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Phospholipase D provides a survival signal in human cancer cells with activated H-Ras or K-Ras

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Abstract

Phospholipase D (PLD) is elevated in rodent fibroblasts expressing activated H-Ras mutants. We therefore examined the PLD activity in human cancer cells with activating Ras mutations. T24 bladder carcinoma cells express an activated H-Ras gene and Calu-1 lung carcinoma cells express an activated K-Ras gene. We report here that both of these cancer cell lines express highly elevated levels of PLD activity and that the PLD activity is dependent upon Ras. We also show that the PLD activity is dependent upon the Ras effector molecules RalA and phosphatidylinositol-3-kinase (PI3K). PLD activity has been shown to provide a survival signal in breast cancer cell lines that suppressed stress-induced apoptosis. Suppression of PLD activity in the T24 and Calu-1 cells resulted in apoptotic cell death in the absence of serum, indicating that the elevated PLD activity provided a survival signal in these cancer cell lines. Suppression of Ras, RalA, or PI3K also led to apoptosis in the absence of serum. These data indicate that a critical component of Ras signaling in human cancer cells is the activation of PLD and that targeting PLD survival signals in cancer cells could be an effective strategy to induce apoptosis in human cancers with activating Ras mutations.

Keywords

Phospholipase D; Ras, Ral; survival signals; apoptosis

1. Introduction

An important aspect of tumorigenesis is the activation of cellular signals that suppress default apoptotic programs that protect against cancer. Signals that suppress apoptosis have been referred to as survival signals because they allow the survival of cells under conditions where the cells would ordinarily undergo a programmed cell death. A common target of survival signals is the mammalian target of rapamycin (mTOR). mTOR is activated in response to signals mediated by phosphatidylinositol-3-kinase (PI3K) and phospholipase D (PLD) [1,2]. Both PI3K and PLD activity have been reported to be elevated in a large number of human cancers [3,4]. Moreover, PI3K and PLD have been shown to suppress apoptosis in many cancer cells [3-5].

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One of the most common gain-of-function mutations found in human cancers are activating mutations to genes encoding Ras family GTPases – most commonly to the K-Ras gene [6]. There are several downstream targets of Ras signaling that includes PI3K, Raf kinase, and the guanine nucleotide exchange factor (GEF) for RalA GTPase [7]. Interestingly, whereas Raf was the most critical downstream target of Ras for the transformation of murine cells, RalA-GEF was the most critical Ras target for the transformation of human cells [8]. RalA-GEF leads to the activation of RalA, which is associated with PLD1 [9] and is required for the activation of PLD activity by epidermal growth factor and Ras [10,11]. RalA has been implicated in cell transformation [12,13], and in transformation of human cells by Ras [14]. The transformation of rat fibroblasts by H-Ras was reported to be dependent upon PLD1 [15], suggesting that a key aspect of RalA in cell transformation by Ras is the activation of PLD.

While activated H-Ras has been reported to stimulate PLD activity in a variety of rodent cell culture systems [16,17], activated K-Ras was unable to stimulate PLD activity in mouse fibroblasts [18]. This is of significance since activating K-Ras mutations are common in human cancers, whereas activating mutations to H-Ras are rare [6]. In the present study we have examined the PLD activity in two human cancer cell lines that have activating mutations to H-Ras or K-Ras. We report here that there are very high levels of PLD activity in both T24 bladder and Calu-1 lung cancer cells that harbor mutations in H-Ras and K-Ras, respectively [19-21]. The PLD activity stimulated by Ras in these cells provided a survival signal that prevented apoptosis in the absence of serum.

2. Materials and methods

2.1. Cells, cell culture conditions and transfection

T24, Calu-1, MDA-MB-231 and MCF-7 cells were obtained from the American Type Culture Collection and were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% bovine calf serum. Transfections were performed using LipofectAMINE 2000 reagent (Invitrogen) according to the vendor's instructions. Transfection efficiency was determined by transfection of pEGFP-C1 (Clontech), which expresses green fluorescent protein. The percentage of green cells was determined microscopically and was routinely in excess of 70%.

2.2. Materials

Rapamycin was obtained from Sigma-Aldrich. Ras antibodies were purchased from Santa Cruz Biotechnology. RalA antibody was obtained from Transduction Laboratories. PLD1 antibodies were from Upstate Biotechnology. Antibodies raised against PLD1 and poly-(ADP-ribose) polymerase (PARP), U0126, and LY294002 were obtained from Cell Signaling Technology. [³H]-myristic acid was obtained from New England Nuclear. Precoated silica 60A thin layer chromatography plates were from Whatman. siRNAs were obtained from Ambion.

2.3. Western blot analysis

Proteins were heated for 3 minutes at 100°C prior to separation by SDS-polyacrylamide gel electrophoresis using an 8~12% acrylamide separating gel. After transferring to nitrocellulose membrane, the membrane filters were blocked with 5% nonfat dry milk in phosphate-buffered saline and incubated with the appropriate antibody. Depending upon the origin of the primary antibodies, either anti-mouse or anti-rabbit IgG conjugated with horseradish peroxidase was used, and the bands were visualized using the enhanced chemiluminescent detection system (Pierce).

2.4. Phospholipase D assays

PLD activity was determined by the transphosphatidyl reaction in the presence of 0.8% butanol as described previously [22]. Cells in 100-mm culture dishes were prelabeled with [³H]-myristate for 4-6 h in Dulbecco's modified Eagle medium containing 0.5% bovine serum. Lipids were extracted and characterized by thin layer chromatography as described previously [10]. Relative levels of PLD activity was then determined by measuring the intensity of the corresponding phosphatidylbutanol band in the autoradiograph using a Molecular Dynamics scanning densitometer and Image-Quant software.

2.5 siRNA

Cells were plated on 35 mm plates at 70% confluence in medium containing 10% serum without antibiotics. After one day, cells were transfected with siRNA using Lipofectamine Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer directions. After 24 hr the media was changed to fresh media containing 10% serum. Three days later cells were lysed and analyzed for expression by Western blot.

3. Results

3.1. PLD activity is elevated in T24 bladder and Calu-1 lung cancer cells

PLD activity is elevated in rodent fibroblasts expressing activated H-Ras [16-18] and PLD activity is required for the transformation of rodent cells by H-Ras [15]. Since activating Ras mutations are present in a large number of human cancers we investigated whether there is elevated PLD activity in human cancer cells with activated H-Ras and K-Ras genes. The T-24 bladder carcinoma expresses an activated H-Ras gene [19,20] and Calu-1 lung carcinoma cells express an activated K-Ras gene [21]. The PLD activity in these two cell lines was evaluated and compared with the PLD activity in two breast cancer cell lines (MDA-MB-231 and MCF7), which express relatively high and low PLD activity respectively [23,24]. As shown in Fig. 1, both the T24 and Calu-1 cells express levels of PLD activity that were substantially higher than that observed in the MDA-MB-231 cells. The MDA-MB-231 cells represent an aggressive malignant cancer cell line that migrates in culture, whereas the MCF-7 cells represent a benign cancer cell line that does not migrate in culture [24]. The ability of the MDA-MB-231 cells to migrate in culture was dependent on PLD activity [24]. Thus, the observation that these two human cancer cell lines with activating mutations in either H-Ras or K-Ras have substantially elevated levels of PLD activity may contribute to their malignant capacity.

3.2. Elevated PLD activity in T24 and Calu-1 cells is dependent upon Ras and RalA

We next examined whether the elevated PLD activity in the T24 and Calu-1 cells was dependent on the activated Ras genes in these cells. To accomplish this, the T24 and Calu-1 cells were transfected with H-Ras and K-Ras siRNAs or a scrambled siRNA control. The PLD activity and Ras protein levels were then determined. As shown in Fig. 2, siRNA for H-Ras and K-Ras suppressed both Ras expression (Fig. 2A) and PLD activity (Fig. 2B) in the T24 and Calu-1 cells.

We demonstrated previously that the activation of PLD by Ras was dependent upon RalA [11,25]. We therefore examined whether the elevated PLD activity in the T24 and Calu-1 cells was dependent upon RalA. Cells were transfected with RalA siRNA and the levels of RalA and PLD activity were examined. As shown in Fig. 3A, the RalA siRNA reduced the level of RalA protein and also reduce the PLD activity in both the T24 and Calu-1 cells. We also investigated whether the Ras-dependent PLD activity was dependent upon two other targets of Ras – phosphatidylinositol-3-kinase (PI3K) and the Raf/MEK/MAP kinase pathway. As shown in Fig. 3B, the PI3K inhibitor LY294002 also suppressed the PLD

activity in both T24 and Calu-1 cells. An inhibitor of MEK, U0126, that suppresses the Raf/MEK/MAP kinase pathway had little or no effect on the PLD activity in either the T24 or Calu-1 cells (data not shown). The data in Figs. 2 and 3 indicate that the elevated PLD activity in these cells is dependent on the Ras-RalA GTPase cascade [5] in these cells. Interestingly, there also appeared to be a dependence on the PI3K pathway.

3.3. Suppression of PLD activity or Ras signaling in T24 and Calu-1 cells results in apoptosis upon serum withdrawal

We reported previously that elevated PLD activity in MDA-MB-231 breast cancer cells provided a survival signal for these cells when deprived of serum [23,27]. To determine whether the Ras-dependent PLD activity stimulated in the T24 and Calu-1 cells was critical for that survival signal generated by Ras, we employed the “alcohol trap” assay [10] whereby primary, but not tertiary, alcohols are preferentially utilized over water in the hydrolysis of phosphatidylcholine to a corresponding inert phosphatidylalcohol rather than phosphatidic acid. As shown in Fig. 4A, primary butanol (1-BtOH), but not tertiary BtOH (t-BtOH) led to apoptosis in serum deprived T24 and Calu-1 cells as indicated by decreased cell viability and increased cleavage of the caspase 3 substrate PARP. To further establish that PLD activity was providing a survival signal, we transfected the T24 and Calu-1 cells with PLD1 siRNA. The T24 and Calu-1 cells were then subjected to low serum and cell viability and PARP cleavage was evaluated. As shown in Fig. 4B, suppression of PLD1 expression made both the T24 and Calu-1 cells sensitive to serum withdrawal as indicated by decreased cell viability and increased PARP cleavage. These data reveal that the PLD activity elevated by H-Ras and K-Ras in the T24 and Calu-1 cells is critical for the Ras-generated survival signals in these cells that suppress apoptosis under the stress of serum withdrawal.

We also examined the effect of suppressing Ras, RalA and PI3K activity on T24 and Calu-1 cells deprived of serum. If these cells were transfected with siRNA for H-Ras (T24) or K-Ras (Calu-1) there was substantial decrease in cell viability and a corresponding increase in PARP cleavage indicating apoptosis (Fig. 5A). Similarly, RalA siRNA (Fig. 5B) and LY294002 (Fig. 5C) also stimulated PARP cleavage in the T24 and Calu-1 cells. These data indicate that suppressing the signals needed for the Ras-dependent PLD activity also induce apoptosis under conditions of low serum.

4. Discussion

During progression to a malignant tumor, several genetic changes must occur that will allow the proliferation cells under conditions where normal cells will either stop proliferating or undergo apoptosis. During the formation of a solid tumor prior to vascularization, a cell must continue to divide and survive in the absence of growth factors. We have provided evidence here that human cancer cells with either an activated H-Ras or K-Ras depend upon highly elevated levels of PLD activity for survival in the absence of serum which contains growth factors. Suppression of PLD activity under these conditions results in apoptosis.

Although the study here examined only two cell lines, it is quite apparent that both H-Ras and K-Ras are capable of stimulating the activation of PLD. The dependence on RalA suggests that it is PLD1 that is being activated in response to both H-Ras and K-Ras, since RalA associates with PLD1 [9,11]. Consistent with this hypothesis, PLD1 siRNA suppressed the survival signals in the T24 and Calu-1 cells. We have demonstrated previously that both PLD1 and PLD2 can work together in signals generated by epidermal growth factor [10] and that both PLD1 and PLD2 can contribute to the transformation of rat fibroblasts [28,29]. Thus, it is possible that PLD2 could also be involved. We have

speculated that activation of PLD1 leads to the activation of PLD2 through stimulation of the production of the PLD co-factor phosphatidylinositol-4,5-bis-phosphate [5].

The finding that both H-Ras and K-Ras strongly activate PLD activity in the human cancer cells was somewhat surprising in that we previously reported that activated H-Ras, but not activated K-Ras, induced PLD activity in NIH-3T3 cells [18]. The Calu-1 lung cancer cells, which have activating mutation in K-Ras had higher levels of PLD activity than the T24 bladder cancer cells, which have an activating mutation in H-Ras. Significantly, the PLD activity in the Calu-1 cells was dependent upon RalA. Several reports have linked RalA to H-Ras via the RalA GEF, which is a direct downstream target of H-Ras [26]. The data reported here implicate RalA in signals mediated by K-Ras as well as K-Ras. Importantly, K-Ras is mutated in many cancers, whereas H-Ras is activated in very few human cancers [6]. Thus, the ability of K-Ras to activate RalA and PLD activity reveals that K-Ras, like H-Ras, take advantage of PLD survival signals in the many cancers that harbor activating K-Ras mutations. Interestingly, the increased PLD activity was sensitive to another downstream target of Ras signaling – PI3K. PI3K has been reported to contribute to PLD activity previously [30], however the mechanism is not known. This finding suggests that targeting PI3K in cancers with Ras mutations may suppress survival signals generated by PLD.

The signals generated by PLD have been shown to impact upon several proteins implicated in tumorigenesis. Elevated PLD activity has been shown to suppress the expression of tumor suppressor p53 [31]. PLD has also been reported to inhibit the activity of another tumor suppressor – protein phosphatase 2A [32]. PLD increases the stability, and consequently the expression of Myc [33,34]. But perhaps the most important target of PLD-generated phosphatidic acid is mTOR. Phosphatidic acid has been reported to interact directly with mTOR in a manner that is competitive with rapamycin [2,35] and consistent with this report, elevated levels of PLD activity lead to rapamycin resistance [36]. mTOR is apparently an important component in tumorigenesis in that survival signals generated by PI3K also target mTOR. These previous studies linking PLD activity with the suppression of tumor suppressors and the activation of Myc and mTOR indicate that activating PLD activity has many outputs that have been shown to promote tumorigenesis. The ability of both activated H-Ras and K-Ras to stimulate PLD activity in human cancer cells may be important for Ras genes to cause cancer. While suppression of Ras expression has been shown to reverse tumorigenesis in mouse models where the tumors were caused by activated Ras genes [37], attempts to target Ras pharmaceutically have been largely disappointing [38]. If PLD is a critical target of Ras as indicated here, then targeting PLD or PLD targets such as mTOR might prove valuable in the large number of human cancers with mutations in Ras genes.

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References

- [1]. Foster DA. Targeting mTOR-mediated survival signals in anticancer therapeutic strategies. *Exp. Rev. Anticancer Ther.* 2004; 4:691–701.
- [2]. Foster DA. Regulation of mTOR by phosphatidic acid? *Cancer Res.* 2007; 67:1–4. [PubMed: 17210675]
- [3]. Foster DA. Phospholipase D survival signals as a therapeutic target in cancer. *Current Signal Transduction Ther.* 2006; 1:295–303.

- [4]. Luo J, Manning BD, Cantley LC. Targeting the PI3K-Akt pathway in human cancer: rationale and promise. *Cancer Cell*. 2003; 4:257–262. [PubMed: 14585353]
- [5]. Foster DA, Xu L. Phospholipase D in cell proliferation and cancer. *Mol. Cancer Res*. 2003; 1:789–800. [PubMed: 14517341]
- [6]. Bos JL. Ras oncogenes in human cancer: a review. *Cancer Res*. 1989; 49:4682–4689. [PubMed: 2547513]
- [7]. Shields JM, Pruitt K, McFall A, Shaub A, Der CJ. Understanding Ras: ‘it ain’t over ‘til it’s over’. *Trends Cell Biol*. 2000; 10:147–154. [PubMed: 10740269]
- [8]. Hamad NM, Elconin JH, Karnoub AE, Bai W, Rich JN, Abraham RT, Der CJ, Counter CM. Distinct requirements for Ras oncogenesis in human versus mouse cells. *Genes Dev*. 2002; 16:2045–2057. [PubMed: 12183360]
- [9]. Luo JQ, Liu X, Hammond SM, Colley WC, Feig LA, Frohman MA, Morris AJ, Foster DA. Ral interacts directly with the Arf-responsive PIP2-dependent phospholipase D1. *Biochem. Biophys. Res. Comm*. 1997; 235:854–859. [PubMed: 9207251]
- [10]. Shen Y, Xu L, Foster DA. Phospholipase D requirement for receptor-mediated endocytosis. *Mol. Cell. Biol*. 2001; 21:595–602. [PubMed: 11134345]
- [11]. Jiang H, Luo JQ, Urano T, Lu Z, Foster DA, Feig LA. Involvement of Ral GTPase in v-Src-induced phospholipase D activation. *Nature*. 1995; 378:409–412. [PubMed: 7477381]
- [12]. Urano T, Emkey R, Feig LA. Ral GTPases mediate a distinct downstream signaling pathway from Ras that facilitates cellular transformation. *EMBO J*. 1996; 16:810–816. [PubMed: 8631302]
- [13]. Lim KH, O’Hayer K, Adam SJ, Kendall SD, Campbell PM, Der CJ, Counter CM. Divergent roles for RalA and RalB in malignant growth of human pancreatic carcinoma cells. *Curr. Biol*. 2006; 16:2385–2394. [PubMed: 17174914]
- [14]. Lim KH, Baines AT, Fiordalisi JJ, Shipitsin M, Feig LA, Cox AD, Der CJ, Counter CM. Activation of RalA is critical for Ras-induced tumorigenesis of human cells. *Cancer Cell*. 2005; 7:533–545. [PubMed: 15950903]
- [15]. Buchanan FG, McReynolds M, Couvillon A, Kam Y, Holla VR, Dubois RN, Exton JH. Requirement of phospholipase D1 activity in H-RasV12-induced transformation. *Proc. Natl. Acad. Sci. USA*. 2005; 102:1638–1642. [PubMed: 15668389]
- [16]. Carnero A, Cuadrado A, del Peso L, Lacal JC. Activation of type D phospholipase by serum stimulation and ras-induced transformation in NIH3T3 cells. *Oncogene*. 1994; 9:1387–1395. [PubMed: 8152799]
- [17]. Jiang H, Lu Z, Luo JQ, Wolfman A, Foster DA. Ras mediates the activation of phospholipase D by v-Src. *J. Biol. Chem*. 1995; 270:6006–6009. [PubMed: 7890731]
- [18]. Xu L, Frankel P, Jackson D, Rotunda T, Boshans RL, D’Souza-Schorey C, Foster DA. Elevated phospholipase D activity in H-Ras-, but not K-Ras-transformed cells by the synergistic action of RalA and Arf6. *Mol. Cell. Biol*. 2003; 23:645–654. [PubMed: 12509462]
- [19]. Santos E, Tronick SR, Aaronson SA, Pulciani S, Barbacid M. T24 human bladder carcinoma oncogene is an activated form of the normal human homologue of BALB- and Harvey-MSV transforming genes. *Nature*. 1982; 298:343–347. [PubMed: 6283384]
- [20]. Taparowsky E, Suard Y, Fasano O, Shimizu K, Goldfarb M, Wigler M. Activation of the T24 bladder carcinoma transforming gene is linked to a single amino acid change. *Nature*. 1982; 300:762–765. [PubMed: 7177195]
- [21]. Shimizu K, Birnbaum D, Ruley MA, Fasano O, Suard Y, Edlund L, Taparowsky E, Goldfarb M, Wigler M. Structure of the Ki-ras gene of the human lung carcinoma cell line Calu-1. *Nature*. 1983; 304:497–500. [PubMed: 6308465]
- [22]. Song J, Foster DA. v-Src activates a unique phospholipase D activity that can be distinguished from the phospholipase D activity activated by phorbol esters. *Biochem. J*. 1993; 294:711–717. [PubMed: 8379928]
- [23]. Zhong M, Shen Y, Zheng Y, Joseph T, Jackson D, Foster DA. Phospholipase D prevents apoptosis in v-Src-transformed rat fibroblasts and MDA-MB-231 breast cancer cells. *Biochem. Biophys. Res. Comm*. 2003; 302:615–619. [PubMed: 12615079]

- [24]. Zheng Y, Rodrik V, Toschi A, Shi M, Hui L, Shen Y, Foster DA. Phospholipase D couples survival and migration signals in response to stress in human breast cancer cells. *J. Biol. Chem.* 2006; 281:15862–15868. [PubMed: 16595654]
- [25]. Luo JQ, Liu X, Frankel P, Rotunda T, Ramos M, Flom J, Jiang H, Feig LA, Morris AJ, Kahn RA, Foster DA. Functional association between RalA and Arf in active phospholipase D complexes. *Proc. Natl. Acad. Sci. USA.* 1998; 95:3632–3637. [PubMed: 9520417]
- [26]. Chien Y, White MA. RAL GTPases are linchpin modulators of human tumor-cell proliferation and survival. *EMBO Rep.* 2003; 4:800–806. [PubMed: 12856001]
- [27]. Chen Y, Rodrik V, Foster DA. Alternative phospholipase D / mTOR survival signal in human breast cancer cells. *Oncogene.* 2005; 24:672–679. [PubMed: 15580312]
- [28]. Lu Z, Hornia A, Joseph T, Sukezane T, Frankel P, Zhong M, Bychenok S, Xu L, Feig LA, Foster DA. Phospholipase D and RalA Cooperate with the EGF Receptor to Transform 3Y1 Rat Fibroblasts. *Mol. Cell. Biol.* 2000; 20:462–467. [PubMed: 10611224]
- [29]. Joseph T, Wooden R, Bryant A, Zhong M, Lu Z, Foster DA. Transformation of cells overexpressing a tyrosine kinase by phospholipase D1 and D2. *Biochem. Biophys. Res. Comm.* 2001; 289:1019–1024. [PubMed: 11741292]
- [30]. Yamada M, Banno Y, Takuwa Y, Koda M, Hara A, Nozawa Y. Overexpression of phospholipase D prevents actinomycin D-induced apoptosis through potentiation of phosphoinositide 3-kinase signalling pathways in Chinese-hamster ovary cells. *Biochem. J.* 2004; 387:649–656. [PubMed: 14640974]
- [31]. Hui L, Abbas T, Pielak R, Joseph T, Bargonetti J, Foster DA. Phospholipase D elevates the level of MDM2 and suppresses DNA damage-induced increases in p53. *Mol. Cell. Biol.* 2004; 24:5677–5688. [PubMed: 15199126]
- [32]. Hui L L, Rodrik V, Pielak RM, Zheng Y, Foster DA. mTOR-dependent suppression of protein phosphatase 2A is critical for phospholipase D survival signals in human breast cancer cells. *J. Biol. Chem.* 2005; 280:35829–35835. [PubMed: 16109716]
- [33]. Rodrik V, Zheng Y, Harrow F, Chen Y, Foster DA. Survival signals generated by estrogen and phospholipase D in MCF-7 breast cancer cells are dependent on Myc. *Mol. Cell. Biol.* 2005; 25:7917–7925. [PubMed: 16107734]
- [34]. Rodrik V V, Gomes E, Hui L, Rockwell P, Foster DA. Myc stabilization in response to estrogen and phospholipase D in MCF-7 breast cancer cells. *FEBS Lett.* 2006; 580:5647–5652. [PubMed: 16996503]
- [35]. Fang Y, Vilella-Bach M, Bachman R, Flanigan A, Chen J. Phosphatidic acid-mediated mitogenic activation of mTOR signaling. *Science.* 2001; 294:1942–1945. [PubMed: 11729323]
- [36]. Chen Y, Zheng Y, Foster DA. Phospholipase D confers rapamycin resistance in human breast cancer cells. *Oncogene.* 2003; 22:3937–3942. [PubMed: 12813467]
- [37]. Pao W, Klimstra DS, Fisher GH, Varmus HE. Use of avian retroviral vectors to introduce transcriptional regulators into mammalian cells for analyses of tumor maintenance. *Proc. Natl. Acad. Sci. USA.* 2003; 100:8764–8769. [PubMed: 12857957]
- [38]. Prendergast GC, Rane N. Farnesyltransferase inhibitors: mechanism and applications. *Expert Opin. Investig. Drugs.* 2001; 10:2105–2116.

Fig. 1.

PLD activity is elevated in T24 bladder and Calu-1 lung cancer cells.

MCF-7, MDA-MB-231, T24 and Calu-1 cells were plated in media containing 10% serum for 24 hr. At that point cells were shifted to media containing 0.5% serum overnight. BtOH was added 20 min prior to harvesting of cells, and the transphosphatidylated product phosphatidylbutanol (PtBt) was determined by thin layer chromatography as described in Materials and Methods. The PLD activity in the MDA-MB-231, T24, and Calu-1 cells was normalized to that observed in the MCF-7 cells which was given a value of 1. Error bars represent the standard deviation for two independent experiments.

Fig. 2.

Elevated PLD activity in T24 and Calu-1 cells is dependent upon Ras. T24 (A) and Calu-1 (B) cells plated at a density of 10^5 cells per 60 mm plate. 24 hr later the cells were transfected with either control (Con) scrambled siRNA or siRNA specific for H-Ras (A) or K-Ras (B) as indicated. The levels of Ras and actin were then determined by Western blot and the PLD activity was determined 48 hr later. PLD activity, determined as in Fig. 1, in the Ras siRNA samples was normalized to the PLD activity in the controls, which was given a value of 1.00. Error bars represent the standard deviation for two independent experiments. The data showing the levels of Ras and actin was representative of an experiment that was repeated at least two times.

Fig. 3.

Elevated PLD activity in T24 and Calu-1 cells is dependent upon RalA and PI3K. (A) T24 and Calu-1 cells were transfected with either control (Con) scrambled siRNA or siRNA specific for RalA as indicated. The levels of RalA and actin were then determined by Western blot and the PLD activity was determined as in Fig. 2. The PLD activity in the RalA siRNA samples was normalized to the PLD activity in the controls, which was given a value of 1.00. (B) T24 and Calu-1 cells were plated at a density of 10^5 cells/60 mm plate. 24 hr later the cells were shifted to media containing 0.5% serum and treated with either DMSO vehicle or LY294004 (50 μ M) as indicated. 4 hr later the levels of PLD activity were determined as in Fig. 1. The PLD activity in the RalA siRNA and LY294002 samples were normalized to the PLD activity in the controls, which was given a value of 1.00. Error bars represent the standard deviation for two independent experiments. The data showing the levels of RalA and actin was representative of an experiment that was repeated at least two times.

Fig. 4.

Suppression of PLD1 expression in T24 and Calu-1 cells results in apoptosis upon serum withdrawal. (A) T24 and Calu-1 cells were plated at a density of 10^5 cells/60 mm plate. 24 hr later the cells were shifted to media containing 0.5% serum. 18 hr later the cells were either untreated (Con), or treated with either tertiary BtOH (t-BtOH) or primary BtOH (1-BtOH) and cell viability (upper graph) and PARP cleavage (lower blot, CI PARP) was determined 6 hr later by Western blot analysis. Error bars represent the standard deviation for duplicate samples from a representative experiment repeated at least two times. (B) T24 and Calu-1 cells were plated at a density of 10^5 cells/60 mm plate. 24 hr later the cells were transfected with either control (Con) scrambled siRNA or siRNA specific for PLD1 as in Fig. 2. 24 hr later the cells were shifted to media containing 0.5% serum. 18 hr later cell viability (upper graph) was determined as in Fig. 4. In the lower panel, PLD1 levels and PARP cleavage (CI PARP) were determined using Western blot analysis. Error bars represent the standard deviation for duplicate samples from a representative experiment repeated at least two times.

Fig. 5.

Suppression of Ras signaling in T24 and Calu-1 cells results in apoptosis upon serum withdrawal. (A) T24 and Calu-1 cells were plated at a density of 10^5 cells/60 mm plate. 24 hr later the cells were transfected with either control (Con) scrambled siRNA or siRNA specific for H-Ras or K-Ras as in Fig. 2. 48 hr later the cells were shifted to media containing 0.5% serum. 18 hr later cell viability (upper graph) and PARP cleavage (lower blot, CI PARP) were determined as described in Materials and Methods. The Western blot for PARP was examined for loading by reprobing with an antibody raised against actin. Error bars represent the standard deviation for duplicate samples from a representative experiment repeated at least two times. (B) T24 and Calu-1 cells were transfected with either control (Con) scrambled siRNA or siRNA specific for RalA as in (A). 48 hr later the cells were shifted to media containing 0.5% serum. 18 hr later PARP cleavage was determined as in (A). (C) T24 and Calu-1 cells were plated at a density of 10^5 cells/60 mm plate. 24 hr later the cells were shifted to media containing 0.5% serum and treated with either DMSO vehicle or LY294004 (50 μ M) as indicated. 18 hr later PARP cleavage was determined as in (A). The data shown are representative of experiments repeated at least two times.