamber method. Equal numbers (2×10^5) of EL4 cells were seeded in serum-free medium in the upper chamber of an 8-μm transwell insert (BD Biosciences, San Jose, CA) coated with 20 μg of Matrigel (BD Biosciences). Medium with 10% FBS was added to the lower wells. The insert membranes were fixed and stained using the Diff-Quik dye kit (Dade Behring Corporation, Deerfield, IL). Cells that invaded into the lower chambers were counted by microscopy. Migration assays were conducted using the same protocol, except that the insert was not coated with Matrigel. In some cases, 25 μM LY294002 was included in the migration assay.

Experimental Metastasis Studies. These experiments were carried out using a protocol described previously (Zhang et al., 1998) and approved by the Institutional Animal Care and Use Committee of the Medical University of South Carolina. Male C57BL/6 mice (National Cancer Institute, Bethesda, MD) were used at 4 weeks of age, with six to seven animals in each group. EL4 cell lines (briefly grown without G418) were collected by centrifugation and resuspended in sterile phosphate-buffered saline. An aliquot of 0.1 ml, containing 3×10^4 cells, was injected into the tail vein of each animal. PBS alone was injected into control animals. Two types of experiments were performed. In survival experiments, mice were maintained until they died or showed signs of distress. In an alternative protocol, all animals were sacrificed 20 days after injection. In both experiments, necropsies were performed to examine the location of tumors, the number of tumors, liver weight, and liver appearance.

Results

Characterization of HA-PLD-Transfected EL4 Cells. For this study, a HA-tagged human PLD2 construct was engineered in a mammalian expression vector driven by the CMV promoter. Full-length PLD2 was generated by reverse transcription-polymerase chain reaction from RNA prepared from PC-3, a human prostate cancer cell line. The resulting DNA was sequenced. There were two conservative differences in amino acid sequences between this DNA and the human sequences entered in GenBank previously: A589G and T869A. These single amino acid substitutions occur in nonhomologous regions, and their significance (if any) is unknown. A single, nine-amino acid HA tag was introduced at the N terminal. A catalytically inactive form of PLD2 (Sung et al., 1997) was generated by site-directed mutagenesis of the catalytic site (K758R) using the same vector.

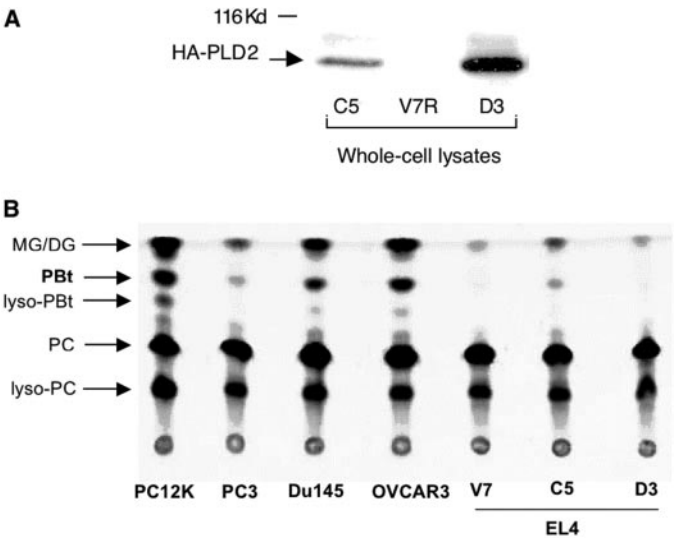

Fig. 1. Stable transfection of PLD2 in EL4 cells. A, EL4-V7 cells were stably transfected with HA-PLD2 (C5), empty vector (V7R), or HA-PLD2-K758R (D3). Membrane extracts, equalized for protein, were immunoblotted using anti-HA antibody. B, membrane PLD activity was assessed using an in vitro assay with a fluorescent substrate in membranes from V7, C5, and D3 EL4 cell lines and from PC12K (rat pheochromocytoma), PC-3 (human prostate cancer), Du145 (human prostate cancer), and OVCAR-3 (human ovarian cancer) cells; equal amounts of membrane protein were used in each assay. PBt production indicates PLD2 activity (Xie and Meier, 2002).

The HA-PLD2 construct, along with a neomycin resistance gene, was introduced by electroporation into EL4-V7 cells. Phorbol ester-induced signal transduction has been extensively studied in EL4 cells, which exists in variants that are either sensitive or resistant to PMA. A series of clonal EL4 cell lines of both phenotypes has been developed and characterized in our laboratory (Ku and Meier, 2000). The V7 cell line is representative of several PMA-resistant EL4 clones. With respect to EL4 cells, PMA resistance refers to a lack of effect of PMA on long-term growth and survival. Sensitive cells undergo growth arrest and death in response to PMA, whereas resistant cells do not (Sansbury et al., 1997). PMA sensitivity is conferred by expression of RasGRP, a phorbol ester binding protein (Han et al., 2007); PMA-induced cell death is dependent on RasGRP-mediated Erk activation (Sansbury et al., 1997). Both sensitive and resistant cells activate PKC isoforms in response to PMA (Ku and Meier, 2000). Stable transfectants were selected for growth in G418 and were then cloned by limiting cell dilution. The resulting clones were screened for PLD2 expression using an in vitro assay for membrane PLD2 activity (Meier and Gibbs, 1999). We selected a cell line designated clone C5, which expressed PLD2 activity, for subsequent characterization. Two other stable V7 transfectants used in this study were V7R (empty vector) and D3 (catalytically inactive HA-PLD2).

As shown in Fig. 1A, an immunoblot for HA detected full-length HA-PLD2 protein (105 kDa) in C5 and D3 cells but not in V7R cells. The catalytically inactive protein (i.e., in D3 cells) was expressed at higher levels than the active protein (i.e., in C5 cells). Both active and inactive HA-PLD2s were most abundant in the membrane, with very little detected in the cytosol (data not shown).

Results presented in Fig. 1B show that membranes prepared from C5 cells exhibited PLD2 activity in an in vitro fluorescent assay in which PLD activity is assessed by production of phosphatidylbutanol (PBt). Membranes from V7 and D3 cells had no detectable PLD activity, consistent with our previous studies of untransfected EL4 cells (Bradshaw et al., 1996). PLD2 activity was present predominantly in the membrane fraction of C5 cells, with very little in the cytosolic fraction (data not shown). It is interesting that despite the use of the strong CMV promoter in the expression construct, the level of PLD2 catalytic activity observed in C5 membranes was modest compared with that seen in several cell lines expressing endogenous PLD2 (PC12K, Du145, and OVCAR-3). However, the level of activity was comparable with that seen in PC-3, a prostate cancer cell line that has been used by our group to study agonist-activated PLD2 activity (Xie et al., 2002). The relatively low level of PLD activity could suggest that high levels of PLD2 are deleterious to EL4 cells, and that HA-PLD2 was integrated in such a manner that the function of the CMV promoter was compromised. On the other hand, the transfected cells may use compensatory mechanisms to keep PLD activity in check.

The ability of HA-PLD2 to confer agonist-induced PLD activity to intact cells was tested, measuring phosphati-

dylethanol (PEt) as the PLD reaction product. Phosphatidylcholine-using PLDs carry out a transphosphatidylation reaction in the presence of simple alcohols, generating a unique phosphatidyl alcohol product that is observed only when the alcohol is present. In untransfected V7 cells, radioactivity recovered as PEt from cells incubated in the presence of ethanol was not significantly higher than that seen in the absence of ethanol, and thus represented assay background (Fig. 2C). Thus, these results indicate an absence of detectable basal PLD activity, consistent with our previous results for EL4 and other cell lines using this method (Gibbs and Meier, 2000). Likewise, basal PLD activity was undetectable in C5 and D3 cells. When C5 cells were treated with PMA, an increase in PEt production was observed (Fig. 2A), indicative of PLD activity. Further illustration of PMA-stimulated PLD activity is presented in Fig. 2C. In this experiment, using only C5 cells, the dual controls of samples without ethanol added (background counts) and with ethanol alone are presented. In this particular experiment, there was a trend toward increased counts in samples incubated with ethanol alone, compared with samples without ethanol, but the difference was not statistically significant. Thus, as mentioned above, we are unable to detect basal PLD activity using these assay conditions. The difference between the control samples without ethanol and the samples treated with PMA in the presence of ethanol, was statistically significant ($p < 0.01$), demonstrating expression of active PLD2. In the same experiment (data not shown), there were no significant differences between any of the three experimental treatments in V7 or D3 cells, again indicating that these cells do not express detectable PLD activity. Thus, the PLD2 expressed in C5 cells is active and responsive to stimulation by PMA.

The ability of HA-PLD2 to be activated by other agonists was explored in HA-PLD2 transfectants. As shown in Fig. 2B, PLD activation was detected after treatment of C5 cells for 30 min with ionomycin (calcium ionophore) or for 30 or 60 min with okadaic acid (serine/threonine phosphatase inhibitor). The increases seen with these agonists were similar to those induced by PMA. The response to concomitant treatment with PMA and ionomycin was comparable with that seen with either agonist alone; no synergistic or additive effects were noted.

In summary, HA-PLD2 was successfully expressed in EL4 cells. The enzyme localizes to membranes, confers PLD activity, and is regulated by agonists.

Signal Transduction Phenotype of PLD2-Expressing EL4 Cells. The signaling phenotype of the C5 cell line was examined and compared with transfected cell lines lacking active PLD2. We first tested whether active PLD2 alters Erk mitogen-activated protein kinase activation in EL4 cells. The effects of PMA and okadaic acid on Erk activation have been characterized previously in EL4 cell lines (Ku and Meier, 2000). As shown in Fig. 3, PMA was able to induce Erk activation to a similar extent in V7 and C5 cells. No differences were detected in either the magnitude or time course of the response between the two cell lines. Okadaic acid induced a low level of Erk activation that was likewise similar between V7 and C5 cells. Thus, although PMA stimulates PLD2 activity in C5 cells, this response does not enhance PMA-induced Erk activation.

To further characterize the role of PLD2 in cell signaling, tyrosine phosphorylation studies were performed. C5 and V7

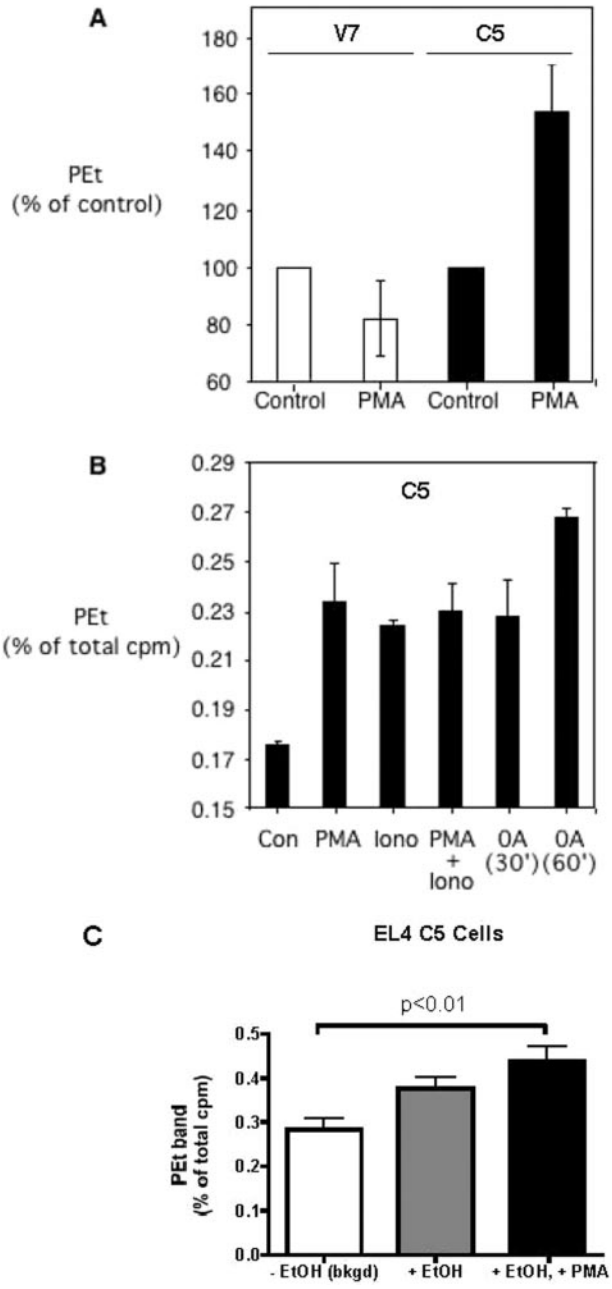

Fig. 2. Effects of agonists on PLD2 activity in EL4 cells. A, PLD activity was assessed in untransfected EL4-V7 cells or HA-PLD2-transfected EL4-C5 cells. Intact metabolically labeled cells were incubated with 100 nM PMA or vehicle control in the presence of 0.5% ethanol for 30 min. PEt production was measured as described under *Materials and Methods*. PEt was calculated as a percentage of total radioactivity recovered; note that the control value reflects the assay background (see text). Next, data were normalized to the value obtained for untreated V7 cells. Each data point represents mean ± S.D. of values obtained from three separate experiments. B, EL4-C5 cells were treated with 100 nM PMA, 10 μM ionomycin, 1 μM okadaic acid, and a combination of PMA and ionomycin in the presence of 0.5% ethanol for 30 min (unless otherwise indicated). PLD activity was assessed in intact cells as described above but is presented as a percentage of the total radioactivity recovered. Each data point represents mean ± S.D. from triplicate samples of cells. C, labeled C5 cells were incubated for 30 min, as described above, in the absence of ethanol, in the presence of 0.5% ethanol alone, or in the presence of ethanol and 100 nM PMA. Each data point represents mean ± S.E.M. from triplicate or quadruplicate samples of cells and is expressed as the percentage of total radioactivity recovered in the PEt band. Statistical significance was assessed by analysis of variance using InStat software (GraphPad Software).

cells were incubated with and without 100 nM PMA (15 and 30 min). Whole-cell extracts were immunoblotted for phosphotyrosine (Fig. 4A). PMA increased phosphorylation of multiple substrates in both cell lines, with no obvious differences in PMA response between V7 and C5 cells. However, a consistent increase in basal phosphorylation of a 125-kDa protein was observed in C5 cells. FAK, a prominent 125-kDa tyrosine kinase, is expressed in PMA-resistant EL4 cells, including V7 (S. M. Knoepp, H. Ku, H. M. Sansbury, Y. Xie, M. S. Chahal, Z. Zhang, S. Tomlinson, and K. E. Meier, submitted). We performed immunoblots for phospho-FAK (Tyr397); phosphorylation of this site is associated with increased catalytic activity of FAK. Figure 4B shows that FAK is phosphorylated to a greater extent, under basal serum-starved conditions, in C5 cells than in V7 (active PLD2) or D3 (inactive PLD2) cells. Levels of p-FAK in V7R (vector control) cells are similar to those in V7 (data not shown).

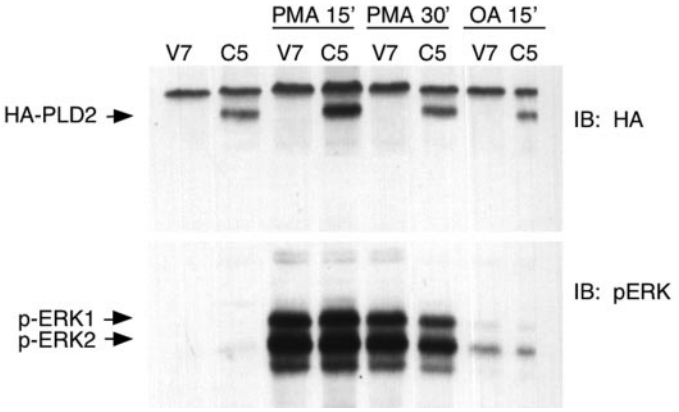

Fig. 3. Effects of active PLD2 on Erk activation in EL4 cells. V7 and C5 cells were incubated with or without 100 nM PMA for 15 or 30 min, or with 1 mM okadaic acid for 15 min. Whole-cell extracts, equalized for protein, were separated by SDS-PAGE and then immunoblotted for HA (top half of gel) and phospho-Erk (bottom half of gel). The uppermost band is a nonspecific band recognized by the anti-HA antibody; this serves as a loading control.

The effects of PLD2 expression on FAK phosphorylation were examined in more detail. The time course of the effects of PMA on phosphorylation of FAK and Erk is presented in Fig. 4C. PMA produced a slight increase in FAK phosphorylation in both cell lines. This may reflect PMA-induced redistribution of FAK from the detergent-insoluble to detergent-soluble fraction, as seen by a slight increase in the apparent level of total FAK. As shown previously, PMA induced ERK phosphorylation similarly in V7 and C5 cells with respect to both magnitude and time course. Taken together, our results indicate that active PLD2 has a positive effect on basal FAK phosphorylation in EL4 cells.

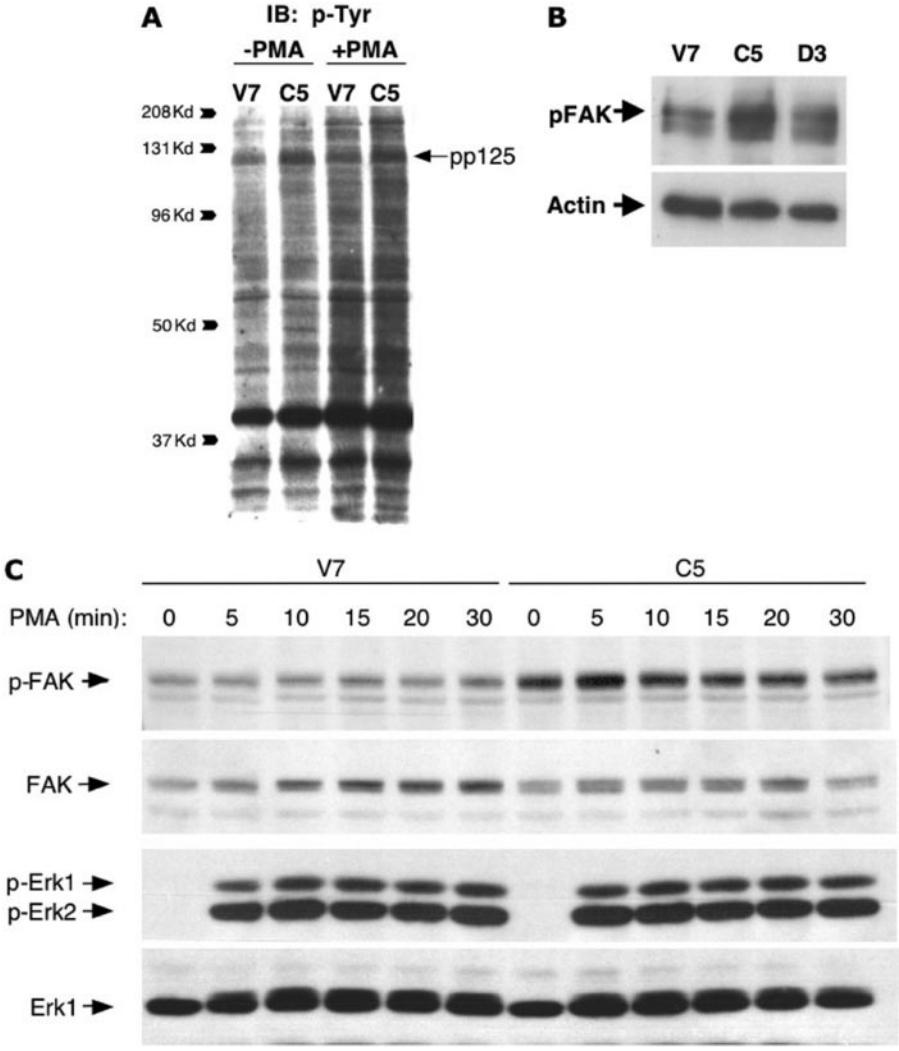

Fig. 4. Effects of active and inactive PLD2 on FAK phosphorylation in EL4 cells. A, V7 and C5 cells were incubated with or without 100 nM PMA for 15 min. Whole-cell extracts, equalized for protein, were immunoblotted for phosphotyrosine. B, whole-cell extracts of serum-starved V7, C5, and D3 cells, equalized for protein, were immunoblotted for phospho-FAK (tyrosine 327) and actin. C, V7 and C5 cells were incubated for the indicated times with 100 nM PMA. Whole-cell extracts, equalized for protein, were immunoblotted for phospho-FAK (397), total FAK, phospho-Erk, and Erk1.

FAK has been shown to activate the PI3K/Akt pathway (Xia et al., 2004). Akt is another protein kinase involved in cell proliferation, survival, and motility. An antibody to the phosphorylated active form of Akt was used to assess Akt activation (Fig. 5). V7 cells exhibit basal Akt phosphorylation; the level of phospho-Akt is elevated in C5 cells expressing active PLD2. The extent of the increase, as quantified by densitometry from separate experiments, is approximately 2-fold. Basal p-Akt levels in D3 cells expressing inactive PLD2 are similar to or lower than that of the parental V7 cell line; a consistent decrease was not observed. Thus, expression of active PLD2 enhances basal Akt activation, whereas inactive PLD2 has no effect on basal Akt activation.

In summary, the signal transduction studies show that active PLD2 has no effect on Erk activation, but enhances FAK phosphorylation and Akt activation. Inactive PLD2 does not affect basal FAK or Akt phosphorylation.

Effects of Active and Inactive PLD2 on EL4 Cell Proliferation. Turning to longer-term cellular responses, we examined proliferation rates for the EL4 cell lines of interest. V7 and C5 proliferated at similar rates (Fig. 6), as did V7R (data not shown). However, the proliferation rate for D3 was significantly reduced at 48 h compared with V7 ($p < 0.01$, Student's t test). Additional studies (data not shown) indicated that proliferation of V7, V7R, and C5 cells was unaffected by 100 nM PMA. Thus, active PLD2 has neither a favorable nor an adverse effect on the proliferation of transfected V7 cells and does not alter sensitivity to PMA. In contrast, expression of inactive PLD2 is associated with a reduced proliferation rate in D3 cells. This was the first observation indicating that inactive PLD2 could exert inhibitory effects on EL4 cell function.

Adhesion, Migration, and Invasion Phenotypes of PLD2-Expressing EL4 Cells. In view of the differences in FAK phosphorylation between cell lines, potential differences in adhesion were assessed. Cells were detached from dishes, allowed to reattach, and then washed and counted. No differences in the kinetics of reattachment were observed between C5 and V7R (Fig. 7). Both cell lines attached readily, similar to the parental V7 cell line (Ku and Meier, 2000). In contrast, D3 cells expressing inactive PLD2 adhered less readily than C5 or V7R cells.

We next examined the morphology of attached cells, using interference contrast photomicroscopy. As shown in Fig. 8A, C5 cells spread on tissue culture plastic to a greater extent than V7, V7R, or D3 cells. Quantification of cell elongation is presented in Fig. 7B, with PMA-sensitive clone WT2 included for purposes of comparison. As described previously (Sansbury et al., 1997; Ku and Meier, 2000), PMA-resistant clones (e.g., V7) attach and spread on tissue culture plastic to a greater extent than PMA-sensitive (e.g., WT2) clones. This is reflected by a decrease in the proportion of V7 cells having an axis ratio of 1 to 2 (rounded) and an increase in cells having ratios of greater than 2 (elongated) compared with WT2 cells. The differences in morphology between the transfected cell lines (Fig. 8B) were striking. A high percentage of C5 cells were elongated, with an axis ratio of greater than 3. V7R cells (vector control) were similar to V7 cells. It is interesting that clone D3 showed a pronounced increase in rounding compared with V7 cells.

The effects of PLD2 expression on cell migration were analyzed. In this assay, cells were incubated in serum-free medium in the top portion of a Boyden chamber with an 8-μm insert. The bottom chamber contained either serum-free medium or medium with 10% fetal bovine serum. As shown in Fig. 9A, V7 and C5 had similar levels of basal migratory activity in the absence of serum. Basal migration was lower ($p < 0.05$) in D3 than in V7 cells. It is most striking that serum significantly increases migration of V7 and C5 cells but not D3 cells. The effects of LY294002, a PI3K inhibitor, were tested. As shown in Fig. 9B, LY294002 inhibited the

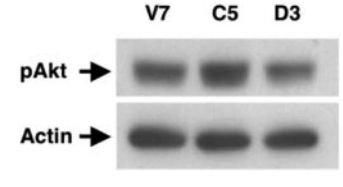

Fig. 5. Effects of active and inactive PLD2 on basal Akt phosphorylation in EL4 cells. Whole-cell extracts were prepared from serum-starved V7, C5, and D3 cells. Equal amounts of protein were immunoblotted for phospho-Akt and actin.

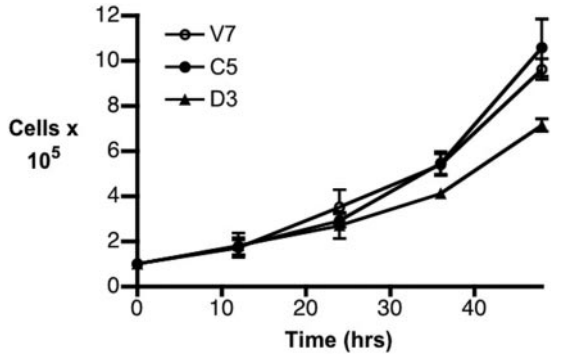

Fig. 6. Effects of active and inactive PLD2 on proliferation of EL4 cells. The indicated EL4 cell lines (V7, untransfected; C5, active HA-PLD2-transfected; D3, inactive HA-PLD2 transfected) were seeded at a density of 1×10^5 cells/well. Live cells were counted by hemacytometer at the indicated times. Each data point represents mean ± S.D. from triplicate wells of cells.

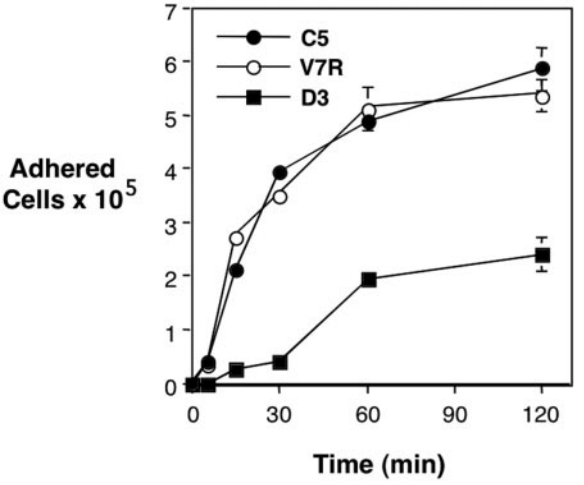

Fig. 7. Effects of active and inactive PLD2 on adhesion of EL4 cells. The indicated cell lines were detached from tissue culture plates using trypsin and then seeded at a density of 1×10^6 cells/well on fresh tissue culture plastic in complete medium. At the indicated time points, attached cells were rinsed three times in PBS, detached with trypsin, and counted using a hemacytometer. Each data point represents the mean ± S.D. of values from triplicate wells.

response to serum in V7, C5, and D3 cells; only inhibition of C5 was statistically significant ($p < 0.001$), which is probably due to the greater extent of migration in this cell line. These results suggest that serum-induced migration is dependent on the PI3K/Akt pathway. Finally, the ability of EL4 cell lines to invade through Matrigel was assessed. The data presented in Fig. 9C show that, in response to serum, C5 cells invade to a significantly greater extent ($p < 0.05$) than do V7 cells. D3 cells do not exhibit an invasive response to serum, indicating that inactive PLD2 inhibits cell invasion.

In summary, catalytically active PLD2 enhances cell spreading and invasion without significantly altering migration or proliferation. In contrast, catalytically inactive PLD2 inhibits spreading, adhesion, migration, invasion, and proliferation.

Experimental Metastasis Studies. EL4 cells can generate liver tumors when injected into the tail vein of syngeneic mice (Zhang et al., 1998). We used this in vivo model to further explore the effects of PLD2 transfection on EL4 cell phenotype. Figure 10A presents the results of a survival study. Survival times for mice injected with V7 and C5 cells were not significantly different (median survival of 21.5 and 23 days, respectively). In contrast, mice injected with D3 cells survived longer compared with those injected with V7 cells (median survival of 28 days; $p = 0.0006$). As described pre-

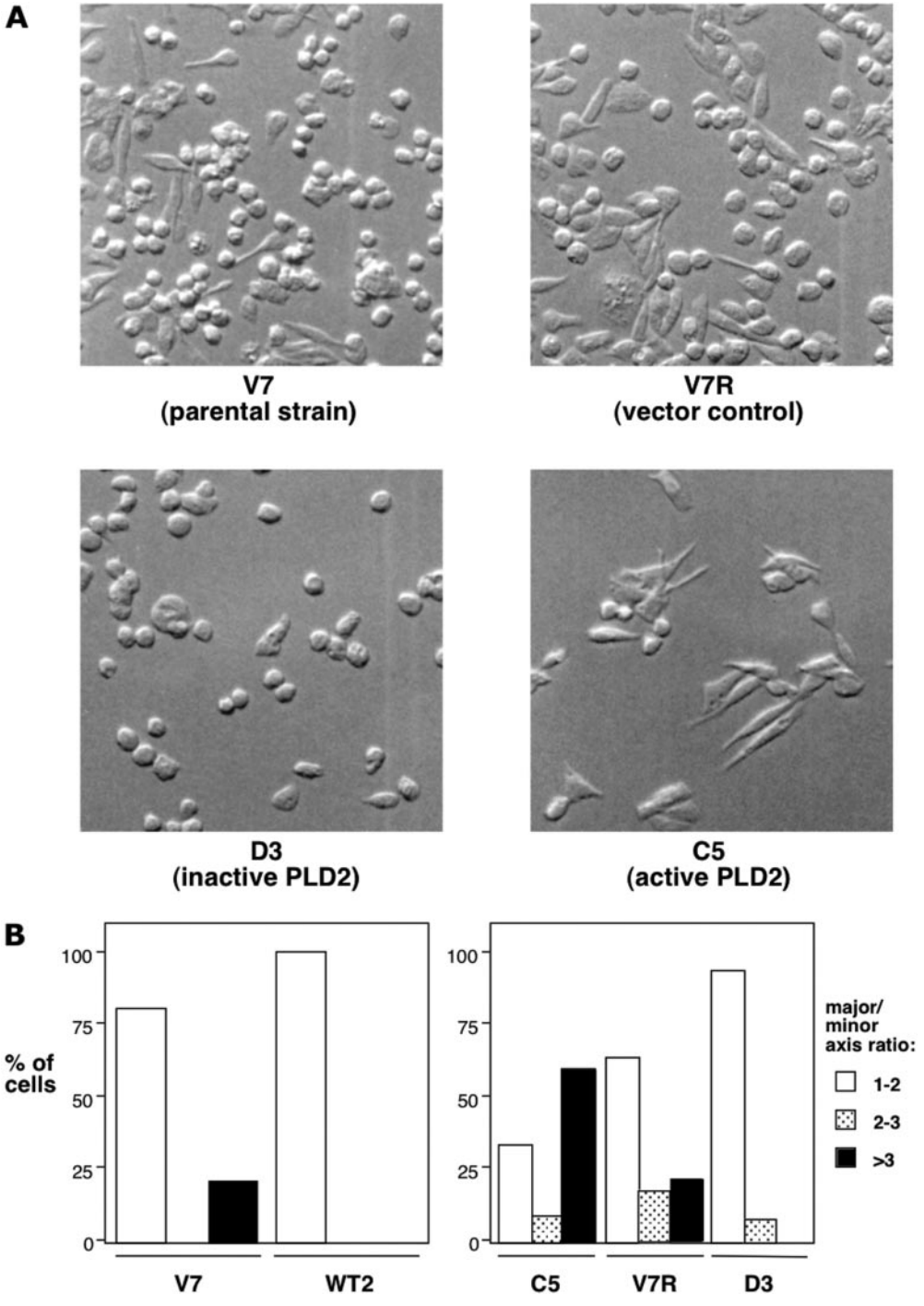

Fig. 8. Effects of active and inactive PLD2 on morphology of EL4 cells. The indicated cell lines were seeded on standard tissue culture plastic. After 1 day in culture, cells were photographed using interference contrast microscopy. A, micrographs of the cells are shown. B, micrographs were analyzed using NIH Image. The ratio of the major to minor axis of each cell was quantified as an index of cell spreading/elongation. Each data point represents the mean value for 35 cells. An index of 1 indicates a round cell; an index of greater than 1 indicates an elongated cell. The left portion of the bottom panel presents data from EL4 cell lines characterized previously (V7, PMA-resistant; WT2, PMA-sensitive); the right portion presents data from transfected EL4-V7 cell lines developed herein (C5, HA-PLD2; V7R, vector control; D3, catalytically inactive HA-PLD2).

viously by others (Zhang et al., 1998), the major site for metastasis is the liver. Figure 10B presents a photograph of livers removed from mice 20 days after injection of PBS ("Control"), V7, C5, or D3 cells. Metastatic nodules are visible on the surface of the livers from mice receiving all three cell types. It is apparent from the figure that liver size is greatest in animals injected with C5 cells. Injection of D3 cells resulted in tumors, but liver size was less than for V7. In addition, hemorrhaging (i.e., red color) was qualitatively less in livers from D3-injected mice. The next two figure parts present quantitative analyses of tumor formation. As shown in Fig. 10C, C5 cells generated a slightly (but not significantly) greater tumor burden (liver mass) than did V7 (p = 0.1), whereas injection of D3 resulted in less tumor burden than for V7 (p = 0.01). Finally, in Fig. 10D, the number of tumor nodules was compared between treatment groups. Compared with V7, nodule number was higher with C5 (p = 0.02). Compared with C5, nodule number was much lower with D3 cell injection (p = 0.00002). The results for D3 were not significantly different from the PBS control. Taken together, these data establish that inactive PLD2 inhibits tumor formation in EL4 cells.

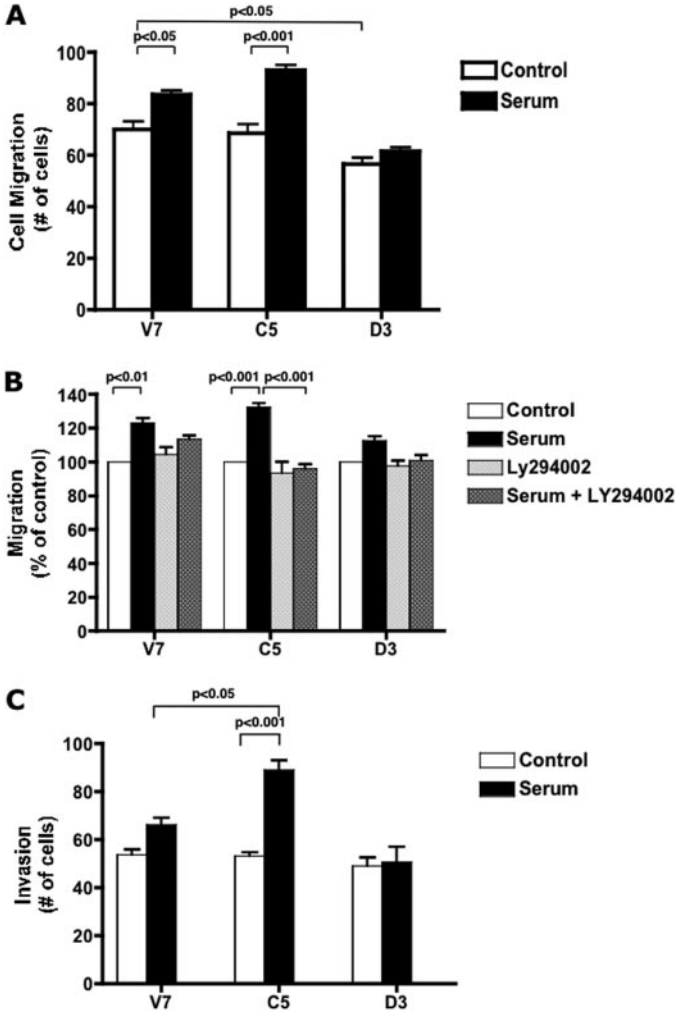

Fig. 9. Effects of PLD2 on migration and invasion of EL4 cells. Equal numbers (2×10^5) of EL4 cells were plated in the upper chamber of an 8-μm transwell insert (BD Biosciences). For the migration assay, the following chemoattractants were added to the bottom chamber: control, serum-free medium; serum, medium with 10% FBS (serum); LY294002, serum-free medium containing 25 μM LY294002; serum + LY294002, medium with 10% FBS containing 25 μM LY294002 (A and B). After 24 h, migrated cells were stained and counted. Data are plotted as the number of cells migrated (A) or as migrated cells as a percentage of untreated control for each cell line (B). Each data point represents mean ± S.D. for two wells of cells (A) or for two separate experiments, each using duplicate wells of cells (B). For the invasion assay (C), the transwell insert was coated with Matrigel (BD Biosciences). Chemoattractants were added to the bottom chamber as follows: control, serum-free medium containing 0.1% bovine serum albumin; serum, medium containing 10% FBS. After 24 h, invaded cells were stained and counted. Data are plotted as the number of cells invaded; each data point represents mean ± S.D. for three wells of cells. For all results, statistical significance was assessed by analysis of variance using InStat software (GraphPad Software).

Discussion

The work described herein has examined several aspects of the role of PLD2 in mammalian cells. Our studies address the regulation of PLD2, its effects on signal transduction, and its effects on cell phenotype. In general, the major findings are that PLD2 enhances processes favorable to tumor formation in EL4 cells, whereas inactive PLD2 inhibits some such processes and also suppresses metastasis. The cellular effects observed with active PLD2 are associated with higher basal levels of FAK and Akt phosphorylation. However, because inactive PLD2 does not reduce basal FAK or Akt phosphorylation, the molecular basis for its inhibitory effects remains to be investigated.

Cell lines stably overexpressing PLD isoforms have, anecdotally, been difficult to generate. One report indicates that inducible overexpression of either PLD1 or PLD2 in fibroblasts is proapoptotic (Zhong et al., 2002). In contrast, fibroblasts stably overexpressing either PLD1 or PLD2 exhibit a transformed phenotype (Min et al., 2001). It is noteworthy that fibroblasts express endogenous PLD1 and PLD2. A study of overexpression of PLD1 and PLD2 in Jurkat T-cells, using transient transfection, concluded that PLD1 (but not PLD2) impairs mitogenic signaling (Diaz et al., 2005). The Jurkat cell line used expresses endogenous PLD2 but low levels of PLD1. The EL4 cells used herein express undetectable PLD1 and PLD2; stably transfected EL4 cells have modest levels of PLD2, on the lower end of the range of endogenous levels seen in other cell types. Thus, the model systems used for PLD2 transfections differ in several important respects.

The regulation of PLD2 in intact cells was initially a matter of debate, because this isoform has higher constitutive activity than PLD1 in vitro. An early report indicated that PLD2 was constitutively active when overexpressed in COS-7 cells, although modest activation was observed in response to PMA in this study (Colley et al., 1997). Later work by our group (Gibbs and Meier, 2000) and others showed that PLD2 is activated in response to PMA. Subsequent studies have shown that PLD2 can be activated via small GTP binding proteins (Kim et al., 2003), epidermal growth factor receptor (Slaaby et al., 1998), and PKCα (Chen and Exton, 2004). Furthermore, PLD2 can be inhibited by heterotrimeric G-proteins (Preininger et al., 2006), and via a G-protein-coupled receptor (Ella et al., 1997). Thus, it is now clear that PLD2 activity is regulated in intact cells. The present study confirms that PLD2 is activated in response to phorbol ester and shows that ionomycin and okadaic acid can stimulate PLD2 activity. The pathways mediating these effects remain to be determined. Calcium-mobilizing agonists

were shown previously to activate both PLD1 and PLD2 in Sf9 cells overexpressing these enzymes (Siddiqi et al., 2000). Okadaic acid, a serine/threonine phosphatase inhibitor, can increase phosphorylation of numerous proteins and thereby activate multiple signaling pathways. Phosphorylation of PLD2 by PKC isoforms, src, or src-related kinases may play a role in its regulation (Ahn et al., 2003; Choi et al., 2004). PKCα-mediated phosphorylation may play a role in inactivating PLD2, whereas direct association with PKCα may be involved in PLD2 activation (Chen and Exton, 2004). EL4 cells express PKCα and other PKC isoforms (Sansbury et al., 1997). Serine/threonine phosphorylation of PLD2 (Chen and Exton, 2004) has been shown to result in mobility shifts on SDS-PAGE. We observed slight gel shifts for both catalytically active and inactive PLD2 in transfected EL4 cells after PMA treatment (e.g., Fig. 4B). The significance of these putative phosphorylation events remains to be determined.

Our study has shown that PLD2 can play a positive role in FAK phosphorylation. PA can stimulate the activities of both phosphatidylinositol phosphate 5-kinase (Fang et al., 2001) and Pak1 (Symons, 2000), which are involved in membrane ruffling and cell spreading, respectively. These proteins may interact with FAK either directly or indirectly through a focal adhesion-localized protein. Mechanisms involving the PI3K/Akt pathway are also possible, as discussed below.

PLD2 activity has been positively correlated with Akt activation in other cell types in a pathway that may involve PA metabolites (Li and Malik, 2005). Consistent with these findings, we observed an increase in basal Akt phosphorylation in cells expressing active PLD2 and a decrease in cells expressing inactive PLD2. It is possible that FAK acts as an intermediate between PLD2 and PI3K activation (Xia et al., 2004). PLD2 may also act downstream of Akt. A direct interaction of PLD2 with raptor, a partner of mTOR, has been demonstrated to activate mTOR in a manner dependent on PLD2 catalytic activity (Heo et al., 2006).

The role of PLD2 in adhesion and cytoskeletal events is of particular interest. An early report characterizing PLD2 described its localization to the plasma membrane and showed that overexpression of PLD2 induced morphological reorganization (Colley et al., 1997). In the same study, it was also observed that PLD2 was redistributed upon serum stimulation. A role for activation of unspecified PLDs in sphingosine-1-phosphate-induced stress fiber assembly has been reported (Porcelli et al., 2002). In an opposing example, overexpression of catalytically active (but not inactive) PLD2 decreased ATP-induced FAK activation and paxillin phosphorylation in PC12 cells (Bae and Ryu, 2001). Another group reported that elevated expression of PLD2 increases cell protrusions in v-Src-transformed fibroblasts, with catalytically inactive PLD2 reducing protrusions (Shen et al., 2002). Both PLD1 and PLD2 can associate with cytoskeletal components. In one study, PLD2 was shown to interact directly with β-actin, resulting in PLD inhibition (Lee et al., 2001). With respect to adhesion, one group has

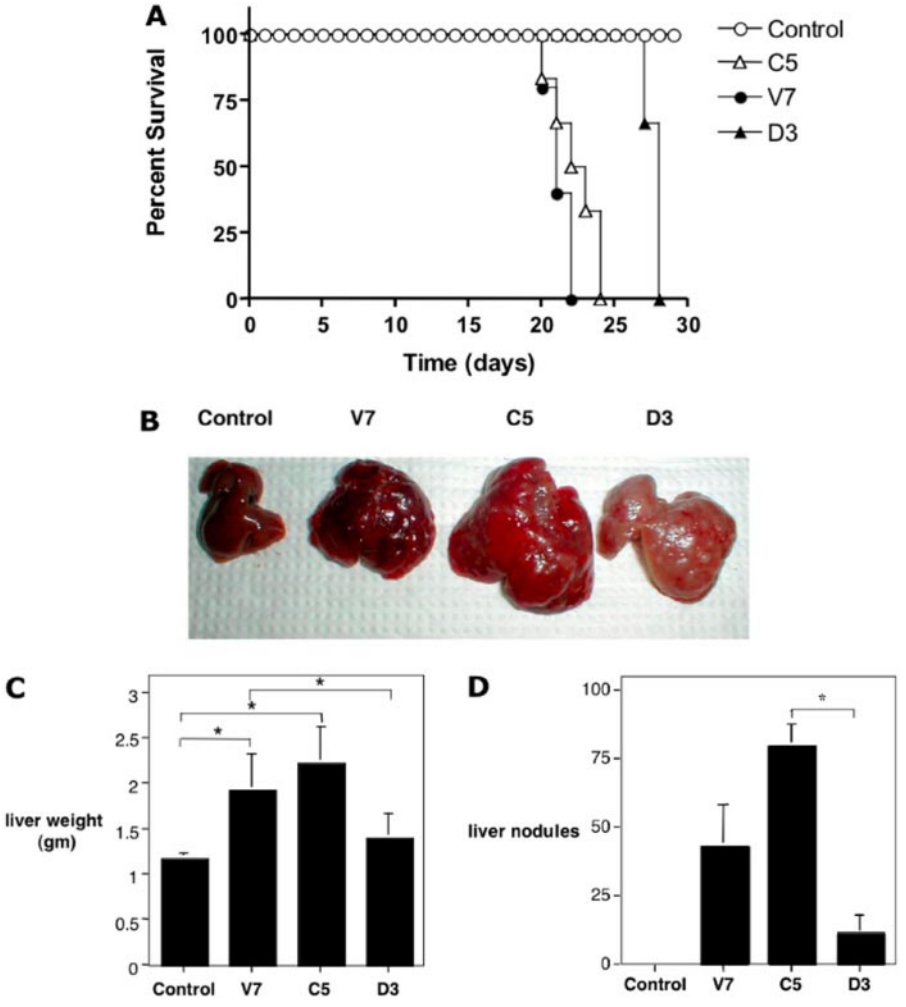

Fig. 10. Experimental metastasis studies. Syngeneic male mice were injected with the indicated EL4 cell lines as described under *Materials and Methods*. A, results of a survival study. Six to seven mice were used in each treatment group; results are expressed as a percentage of the initial group surviving at each time point. B, livers from the injected mice, photographed 20 days after injection. C and D, liver weight and number of surface nodules were quantified 20 days after injection. Each data point represents the mean ± S.E.M. of values from five animals. In C, the asterisks indicate values that were significantly different from the control as determined by Student's *t* test (control versus V7, $p = 0.002$; control versus C5, $p = 0.00001$; D3 versus V7, $p = 0.01$). In D, the asterisk indicates values that were significantly different from each other by Student's *t* test (C5 versus D3, $p = 0.00002$).

shown that PLD2-generated PA enhances integrin-mediated adhesion by stimulating production of phosphatidylinositol 4,5-bisphosphate (Powner et al., 2005).

The potential roles of PLD isoforms in tumor biology are only beginning to be explored. One report indicated that stable overexpression of either PLD1 or PLD2 in fibroblasts causes transformation and enhances tumor formation (Min et al., 2001). In another study, overexpression of PLD2 resulted in transformation of fibroblasts overexpressing the epidermal growth factor receptor or c-Src (Joseph et al., 2001). Elevations of PLD2 activity and protein have been observed in human renal cancers (Zhao et al., 2000). It is interesting that one report indicates that a polymorphism in the PLD2 gene is associated with increased risk of colorectal cancer, even though this polymorphism does not alter PLD2 catalytic activity (Yamada et al., 2003). A role for PLD in breast cancer has been proposed (Foster and Xu, 2003). In breast cancer cells, PLD2 overexpression confers a survival signal attributed to an increase in basal mTOR activity (Rodrik et al., 2005). The results of our work emphasize the effects of PLD2 on the FAK/Akt axis and suggest that this pathway plays a major role in PLD2-enhanced cell survival and/or proliferation.

Our observation that catalytically inactive PLD2 can exert inhibitory effects on some responses in EL4 cells was unexpected. The inactive construct could potentially act as a dominant-negative inhibitor of endogenous PLDs. However, PLD1 and PLD2 are expressed at levels lower than our limit of detection in EL4 cells (Gibbs and Meier, 2000). A second possibility is that PLD2 affects signal transduction in a manner independent of its catalytic activity (e.g., by binding to proteins or lipid). Consistent with this latter hypothesis, one report indicates that the pleckstrin homology domain of PLD2 can exert signaling effects (Lee et al., 2006). PLD2 is also capable of interacting with the adaptor protein Grb2 (Di Fulvio et al., 2007). Our studies suggest that the effects of the inactive PLD2 construct would be of benefit in cancer therapy. Specifically, inactive PLD2 inhibits cell adhesion, migration, and invasion and reduces tumor formation.

In conclusion, our studies indicate that expression of PLD2 enhances FAK phosphorylation, Akt activation, and serum-induced invasion in EL4 cells. Inactive PLD2 exerts inhibitory effects on adhesion, proliferation, migration, invasion, and tumor formation in these cells. Further studies of the signaling roles of PLD2 and the effects of inactive PLD2 in tumors of hematopoietic origin are warranted.

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