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Novel Phospholipase D / mTOR Survival Pathway in Human Breast Cancer Cells

by

Yuhong Chen

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

2004

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This manuscript has been read and accepted for the Graduate Faculty in Biology in Satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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ABSTRACT

Novel Phospholipase D / mTOR Survival Pathway in Human Breast Cancer Cells

by

Yuhong Chen

Adviser: Dr. David A. Foster

It was recently reported that phosphatidic acid (PA) generated by phospholipase D (PLD) activates mTOR (mamalian target of rapamycin), a protein kinase that regulates cell cycle progression, cell growth and survival signaling. PA binds to the same domain of mTOR as rapamycin (a specific inhibitor of mTOR). The effect of rapamycin on breast cancer cell proliferation was investigated. Initial studies revealed that MCF-7 cells, with relatively low levels of PLD activity, were sensitive to the growth-arresting effects of rapamycin, whereas MDA-MB-231 cells with 10-fold higher PLD activity comparing to MCF-7 cells, were resistant to rapamycin. Elevating PLD activity in MCF-7 cells led to rapamycin resistance; and inhibition of PLD activity in MDA-MB-231 cells increased rapamycin

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sensitivity. The conclusion from this study was that the PA generated by PLD was able to compete with rapamycin for binding to mTOR, therefore elevated phospholipase D activity confers resistance to rapamycin in human breast cancer cell lines. These data provided the first evidence linking mTOR with the mitogenic effects of PLD.

In serum free condition, Rapamycin induced apoptosis in MDA-MB-231 cell. MDA-MB-435S cells, which has relative very low levels of PLD activity, was far less sensitive to rapamycin. These cells were however sensitive to LY294002, a compound that blocks phosphatidylinositol (PI)-3 kinase (PI3K), an enzyme generating PIP_3. PIP_3 recruits and activates Akt. The PI3K/Akt pathway has emerged as a critical survival pathway for many cancers. The MDA-MB-231 cells with an active PLD/mTOR survival pathway were resistant to LY294002, indicating that the PLD/mTOR apparently constitutes an alternative survival signal that is active in MDA-MB-231 cells.

In summary, the evidence provided in this thesis further establishes that there is a critical dependence of PLD survival signaling upon mTOR. The PLD/mTOR pathway represents an alternative survival signaling strategy to the established PI3K/Akt pathway.

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I would like to thank my mentor Dr. David Foster for all his supervision and help in my research. It is because him I know how to think in scientific way, and have a wonderful beginning of my scientific career.

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LIST OF ABBREVIATION

eIF-4E: eukaryotic initiation factor 4E

4EBP1: eIF-4E binding protein-1

FRB domain: FKBP12-rapamycin complex binding domain

IC50: 50% inhibition concentration

mTOR: Mammalian target of rapamycin

PA: Phosphatidic acid

PARP: Poly-(ADP-ribose) polymerase

PC: Phosphatidylcholine

PH domain: Pleckstrin homology domain

PLD: Phospholipase D

PI-4-P-5-K: Phosphatidylinositol 4-phosphate 5-kinase (PI-4-P-5- kinase)

PI3K: Phosphatidylinositol-3-kinase

PIP2: Phosphatidylinositol 4,5-bisphosphate, PtdIns(4,5)P2

PIP3: Phosphatidylinositol 3,4,5-trisphosphate, PtdIns(4,5)P3

PKCα: Protein kinase C α

PTEN: Phosphatase and *TEN*sin homologue deleted on chromosome *TEN*

S6K: Kinase of the S6 protein of the 40S ribosomal complex

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INTRODUCTION

In recent years we have come to understand that phospholipids not only play a role as structural components of cellular membranes, but also are important mediators of mitogenic signal transduction. Phosphatidylcholine (PC) is the major phospholipid of mammalian cell membranes and the substrate of Phospholipase D (PLD). PLD catalyzes PC hydrolysis to generate phosphatidic acid (PA) and choline (Fig. 1). It is widely believed that PA is the critical metabolite and the phospholipid mediator for the cellular functions of PLD. These functions include membrane trafficking, cytoskeletal reorganization, receptor endocytosis , exocytosis, cell migration (Exton, 2002a; 2002b), cell proliferation and cell survival.

I. Phospholipase D Isoforms

Two mammalian PLD genes (PLD1 and PLD2) have been reported (Colley et al., 1997; Hammond et al., 1995; 1997). There is considerable homology between the two genes, however there are significant differences in the regulation and subcellular distribution of PLD1 and PLD2. PLD1 can be activated by the ARF- and Rho-family GTPases, as well as by PKCα (Colley et al., 1997). Both PLD1 and PLD2 require PIP2 for activation.

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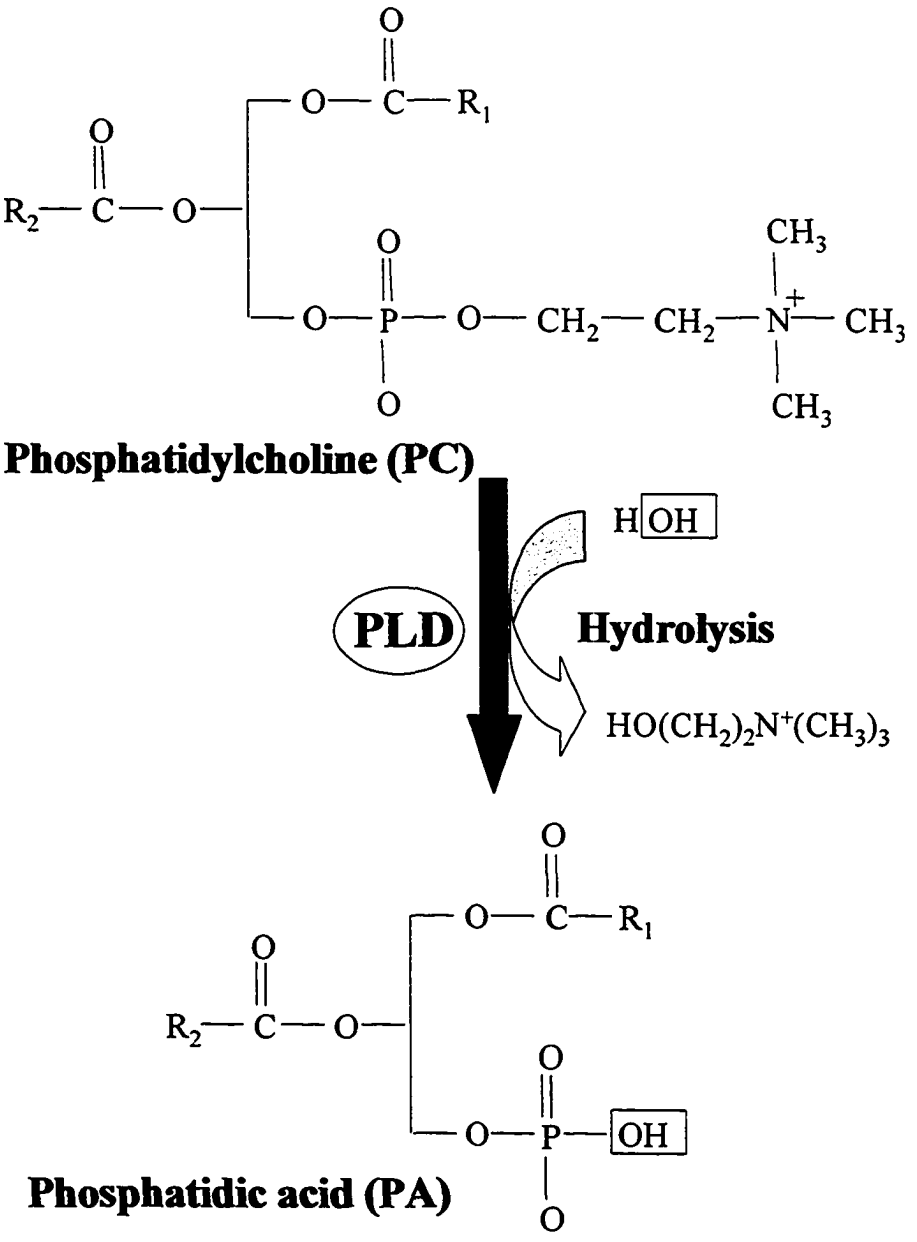

Fig. 1. Enzymology of Phospholipase D
Phospholipase D catalyzes PC hydrolysis reaction to generate PA and free choline. PA is the important metabolite and mediator for PLD to regulate cell proliferation.

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PLD2 is constitutively active in vitro and this activity is unaffected by GTPases or PKCα (Hammond et al., 1995). Studies on the subcellular distribution of PLD1 and PLD2 showed that there are differences in the distribution of the two isoforms. PLD1 is found throughout the cell, but primarily in perinuclear, Golgi, and heavy membrane fractions. In contrast, PLD2 is localized almost exclusively on the plasma membrane in light membrane fractions that co-fractionate with caveolin (Xu et al., 2000).

II. The Regulation of PLD

a. Activation by Protein Kinase Cα

Protein kinase Cα (PKCα) has been implicated as a regulator of PLD1 (Hammond et al., 1995; 1997), however it is not clear how PKCα regulates PLD1. In vitro, PKCα activates PLD1 in a kinase-independent manner (Conricode et al., 1992); but in vivo, PLD1 is phosphorylated by PKCα. Interestingly, the phosphorylated PLD1 localizes to caveolin-enriched light membrane fractions (Han et al., 2002), where mitogenic PLD activity is largely restricted (Xu et al., 2000). Blocking the kinase activity of PKCα in vivo blocks EGF-induced PLD activity (Hornia et al., 1999),

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therefore, in vivo the kinase requirement for PKCα may reflect a phosphorylation requirement for translocation of PLD1.

b. Activation by Inositol Phospholipids

Both PLD1 and PLD2 contain a pleckstrin homology (PH) domain. The PH domain of PLD is selective for bisphosphorylated inositides PtdIns(4,5)P2 (PIP2) (Hodgkin et al., 2000). Binding of PIP2 to the PH domain of PLD has two functions, firstly mutation of the PH domain reduces or inhibits activation of PLD, but secondly complete removal of the PH domain both ablates PLD activity and prevents membrane association of PLD (Hodgkin et al., 2000). Interestingly, phosphatidylinositol 4-phosphate 5-kinase (PI-4-P-5-kinase), which generates PIP2 from PIP, is a downstream target of PA (Moritz et al., 1992; Jenkins et al., 1994), this suggests a positive feedback loop of PLD activation.

c. Activation by Small GTPases

Several factors have been reported to regulate PLD activity, including the Rho-, ARF- and Ras family GTPases. Rho- and ARF-family GTPases can activate PLD1 activity directly in vitro (Brown et al., 1993; Colley et al 1997; Hammond et al., 1997). RalA, a Ras family GTPase, directly interacts with PLD1 (Luo et al., 1997), but does not activate PLD1.

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RalA can work synergistically with ARF1 to elevate PLD1 activity in vitro (Kim et al., 1998). Recently it has been reported that a combination of activated RalA and activated ARF6 was sufficient to elevate PLD activity in vivo (Xu et al., 2003).

III. Downstream Targets of PLD

While much is known about upstream regulation of PLD, relevant downstream PLD targets have been difficult to establish.

1. PI-4-P-5-kinase: PA is required for the activation of PI-4-P-5-kinase (Moritz et al., 1992). Which generates PIP2, a critical co-factor for PLD. As proposed above, the generation of PIP2 may be a positive feedback mechanism and contribute to the activation of PLD2 in lipid rafts.

2. Raf: has a PA binding site (Ghosh et al., 1996) and it has been proposed that the generation of PA facilitates the recruitment of Raf to the plasma membrane, where it can participate in activation of the MAP kinase pathway (Ghosh et al., 1996; Rizzo et al., 1999). Activation of the MAP kinase pathway has been implicated in almost all mitogenic signaling and therefore contributing to Raf activation almost certainly contributes to the mitogenic properties of PLD (Fig. 2).

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3. mTOR: It was recently reported that PA is required for the activation of mTOR (mammalian target of rapamycin) (Brown et al., 1994; Chiu et al., 1994; Sabatini et al., 1994; Sabers et al., 1995) signaling and PA interacted with the FKBP12-rapamycin-binding (FRB) domain of mTOR (Fang et al., 2001; Chen et al., 2002) (Fig. 2, 3). mTOR is a protein kinase that regulates cell cycle progression and cell growth (Kuruvilla et al., 1999; Schmelzie et al., 2000) (Fig. 2). mTOR controls these processes by regulating translation, transcription, membrane traffic and protein degradation (Schmelzie et al., 2000) (Fig. 4). Blocking mTOR pathway by rapamycin induced apoptosis in human rhabdomyosarcoma cell line (Thimmaiah et al., 2003). mTOR is also a target of survival signals generated by the PI3K/Akt survival pathway (Nave et al., 1999; Sekulic et al., 2000). S6K (the downstream target of mTOR) provides survival signal by phosphorylating and inhibiting BAD (Harada et al., 2001). BAD is a proapoptotic protein. These data suggest mTOR pathway is involved in cell survival signaling (Fig. 2). Rapamycin, a complex lipophilic macrolide isolated from a strain of *Streptomyces hygroscopicus* (Sehgal et al., 1975; Vezina et al., 1975), is a specific inhibitor of mTOR (Cafferkey et al., 1993; Brown et al., 1994; Sabatini et al., 1994) and has been widely used to block cell proliferation. Rapamycin is also in phase 1 clinical trials for treatment of breast and other cancers

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(Hidalgo et al., 2000). Rapamycin acts by binding to the immunophilin FK506 binding protein 12 (FKBP12) (Fig. 3), and the resultant complex binds mTOR with high affinity and inhibits mTOR function in a poorly understood fashion (Chen et al., 2002).

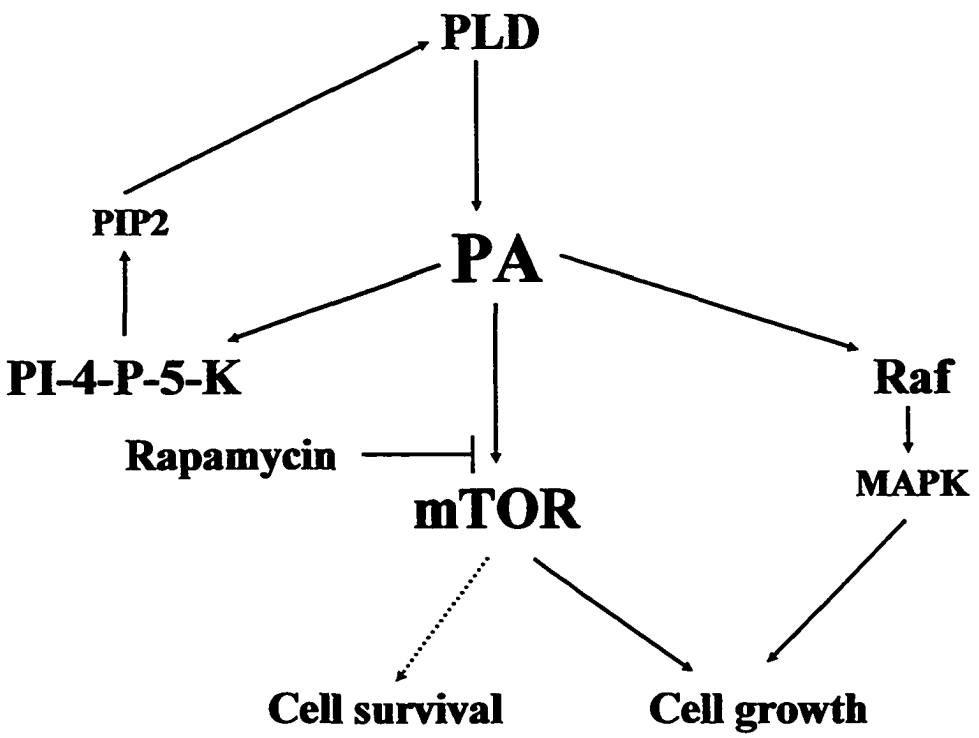

Fig. 2. Downstream targets of PA
PA is required for the activation of PI-4-P-5-kinase, Which generates PIP2, a critical co-factor for PLD. Raf has a PA binding site, PA facilitates the recruitment of Raf to the plasma membrane, where it can participate in the activation of MAP kinase pathway which regulates the cell growth and proliferation. Recently, it is reported that PA is required for mTOR signaling activation. mTOR is an important regulator of cell survival and growth.

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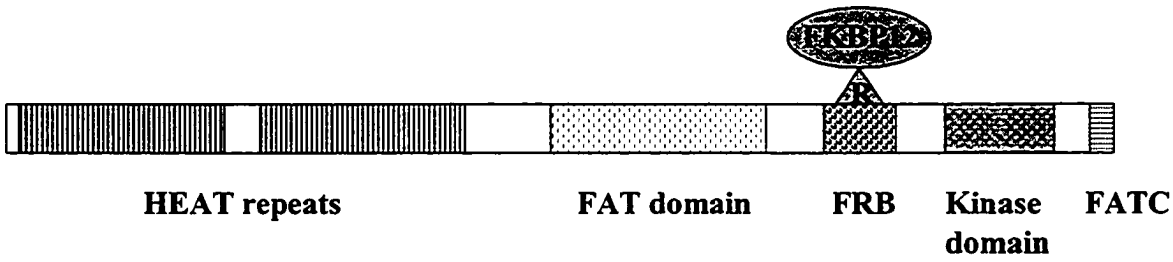

Fig. 3. Structure of mTOR kinase
mTOR protein contains up to 20 tandemly repeated HEAT motifs at their N terminus, roughly grouped into two blocks. HEAT motifs have been proposed to mediate protein-protein interactions in multiprotein complexes. FAT domain function is not clear. FRB is FKBP-rapamycin complex binding domain. PA binds to this same domain and competes with FKBP-rapamycin complex. Catalytic kinase domain is at the C terminal of mTOR. FATC domain occurs only in combination with the FAT domain and maybe important for catalytic activity of mTOR kinase.

IV. Physiological Significance of PLD

a. Vesicle Transport

Vesicle budding in the Golgi network was shown to be mediated in part by ARF family GTPase (Moss et al., 1998). It has been reported that ARF family GTPases regulate PLD activity, therefore a function of PLD in vesicle transport is possible. Consistent with this idea, PA generated by PLD has been reported to be required for the formation of Golgi vesicles and for the transport of vesicles from the ER to the Golgi complex. Endocytosis of

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the EGF receptor is required for EGF-induced MAP kinase activation (Vieira et al., 1996; Kranenburg et al., 1999). Both PLD1 and PLD2 were required for ligand-induced endocytosis of the EGF receptor (Shen et al., 2002). Blocking PLD activity prevented the activation of MAP kinase. This observation was consistent with previous reports indicating that endocytosis is a requirement for signals produced by EGF (Vieira et al., 1996; Kranenburg et al., 1999). BFA, which prevents PLD1 activation by inhibiting ARF GTP/GDP exchange, inhibited MAP kinase activation and receptor internalization in response to insulin (Rizzo et al., 1999), suggesting PLD activity is also involved in the regulation of the endocytosis of insulin receptor.

b. Cell Proliferation

A role for PLD in cell proliferation is indicated from reports showing that PLD activity is upregulated in response to PDGF (Plevin et al., 1991), EGF(Song et al., 1994) insulin (Karnam et al., 1997) and insulin-like growth factor1 (IGF1) (Banno et al., 2003). PLD activity also is elevated in cells transformed by v-Src (Song et al., 1991), v-Ras (Carnero et al., 1994; Jiang et al., 1995), v-Fps (Jiang et al., 1994) and v-Raf (Frankel et al., 1999). PA the critical metabolite of PLD directly interacts with Raf and recruits it to

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plasma membrane, where Raf can participate in the activation of MAP kinase pathway (Ghosh et al., 1996; Rizzo et al., 1999). The activation of MAP kinase pathway has been implicated in almost all mitogenic signaling.

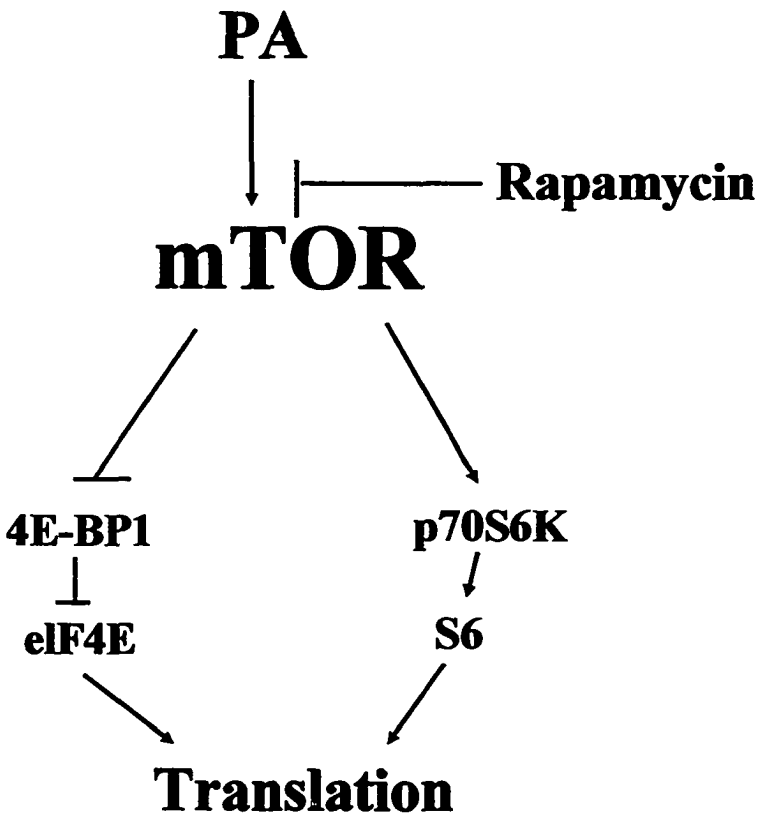

Fig. 4. mTOR is a regulator of translation
mTOR controls the mammalian translation machinery by activation of the P70S6K and inhibition of the 4E-BP1 (eLF4E inhibitor). Phospholipase D activates mTOR signaling pathway by generating PA.

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Moreover PA activates mTOR signaling pathways (Chen et al., 2002; Fang et al., 2001) and mTOR is an important regulator for cell proliferation. These data suggest Raf and mTOR are important downstream mediators for PLD to control cell proliferation. Some reports from our lab show that elevated expression of either PLD1 or PLD2 induces anchorage-independent growth in cells overexpressing a tyrosine kinase (c-Src, EGFR) (Lu et al., 1997; 2000; Joseph et al., 2001), indicating that PLD activity contributes to cell transformation. Interestingly, Studies of human cancer cells showed PLD protein expression level and activity are upregulated in human breast, renal and gastric cancers (Uchida et al., 1997; 1999; Noh et al., 2000; Zhao et al., 2000), suggesting that PLD may be involved in the regulation of the proliferation in human cancer cells.

c. Prevents Cell Cycle Arrest and Provides Survival Signal

In addition to regulating cell growth, and transformation, elevated PLD expression also could prevent cell cycle arrest and apoptosis induced by high intensity Raf signaling (Joseph et al., 2002). Similarly, fibroblasts overexpressing c-Src undergo apoptosis in response to growth factor deprivation, either PLD1 or PLD2 could provide a survival signal to prevent apoptosis in these fibroblasts (Zhong et al., 2003). Under serum starvation

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conditions, v-Src transformed fibroblasts didn't undergo apoptosis, but inhibiting PLD activity induced apoptosis in these cells. In human breast cancer cells, PLD plays important role for survival signaling, blocking PLD activity induced apoptosis in MDA-MB-231 cells deprived of serum (Zhong et al., 2003). PLD activity and PLD protein levels have also been reported to be elevated in a majority of breast cancer tissues that were examined (Noh et al., 2000). Downstream targets of PA have been described above, including PI-4-P-5-kinase (Moritz et al., 1992; Jenkins et al., 1994) and Raf (Ghosh et al., 1996; Rizzo et al., 1999). PA activating Raf may certainly contribute to the mitogenic properties of PLD, the ability of PLD to suppress cell cycle arrest and overcome apoptosis (Joseph et al., 2002) was not usually attributed to Raf. Thus, the recent demonstration of a PA requirement for mTOR activation (Fang et al., 2001) may be highly significant. mTOR is a target of survival signals generated by the PI3K/Akt survival pathway (Nave et al., 1999; Sekulic et al., 2000). Blocking mTOR pathway by rapamycin induced apoptosis in human rhabdomyosarcoma cell line (Thimmaiah et al., 2003). It has been reported S6K (the downstream target of mTOR) phosphorylated and inhibited BAD (Harada et al., 2001). BAD is a proapoptotic protein. These data suggested that mTOR could be the critical downstream mediator for PLD providing survival signal.

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EXPERIMENTAL PROCEDURE

I. Cells and cell culture conditions

MCF-7, MDA-MB-231 and MDA-MB-435S 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.

II. Transient transfection and plasmid vectors

Transfections were performed by using LipofectAMINE PLUS™ reagent (GIBCO) according to the vendor's instructions, in which PLUS™ reagent was included to enhance the transfection efficiency of LipofectAMINE. Transfection efficiency was determined by transfection of pEGFP-C1 (Clonetech), which expresses green fluorescent protein. The percentage of green cells was determined microscopically and was typically in excess of 60%. Plasmid expression vectors for PLD2 (pCGN-mPLD2) (Colley et al., 1997), and mPLD2-K758R (pCGN-mPLD2- K758R) (Sung et al., 1997; 1999) were the generous gift of Dr. Michael Frohman (SUNY-Stony Brook).

III. Materials

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Rapamycin was obtained from Sigma-Aldrich, LY294002 was obtained from Calbiochem. [^{3}H]-myristic acid was obtained from New England Nuclear. Precoated silica 60A thin layer chromatography plates were from Whatman. Stock solutions of MTS and PMS were purchased from Promega Corp. Polyclonal antibody specific for cleaved poly-(ADP-ribose) polymerase (PARP) (human specific), phosphorylated Akt (ser 473), Akt, phosphorylated S6K (Thr 389) and S6K were obtained from Cell Signaling Technology. Antibody to c-myc and β-tubulin were obtained from Santa Cruz Biotechnology. The antibody specific for p53 is the generous gift of Dr. Jill Bargonetti. The Plasmid expression vectors for PLD2 (pCGN-mPLD2) and mPLD2-K758R (pCGN-mPLD2- K758R) were the generous gift of Dr. Michael Frohman (SUNY-Stony Brook). The plasmid expression vector for Akt1 (K179M) in pUSEamp(+) is from Upstate Biotechnology.

IV. Cell proliferation assay

Cell proliferation was measured using Promega's CellTiter 96 AQueous cell proliferation assay according to the manufacturer's protocol (Promega). In this assay, metabolically active cells are detected by their ability to convert MTS reagent to formazan, which can be measured by absorbance at 490 nm. Cells are plated in 96-well plates in DMEM with 10% serum for 24 h, at

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which time cells were treated with rapamycin or as indicated. Two days after drug treatment, viable cell densities were determined by measuring metabolic conversion of the dye MTS. The reagents were added into medium and incubated for 2 h. The intensity of the color was measured at 490nm using a 96-well plate reader.

V. Western blot analysis

For PARP cleavage analysis, collecting cells on the culture dish and in the supernatant as well. Using RIPA buffer to lyse cells. Equal amount of protein from cell lysate were subjected to sodium dodecyl sulfate-polyacrylamide (SDS) gel electrophoresis. The percentage of acryamide in separating gel was determined by the molecular weight of the protein examined. Transferred to nicrocellulose filters, and blocked at room temperature for 1h with 5% nonfat dry milk in PBST(0.05% tween 20). The nicrocellulose filters were then incubated with primary antibodies as described in the figure legends. Depending on the origin of the primary antibodies, either anti-mouse or anti-rabbit IgG was used. For detection using the ECL system (Amersham) and SuperSignal™ western blotting reagent (Pierce).

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VI. Phospholipase D Assay

PLD activity was assayed as follows: Cells were grown in DMEM supplemented with 10% bovine calf serum (HyClone) in 60 mm culture dish. Subconfluent cells were then made quiescent by shifting into DMEM containing 0.5% bovine calf serum for overnight and then prelabeled for 4 h with [^{3}H]-Myristic acid, 3 μCi (40 Ci/mmol) in 3 ml of medium. PLD catalyzed transphosphatidylation in the presence of 0.8% 1-BtOH. Extraction and characterization of lipids by thin layer chromatography were performed as previously described (Shen et al., 2001).

VII. Cell viability assay

Cell viability was determined by trypan blue exclusion. After various treatments, cells were harvested, washed and treated with trypan blue at a concentration of 0.4% w/v. After 10 min, trypan blue uptake (dead cells) was determined by counting on a hemocytometer.

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I

PHOSPHOLIPASE D CONFERS RAPAMYCIN RESISTANCE IN HUMAN BREAST CANCER CELLS

Introduction

Some reports indicate PLD is an important regulator in cell proliferation. PLD is elevated in response to most mitogenic and oncogenic signals. Previous works from our Lab show that PLD could prevent cell cycle arrest, apoptosis (Joseph et al., 2002); and fully transform cells with weakly transformed phenotypes (Lu et al., 2000; Joseph et al., 2001; Min et al., 2001). PLD protein expression level and activity are elevated in a majority of breast cancer (Noh et al., 2000). Interestingly, it has been reported lately, that mTOR requires phosphatidic acid (PA) for signaling activation (Fang et al., 2001). PA is generated in response to mitogenic signals by the phospholipase D (PLD)-catalyzed hydrolysis of phosphatidylcholine (Exton, 1999). mTOR (mammalian target of rapamycin) (Brown et al., 1994; Chiu et

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al., 1994; Sabatini et al., 1994; Sabers et al., 1995) is a protein kinase that regulates cell cycle progression and cell growth (Kuruvilla et al., 1999; Schmelzie et al., 2000). Rapamycin, a highly specific inhibitor of mTOR (Cafferkey et al., 1993; Brown et al., 1994; Sabatini et al., 1994), has been widely used to block cell proliferation and is in clinical trials for treatment of breast and other cancers (Hidalgo et al., 2000). Rapamycin acts by binding to the immunophilin FK506 binding protein 12 (FKBP12), which then binds mTOR and inhibits its function in a poorly understood fashion (Chen et al., 2002). PA interacts with the FKBP12-rapamycin-binding (FRB) domain of mTOR and rapamycin competes with PA binding to mTOR (Fang et al., 2001). I have investigated the effects of rapamycin on the proliferation of human breast cancer cell lines with different levels of PLD activity. In the following, I will provide evidence that elevated PLD activity leads to rapamycin resistance in human breast cancer cells.

Results

1. Rapamycin resistance in breast cancer cells correlates with the level of PLD activity

Rapamycin has been reported to have differential efficacy on the proliferation of breast cancer cell lines (Yu et al., 2002). The effects of

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rapamycin upon MCF-7, MDA-MB-231 and a fast growing MCF-7 cell line variant (MCF-7R) were examined. As shown in Fig. 5A, the proliferation of MCF-7 cells was sensitive to low nanomolar concentrations of rapamycin, whereas MDA-MB-231 cell proliferation was resistant to micromolar concentrations of rapamycin. MCF-7R cells had an intermediate sensitivity to rapamycin. The IC_{50} for rapamycin on MCF-7 cells was about 20 nM, whereas the IC_{50} for MDA-MB-231 cells was about 10 μM; MCF-7R cells had an IC_{50} of about 600 nM (Fig. 5B). PLD activity in these cells was then determined, and as shown in Fig. 5C, the level of PLD activity in these three cell lines correlated with the resistance to rapamycin, with MDA-MB-231 cells having 10-fold greater PLD activity than MCF-7 cells. The fast growing MCF-7R cells had 5-fold elevated PLD activity relative to MCF-7 cells. These data are consistent with the hypothesis that elevated PA generated by PLD is competing with rapamycin for mTOR (Fang et al., 2001; Chen et al., 2002).

2. Elevated PLD activity in MCF-7 cells confers resistance to rapamycin

Data presented in Fig. 5 showed that MCF-7R cells with elevated PLD activity were resistant to rapamycin. To test whether it was the PLD activity

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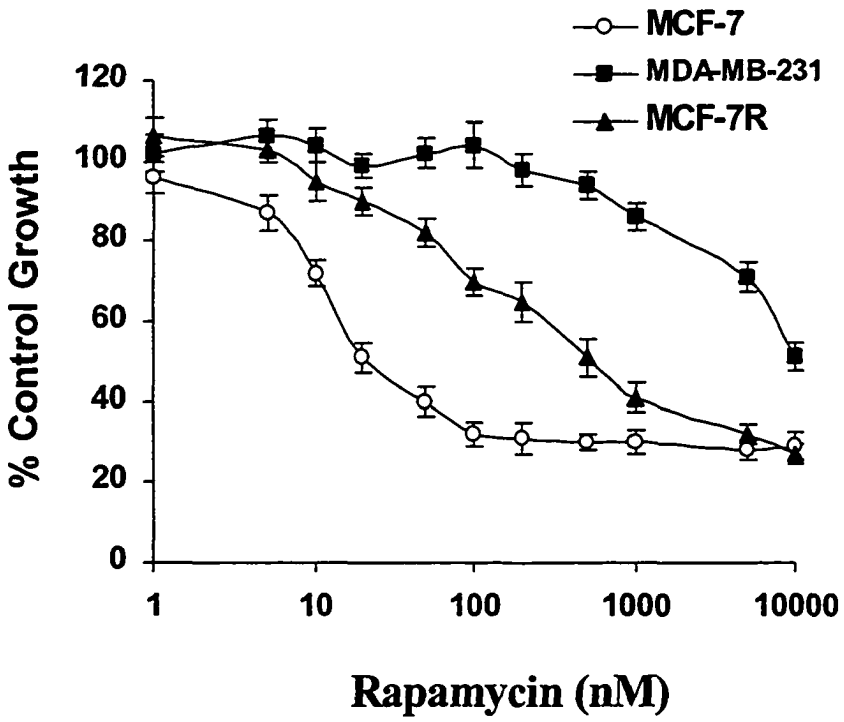

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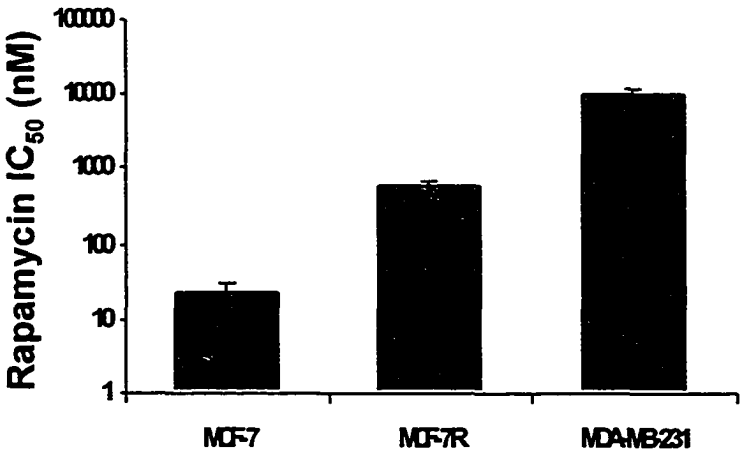

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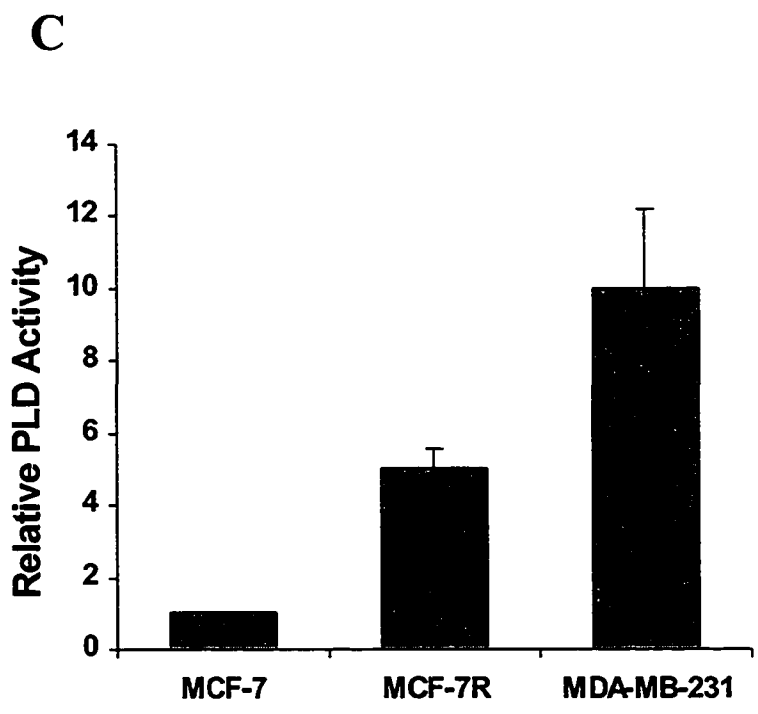

Fig. 5. Increasing PLD activity in breast cancer cells correlates with resistance to rapamycin

(A) MCF-7, MCF-7R, and MDA-MB-231 cells were plated in 96-well culture plates in Dulbecco's modified Eagle's medium with 10% bovine calf serum. MCF-7 cells were plated at 6000 cells per well; MCF-7R and MDA-MB 231 cells were plated at 3000 cells per well. After 24 hr, rapamycin was added with increasing concentration as shown. Two days later, metabolically active cells were determined using Promega's CellTiter 96 AQueous cell proliferation assay according to the manufacturer's protocol (Promega). The effect of rapamycin on cell proliferation was calculated as the percentage of metabolically active cells relative to the untreated controls, which were given a value of 100%. The data shown are the average of 3 independent experiments with error bars representing the standard deviation. (B) IC_{50} values, the concentration of rapamycin required to inhibit proliferation by 50%, were averaged over 3 independent experiments. Error bars represent the standard deviation. (C) PLD activity in MCF-7, MCF-7R, and MDA-MB 231 cells was determined as described previously (Shen et al., 2001). The PLD activity in the cell lines was normalized to that in MCF-7 cells, which was given a value of 1. Error bars represent the standard deviation for 3 independent experiments.

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that conferred the resistance to rapamycin, an expression vector for PLD2 was transiently transfected into the rapamycin-sensitive MCF-7 cells. PLD2 was used in these studies because PLD2 expression is tolerated better than PLD1 (Joseph et al., 2001; Shen et al., 2001). However as described previously, both PLD1 and PLD2 transform cells with an overexpressed tyrosine kinase (Joseph et al., 2001). Transient expression of PLD2 in MCF-7 cells resulted in a three-fold increase in PLD activity (Fig. 6A). This also resulted in increased resistance to rapamycin (Fig. 6B) as indicated by a tenfold increase in the IC_{50} for inhibition of cell proliferation (Fig. 6C). These data further suggest that elevated PLD activity leads to rapamycin resistance and indicate that the increased rapamycin resistance seen in MCF-7R cells was likely due to the elevated PLD activity in these cells.

3. Suppression of PLD activity in MDA-MB-231 cells confers sensitivity to rapamycin

Next I investigated whether inhibiting PLD activity in the rapamycin-resistant MDA-MB-231 cells could increase the sensitivity to rapamycin. Catalytically inactive mutants of PLD have been generated (Sung et al., 1997; 1999) that it has been used previously as dominant negative mutants

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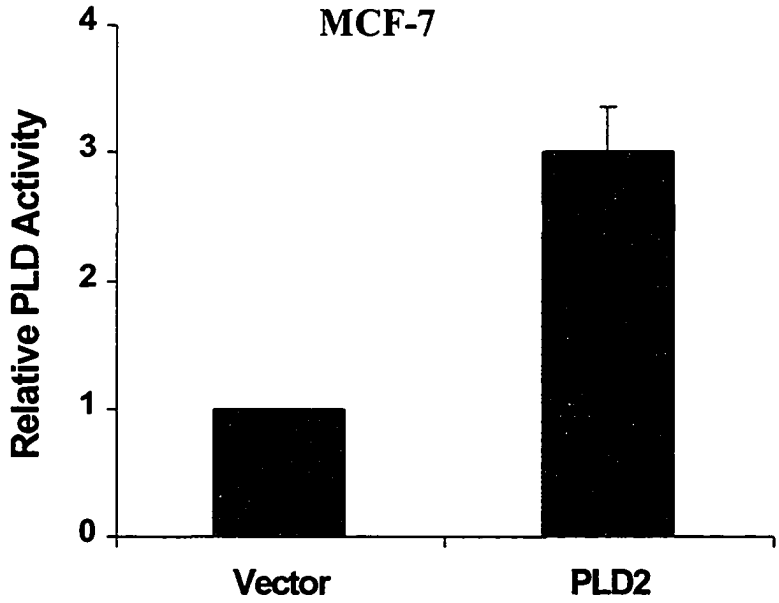

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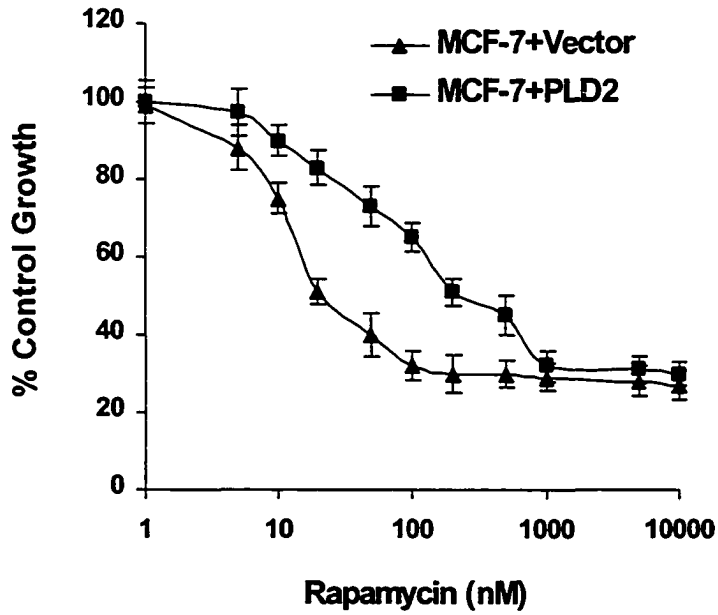

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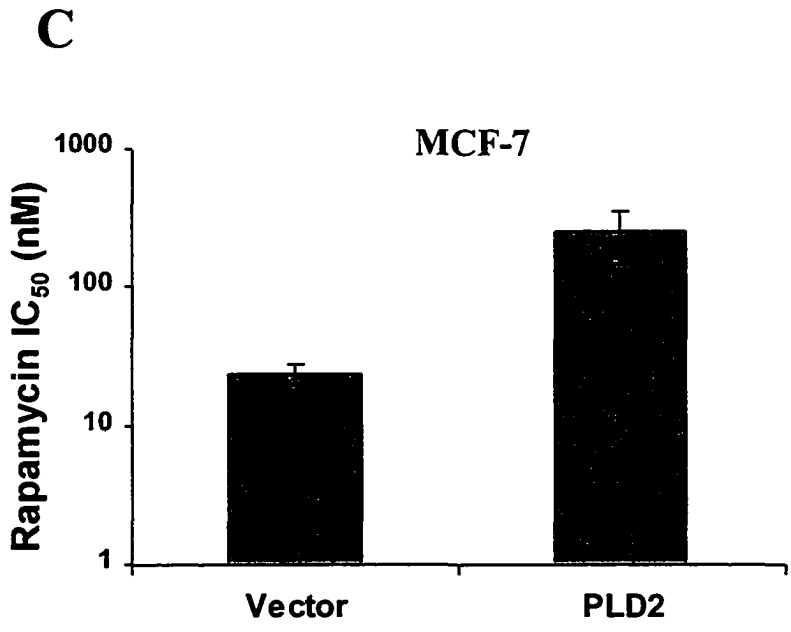

Fig. 6. Elevated expression of PLD2 confers resistance to rapamycin in MCF-7 cells (A) MCF-7 cells were plated in 60mm culture dish at 2 x 10^5 cells per dish. 24 hr later, the cells were transfected with either pCGN-mPLD2, which expresses wild type PLD2 (Colley et al., 1997) or the pCGN empty vector. 30 hr later, cells were placed in media containing 0.5% bovine calf serum for overnight and the relative PLD activity in MCF-7 cells transfected with pCGN-mPLD2 relative to those transfected with the empty vector was determined as in Fig. 5. The data were normalized to the PLD activity in MCF-7 cells transfected with the empty vector, which was given a value of 1. (B) MCF-7 cells were plated in 96-well culture plates at 6,000 cells per well. 24 hr later the cells were transiently transfected with either pCGN-mPLD2 or the pCGN empty vector. Three hours later rapamycin was added at increasing concentrations and the effect of rapamycin on cell proliferation was calculated as the percentage of metabolically active cells relative to the untreated controls, which were given a value of 100% as in Fig. 5. (C) IC_{50} values were determined for 3 independent experiments and averaged. Error bars represent the standard deviation. Transfections were performed by using LipofectAMINE PLUS™ reagent (GIBCO) according to the vendor's instructions. Transfection efficiency was determined by transfection of pEGFP-C1 (Clonetech), which expresses green fluorescent protein. The percentage of green cells was determined microscopically and was routinely in excess of 60%.

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to block PLD signaling (Shen et al., 2001). Introduction of the catalytically inactive PLD2 mutant into MDA-MB-231 cells reduced the PLD activity by greater than 60% (Fig. 7A). Along with this reduction in PLD activity, resistance to rapamycin was reduced (Fig. 7B) as indicated by a reduction in the IC_{50} from 10 μM to 100 nM (Fig. 7C). Thus, reducing PLD activity in MDA-MB-231 cells increased sensitivity to rapamycin.

4. The rapamycin-induced inhibition of S6 kinase phosphorylation is overcome by elevated PLD activity

Ribosomal subunit S6 kinase (S6K) is an important downstream target for mTOR regulating translation (Burnett et al., 1998). I examined the effect of rapamycin upon the phosphorylation of S6K in MCF-7 and MDA-MB-231 cells. In Fig. 8A, it can be seen that S6K phosphorylation was blocked by rapamycin at about 20 nM in MDA-MB-231 cells; whereas in MCF-7 cells, was similarly blocked at about 0.5nM rapamycin (Fig. 8B). Introduction of the catalytically inactive PLD2 into MDA-MB-231 cells increased sensitivity to rapamycin (Fig. 8A); and introduction of wild type PLD2 into MCF-7 cells increased resistance to rapamycin (Fig. 8B). For reasons that are not clear, S6K phosphorylation is more sensitive to rapamycin than

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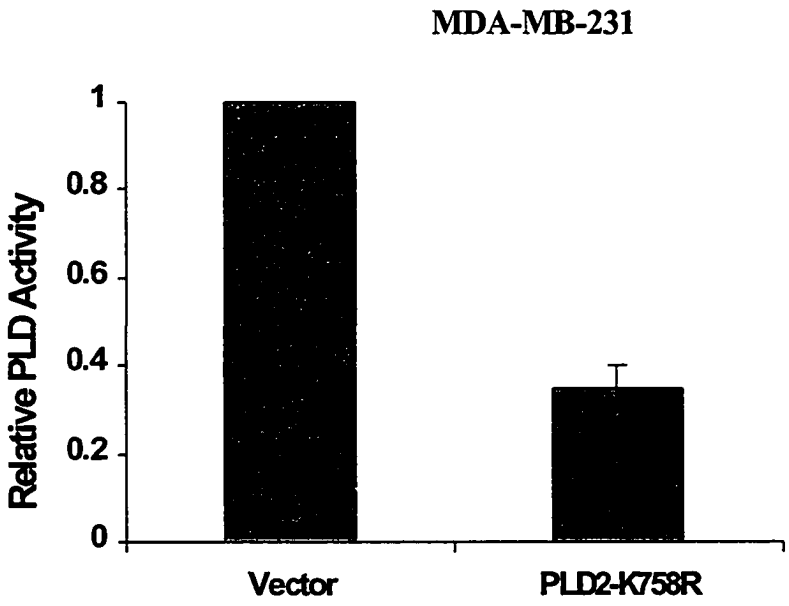

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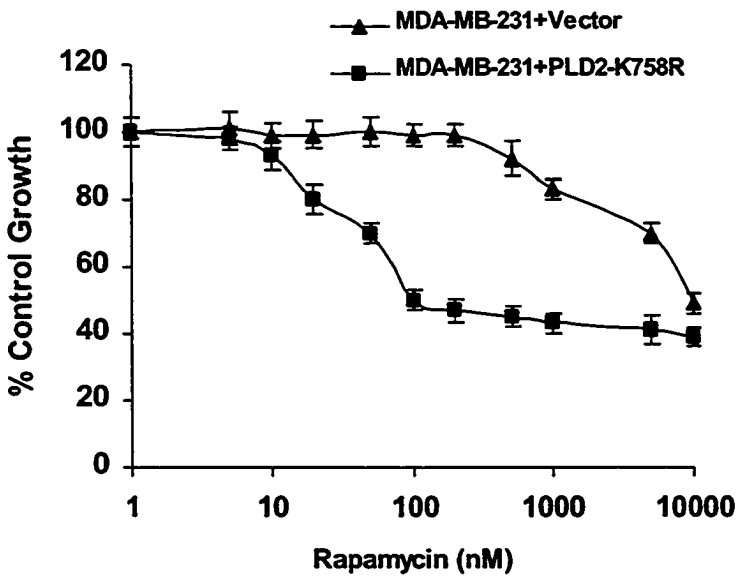

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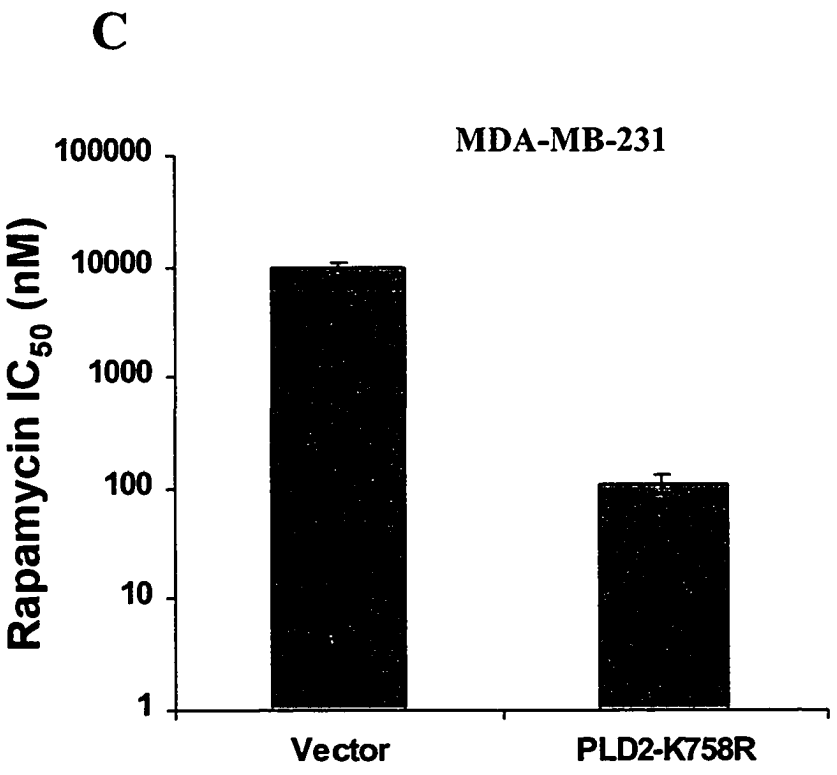

Fig. 7. Inhibiting PLD activity in MDA-MB-231 cells increases sensitivity to rapamycin

(A) MDA-MB-231 cells were plated in 60mm culture dish at 10^5 cells per dish. 24 hr later, the cells were transfected with either pCGN-mPLD2- K758R, which expresses a catalytically inactive PLD2 (Colley et al., 1997) or the pCGN empty vector. 30 hr later, cells were placed in media containing 0.5% bovine calf serum for overnight and the relative PLD activity in MDA-MB-231 cells transfected with pCGN-mPLD2- K758R relative to those transfected with the empty vector was determined as in Figure 5. The data were normalized to the PLD activity in MDA-MB-231 cells transfected with the empty vector, which was given a value of 1. (B) MDA-MB-231 cells were plated in 96-well culture plates at 3,000 cells per well. 24 hr later the cells were transiently transfected with either pCGN- mPLD2-K758R or the pCGN empty vector. Three hours later rapamycin was added at increasing concentrations and the effect of rapamycin on cell proliferation was calculated as the percentage of metabolically active cells relative to the untreated controls, which were given a value of 100% as in Fig. 5 and 6. (C) IC_{50} values were determined for 3 independent experiments and averaged. Error bars represent the standard deviation.

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proliferation. At the concentration of rapamycin that can dramatically block S6K phosphorylation, the cells were still able to proliferate, indicating that the effect of rapamycin upon S6K phosphorylation was not sufficient to block translation and cell growth. The nature of the differential effect of rapamycin on proliferation and S6K phosphorylation may reflect differential effects of the rapamycin on different downstream targets of mTOR.

5. PLD reduces the sensitivity of serum-induced Myc expression to rapamycin

Expression of Myc is critical for progression through the cell cycle and for cell proliferation (Grandori et al., 2000). Moreover, mTOR has been implicated in Myc expression (West et al., 1998). The sensitivity of c-Myc expression to rapamycin was examined in MCF-7, MCF-7R and MDA-MB-231 cells. As shown in Fig. 9A, c-Myc expression in the absence of serum was detected in MCF-7R and MDA-MB-231 cells, but not in MCF-7 cells. In MCF-7 cells, serum induced c-Myc expression and this induction was sensitive to 50 nM rapamycin (Fig. 9A). In MCF-7R cells, serum induced c-Myc expression was resistant to 50 nM rapamycin. c-Myc expression in MDA-MB-231 cells was completely insensitive to 50 nM rapamycin (Fig. 9A). When PLD2 was introduced into MCF-7 cells, the serum-induced

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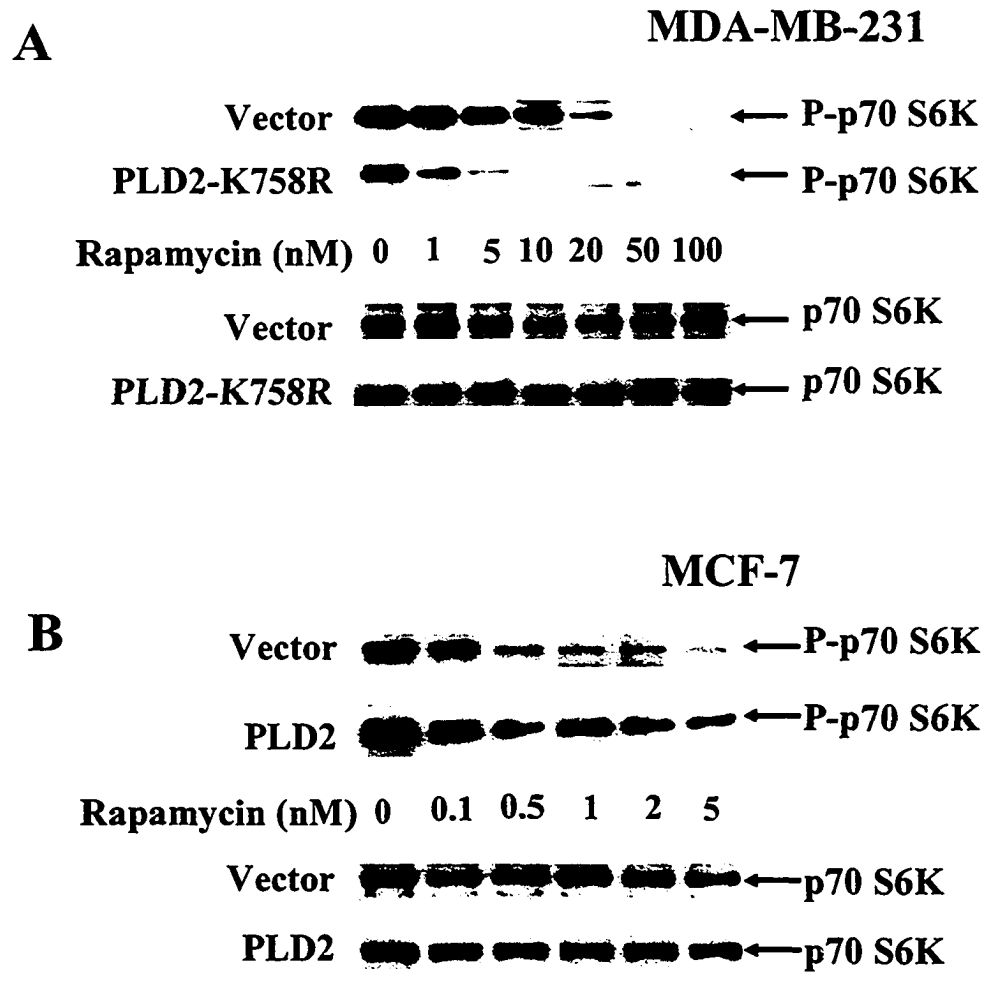

Fig. 8. The rapamycin-induced inhibition of S6 kinase phosphorylation is overcome by elevated PLD activity

(A) MDA-MB-231 and (B) MCF-7 cells were plated in 60mm culture dish at 10^5 and 2 x10^5 cells per dish respectively. 24hr later MDA-MB-231 and MCF-7 cells were transiently transfected with either mPLD2-K758R (MDA-MB-231), pCGN-mPLD2 (MCF-7), or the pCGN empty vector. The next day, cells were placed in media containing 0.5% serum with increasing concentrations of rapamycin for 16 hr as indicated. Cell extracts were prepared and analyzed for phosphorylated S6K or S6K protein by Western Blot analysis as described previously (Shen et al., 2001). Antibodies specific for S6K and phosphorylated S6K (Thr389) were obtained from Cell Signaling Technology. The data presented in A and B are representative experiments repeated twice.

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Myc expression became resistant to 50 nM rapamycin (Fig. 9B). There was also a slight increase in Myc expression in the absence of serum when PLD2 was introduced. The concentration of rapamycin required for inhibition of Myc expression was closer to that required to inhibit proliferation than for the inhibition of S6K observed above in Fig. 8. The data in Fig. 8 and 9 provide evidence that in addition to inhibiting the effects of rapamycin upon cell proliferation, PLD also interferes with the ability of rapamycin to inhibit S6K phosphorylation and the expression of a gene that is critical for cell proliferation.

Discussion

The evidence provided demonstrates that elevated PLD activity leads to rapamycin resistance in human breast cancer cells. Other mechanisms of resistance to rapamycin have been described previously (Aoki et al., 2001). Mutations that prevent rapamycin from interacting with either FKBP12 or mTOR can confer resistance, as can mutations to downstream targets of mTOR. However, most mutations of this nature would be loss of function mutations and should, in principle, require two mutations and therefore be relatively rare. Elevation of PLD activity is a gain of function and therefore should only require mutations to a single allele of either PLD or a regulator

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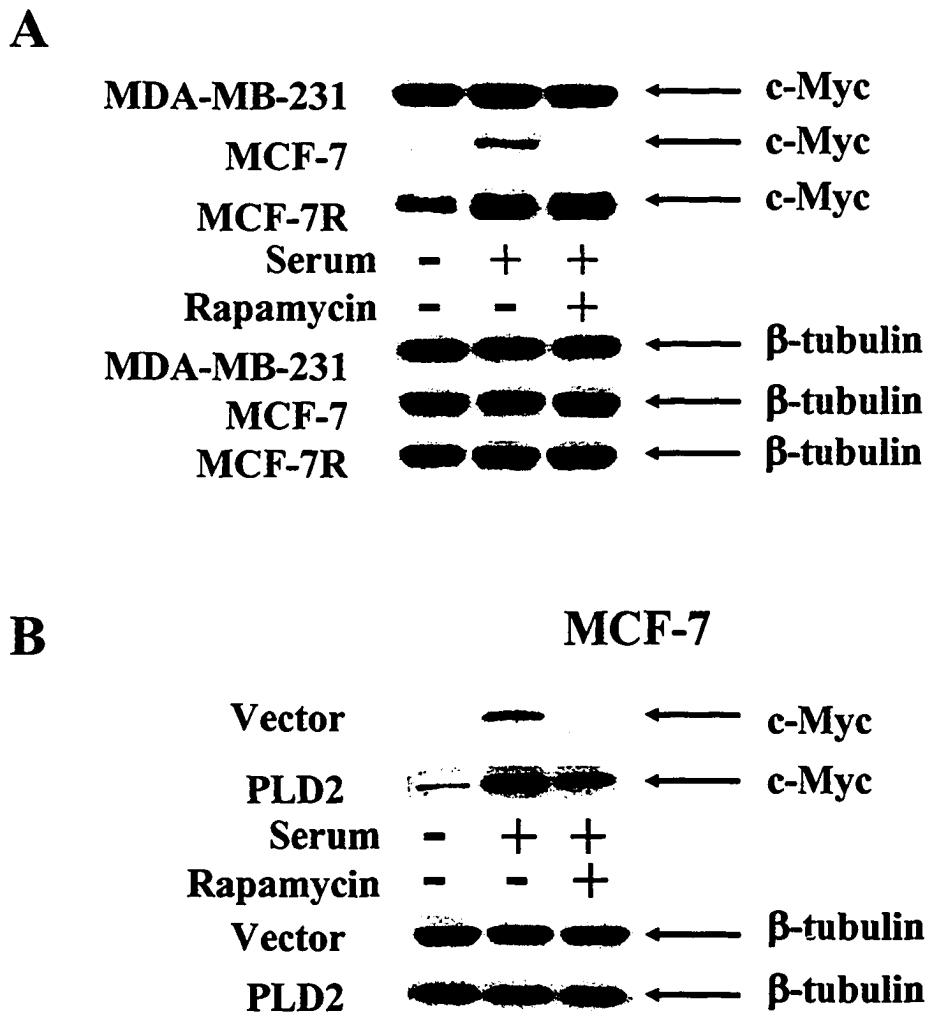

Fig. 9. PLD reduces the sensitivity of serum-induced Myc expression to rapamycin (A) MCF-7 cells were plated at 2 x 10^5 cells per 60mm culture dish; MCF-7R cells and MDA-MB-231cells were plated at 10^5 cells per 60mm culture dish. Two days later, cells were placed in serum free media for 24 hr. Serum was then added, where indicated, to a final concentration of 10%. The cells were incubated for an additional 4 hours with or without rapamycin (50nM) (rapamycin was added 15 min before serum stimulation). Levels of c-Myc were determined by subjecting cell lysates to Western Blot analysis using an anti-Myc antibody (Santa Cruz Biotechnology). (B) MCF-7 cells were plated in 60mm culture dish at 2 x 10^5 cells per dish. 24hr later, the cells were transiently transfected with either pCGN-mPLD2 or pCGN empty vector. The next day, cells were placed in serum free media for 24 hr. Serum was then added to a final concentration of 10% where indicated. The cells were incubated for an additional 4 hours with or without rapamycin (50nM) (the rapamycin was added 15 min before serum stimulation). The levels of c-Myc expression were determined as in A. Loading controls for A and B were performed by stripping the membranes and reprobing with an antibody to β-tubulin (Santa Cruz Biotechnology).

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of PLD. In breast cancer, where rapamycin is in clinical trials (Hidalgo et al., 2000), PLD activity was found to be elevated in a substantial majority of the cases examined (Noh et al., 2000). Thus, it would be predicted that rapamycin would not be as effective in cases where there was elevated PLD activity. Interestingly, rapamycin has been reported to be especially effective in cells where the phosphatidylinositol-3-kinase (PI3K)/Akt survival pathway is activated (Aoki et al., 2001; Mills et al., 2001; Neshat et al., 2001; Podsypanina et al., 2001), which also occurs frequently in breast cancer (Fry, 2001). Since Akt phosphorylation and PLD activity/protein levels are easily determined, the status of PI3K/Akt signaling and PLD levels in a given breast cancer should give a good molecular indication of the likely efficacy of rapamycin. S6K is a well known downstream target of mTOR, it is an important initiator of translation (Burnett et al., 1998). However, the data presented show that S6K phosphorylation is more sensitive to rapamycin than proliferation. At the concentration of rapamycin that can dramatically block S6K phosphorylation, the cells were still able to proliferate, indicating that the effect of rapamycin upon S6K phosphorylation was not sufficient to block translation and cell growth. These data suggest there are other downstream targets of PLD/mTOR pathway involving in the regulation of cell proliferation.

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PLD activity is elevated in cells transformed by v-Src (Song et al., 1991), v-Ras (Carnero et al., 1994; Jiang et al., 1995), and v-Raf (Frankel et al., 1999). PLD activity is also elevated in response to mitogens including PDGF (Plevin et al., 1991), EGF (Song et al., 1994), and insulin (Karnam et al., 1997). More recently, PLD activity has been shown to cooperate with overexpression of a tyrosine kinase to transform rat fibroblasts (Lu et al., 2000; Joseph et al., 2001) and to overcome cell cycle arrest and apoptosis in cells with high intensity Raf signals (Joseph et al., 2002). In addition, many proteins that regulate PLD activity have also been implicated in regulating cell cycle progression and cell transformation, including RalA (Jiang et al., 1995), Rho family GTPases (Exton, 1999), and PKCα (Hornia et al., 1999). Thus, evidence supporting a role for PLD in regulating both growth factor- and oncogene-induced cell proliferation is substantial, and therefore the elevation of PLD activity in a majority of breast cancer tissues examined (Noh et al., 2000) is likely a contributing factor in breast cancer progression.

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II

NOVEL PHOSPHOLIPASE D / mTOR SURVIVAL PATHWAY IN HUMAN BREAST CANCER CELLS

Introduction

In the progression of a normal cell to a malignant cancer there are a number of obstacles that must be overcome in order for the cancer cells to divide and spread (Hanahan et al., 2000). One of these obstacles is default apoptosis programs that kick in when there is inappropriate proliferation signals or damage to the genome. This can be accomplished by the activation of survival signals that suppress apoptosis and allow progression through critical cell cycle checkpoints (Igney et al., 2002). The best-studied survival signals are those generated by elevating the activity of phosphatidylinositol (PI)-3-kinase (PI3K). PI3K generates PI-3,4,5-tris-phosphate (PIP3), which leads to the activation of Akt protein kinase. Akt then phosphorylates and inactivates many substrates proteins that negatively regulate cell cycle progression and/or facilitate apoptosis (Vivanco et al., 2002). This survival signal pathway is activated in many cancers, frequently by the loss or

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inactivation of PTEN, a phosphatase that dephosphorylates PIP3 to PIP2 (Vivanco et al., 2002; Fry, 2001).

PLD activity is elevated in response to most mitogenic signals, and has been reported to prevent cell cycle arrest and apoptosis (Joseph et al., 2002); and fully transform cells with weakly transformed phenotypes (Lu et al., 2000; Joseph et al., 2001; Min et al., 2001; Ahn et al., 2003). Downstream targets of PA have been described including phosphatidylinositol-4-phosphate-5-kinase (Moritz et al., 1992; Jenkins et al., 1994) and Raf (Ghosh et al., 1996; Rizzo et al., 1999). While impacting upon Raf activation may certainly contribute to the mitogenic properties of PLD, the ability of PLD to suppress cell cycle arrest and overcome apoptosis (Joseph et al., 2002) was not usually attributed to Raf. Thus, the recent demonstration of a PA requirement for mTOR activation (Fang et al., 2001) may be highly significant. mTOR (mammalian target of rapamycin), a protein kinase that regulates cell cycle progression and cell growth (Kuruvilla et al., 1999; Schmelzie et al., 2000). Rapamycin is a highly specific inhibitor of mTOR (Cafferkey et al., 1993; Brown et al., 1994; Sabatini et al., 1994); has been widely used to block cell proliferation; and is in clinical trials for treatment of breast and other cancers (Hidalgo et al., 2000). Rapamycin acts by

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binding to FKBP12, which then binds mTOR and inhibits its function in a poorly understood fashion (Chen et al., 2002). mTOR has a requirement for phosphatidic acid (PA) for activation and PA interacts with the FKBP12-rapamycin-binding (FRB) domain of mTOR (Fang et al., 2001; Chen et al., 2002). It was proposed that rapamycin competes with PA binding to mTOR (Fang et al., 2001). PA is generated in response to mitogenic signals by the phospholipase D (PLD)-catalyzed hydrolysis of phosphatidylcholine (Exton, 1999). And consistent with the hypothesis that rapamycin competes with PA for binding to mTOR, breast cancer cells with elevated PLD activity require higher levels of rapamycin to block cell proliferation (Chen et al., 2003).

It has been reported that the PI3K/Akt pathway generates survival signals through mTOR (Nave et al., 1999; Sekulic et al., 2000). Blocking mTOR pathway by rapamycin induced apoptosis in human rhabdomyosarcoma cell line (Thimmaiah et al., 2003). S6K (the downstream target of mTOR) phosphorylated and inhibited BAD (Harada et al., 2001). BAD is a proapoptotic protein. Since both PLD and mTOR have been implicated in survival signaling and there is a PA (the important metabolite of PLD) requirement for mTOR activation, mTOR is a strong candidate for a

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downstream target of PLD responsible for the survival signals. PLD activity and expression have been reported to be elevated in a majority of breast (Noh et al., 2000) and renal (Zhao et al., 2000) cancers examined. MDA-MB-231 breast cancer cells have very highly elevated levels of PLD activity relative to other breast cancer cells, and this PLD activity is critical for the survival of this breast cancer cell line when subjected to the apoptotic stress of serum withdrawal (Zhong et al., 2003).

Recently the most strategies of developing anti-cancer medicine are targeting the growth factor signal transduction pathways. For instance EGF, VEGF, Insulin. However some cancers are resistant to these anticancer medicine. Understanding survival signals activated in those resistant human cancers offers great therapeutic opportunities, since suppression of these signals, in principle, will induce cancers to kill themselves. I have studied the survival pathways in different human breast cancer cell lines resistant to the apoptotic stress of serum withdrawal. In the following, I will provide evidence to show a novel survival signal generated by PLD / mTOR that may be elevated in cancers where the well-characterized PTEN/PI3K/Akt survival pathway has not been activated.

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Results

1. Resistance of breast cancer cell lines to serum withdrawal

A hallmark of cancer cells is the ability to generate survival signal that overcome default apoptosis signals (Hanahan et al., 2000). Many cells undergo apoptosis in the absence of growth factors that stimulate survival signals. MDA-MB-231 and MCF-7 breast cancer cell lines have frequently been used to represent highly malignant and benign cancer cells respectively (Yu et al., 2002). Consistent with this paradigm, MCF-7 cells undergo apoptosis when subjected to serum withdrawal, whereas MDA-MB-231 cells are resistant to this stress (Fig. 10). Two additional breast cancer cell lines were also examined for tolerance to serum withdrawal, MDA-MB-435S and MCF-7R. MCF-7R cells are a fast growing derivative of MCF-7 cells (Chen et al., 2003). As shown in Fig. 10, both of these cell lines are resistant to the apoptotic stress of serum withdrawal as indicated by cell viability (Fig. 10A) and the cleavage of PARP (Fig. 10B).

2. Survival signaling in breast cancer cells

Survival signals are frequently generated through the PI3K/Akt signaling pathway. Therefore I examined the phosphorylation level of Akt in these

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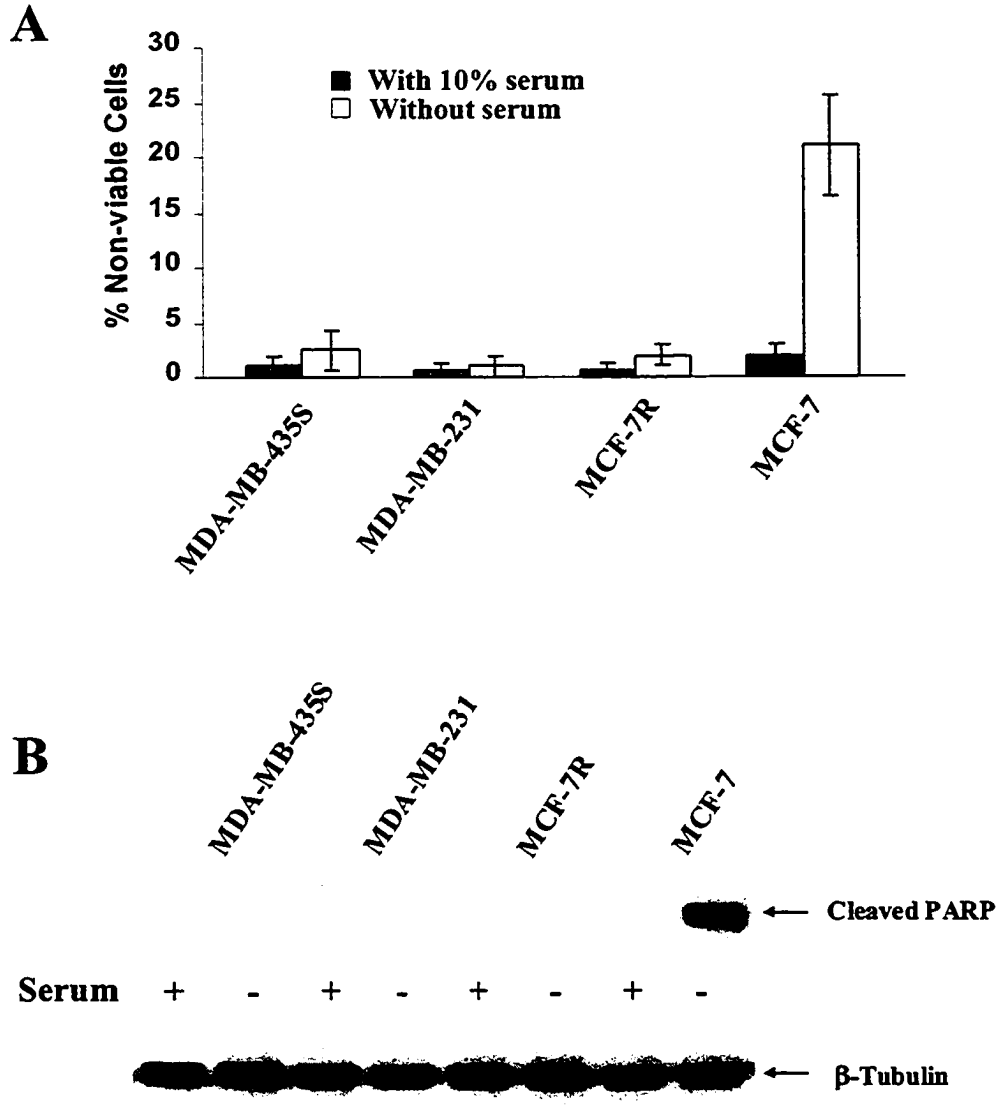

Fig. 10. Resistance of breast cancer cell lines to serum withdrawal
(A) MDA-MB-435S and MCF-7 cells were plated at $4x10^5$ cells/60 mm culture dish; MDA-MB-231 and MCF-7R cells were placed at $2x10^5$ cells/60 mm culture dish. At the next day, the culture media was replaced with fresh media containing either 10% or 0% serum (as indicated). 24hr later the percentage of non-viable cells was determined by trypan blue exclusion. Error bars represent the standard deviation for 3 independent experiments. (B) MDA-MB-435S, MDA-MB-231, MCF-7 and MCF-7R cells were prepared as in A and examined for the level of cleaved PARP using Western Blot analysis as described previously. Blots were stripped and reprobed with an antibody to β-tubulin to control for loading. The experiment shown is representative of three independent experiments.

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four breast cancer cell lines. As shown in Fig. 11A, there were elevated levels of Akt phosphorylation in MDA-MB-435S cells relative to MDA-MB-231, MCF-7, and MCF-7R. It has been shown previously that there were elevated levels of PLD activity in MDA-MB-231 and MCF-7R cells (Chen et al., 2003) and since PLD has also been reported to generate survival signals (Joseph et al., 2002; Zhong et al., 2003), I compared the levels of PLD activity in MDA-MB-231 and MCF-7R cells with that in MDA-MB-435S cells. As shown in Fig. 11B, there were very low levels of PLD activity in MDA-MB-435S cells relative to MCF-7R and MDA-MB-231 cells. In fact, there was more PLD activity in MCF-7 cells than in MDA-MB-435S cells (Fig. 11B). These data suggested that alternative survival signals were being generated in the breast cancer cell lines resistant to the apoptotic stress of serum withdrawal.

3. Differential sensitivity of breast cancer cells to the inhibition of PI3K/Akt survival pathway

Akt phosphorylation is dependent upon PI3K, which generates PIP3. PIP3 recruits both Akt and PDK1 to the plasma membrane, and facilitates phosphorylation of Akt by PDK1 (Vivanco et al., 2002). To characterize the dependence of survival signaling upon PI3K and Akt, I examined the effect of PI3K inhibitor LY294002 on cell survival in the absence of serum.

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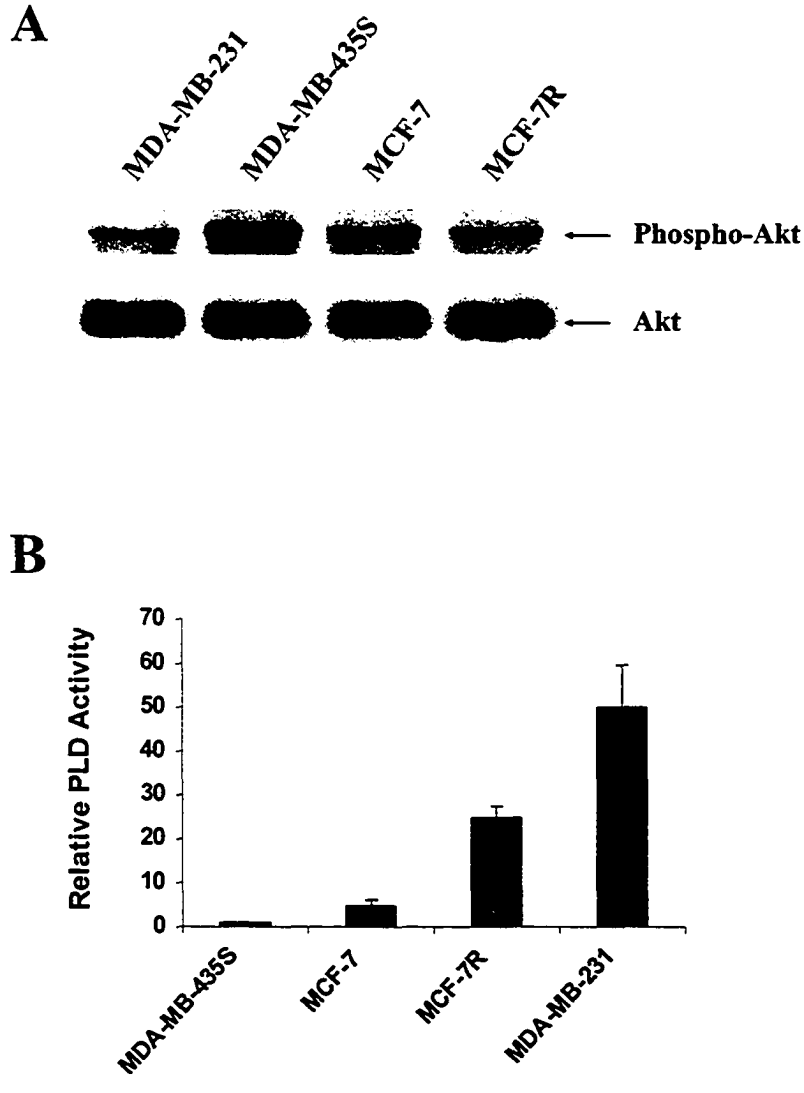

Fig. 11. Survival signaling in breast cancer cells
(A) MDA-MB-435S, MDA-MB-231, MCF-7 and MCF-7R cells were prepared as in Fig. 10. At the next day, the culture media was replaced with fresh media lacking serum. 24 hr later the levels of phosphorylated Akt were examined by Western Blot analysis using an antibody specific for the phosphorylated form of Akt (upper row). Total Akt protein levels (lower panel) were determined using an anti-Akt antibody. The experiment shown is representative of three independent experiments. (B) PLD activity in these breast cancer cells examined in A was determined as described previously. PLD activity was normalized to the PLD activity in MDA-MB-435S cells, which was given a value of 1. Error bars represent the standard deviation for 3 independent experiments.

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LY294002 reduced cell viability (Fig. 12A), induced PARP and caspase 3 cleavage (Fig. 12B and 12E) in MDA-MB-435S cells. Interestingly, both MDA-MB-231 and MCF-7R cells were largely resistant to LY294002 in the absence of serum (Fig. 12A and 12B). As shown in Fig. 12C, LY294002 strongly reduced Akt phosphorylation in MDA-MB-435S cells where there were elevated levels of activated Akt. BAD is a proapoptotic protein, it has been reported that PI3K/Akt provided survival signals by phosphorylating and inhibiting BAD (Datta et al., 1997). The effect of LY294002 upon the phosphorylation state of BAD was examined; and as shown in Fig. 12D, LY294002 significantly reduced the phosphorylation level of BAD. Overexpressing dominant negative Akt1 into MDA-MB-435S cells decreased the level of activated Akt and induced apoptosis as shown in Fig 12F and G. These data reveal that in cells where there is elevated Akt phosphorylation, survival is dependent upon PI3K in the absence of serum, which was the expected result. More significantly however, the breast cancer cell lines with elevated PLD activity were not dependent upon PI3K for survival in the absence of serum. These data support the hypothesis that alternative survival signals are operational in different breast cancer cells.

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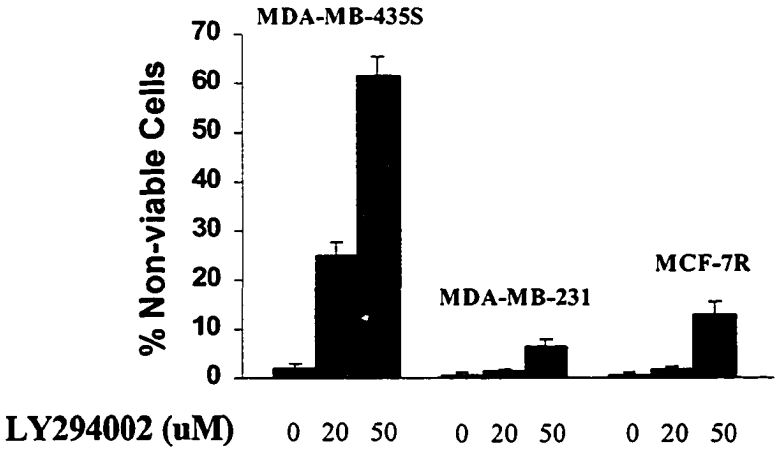

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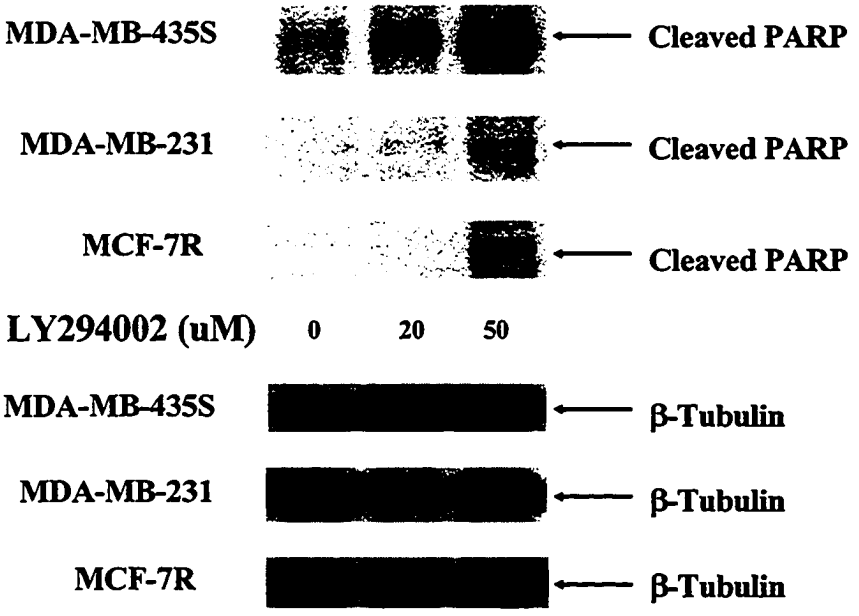

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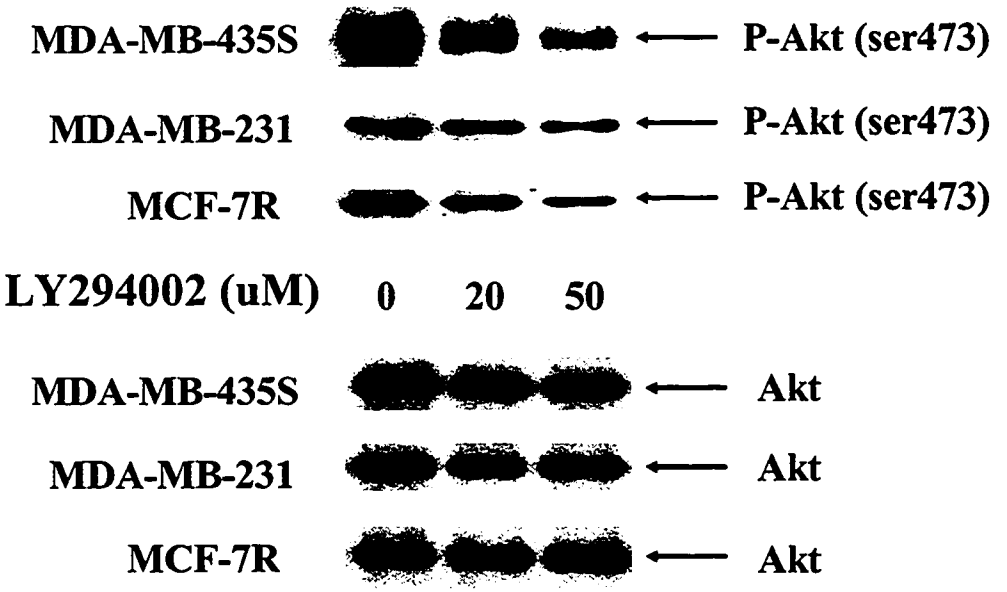

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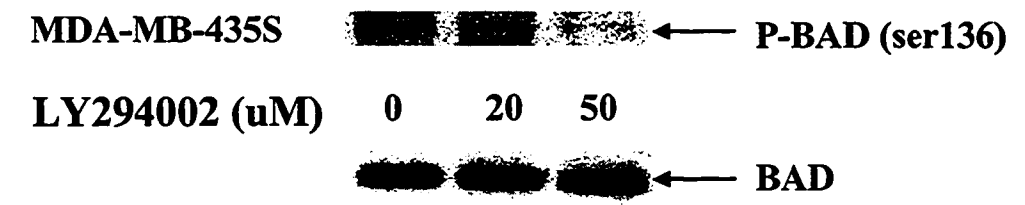

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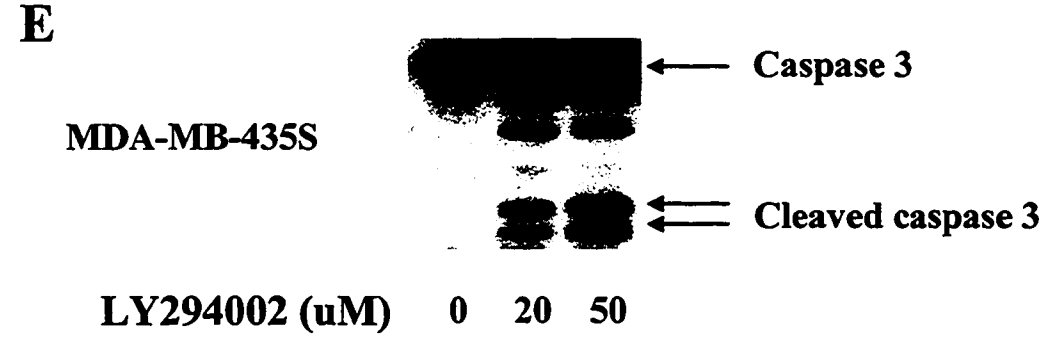

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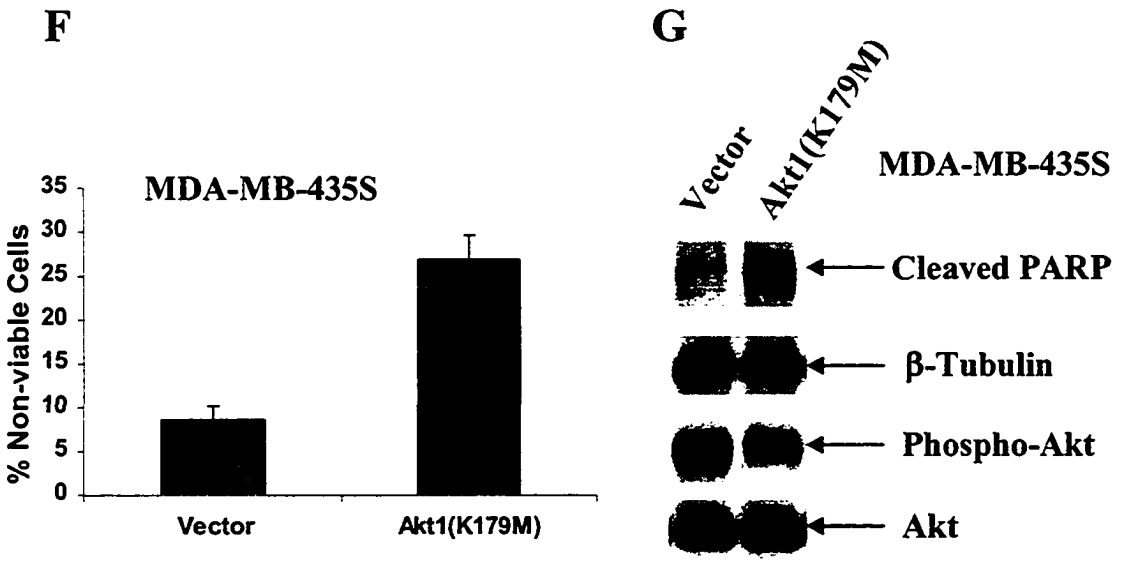

Fig. 12. Differential sensitivity of breast cancer cells to the inhibition of PI3K/Akt survival pathway

(A) MDA-MB-435S, MDA-MB-231, and MCF-7R cells were plated as described in Fig. 10. 24 hr later the cells were shifted into media lacking serum in the presence of the indicated concentrations of LY294002. At the next day, the % of non-viable cells was determined as in Fig. 10. Error bars represent the standard deviation for 3 independent experiments. (B) MDA-MB-231, MCF-7R, and MDA-MB-435S cells were prepared as in A, and the level of cleaved PARP was determined by Western Blot analysis as in Fig. 10. Blots were stripped and reprobed with an antibody to β-tubulin to control for loading as in Fig. 10. (C) MDA-MB-231, MCF7R, and MDA-MB-435S cells were prepared as in A, the levels of phosphorylated Akt were determined as in Fig. 11A. (D) MDA-MB-435S cells were prepared as in A, the levels of phosphorylated BAD and total BAD were determined. (E) MDA-MB-435S cells were prepared as in A, the levels of caspase 3 and cleaved caspase 3 were determined using an antibody specific for caspase 3. The experiment shown in B, C, D, and E is representative of three independent experiments. (F)) MDA-MB-435S cells were plated in 60mm culture dish at 2 x 10^5 cells per dish. 24 hr later, the cells were transfected with either Akt1 (K179M) in pUSEamp(+), which expresses dominant negative Akt; or the pUSEamp(+) empty vector. At the next day, cells were shifted into media without serum for 24hr. The % of non-viable cells was determined as in Fig. 10. Error bars represent the standard deviation for 3 independent experiments. (G) MDA-MB-435S cells were prepared as in F, and the levels of cleaved PARP, phospho-Akt and Akt were determined by Western Blot analysis as in B and C. Blots were stripped and reprobed with an antibody to β-tubulin to control for loading as in Fig. 10. The experiment shown in G is representative of three independent experiments.

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4. mTOR dependent survival signal in breast cancer cells with elevated PLD activity

The data in Fig 12 indicated in the absence of serum the survival signals in MDA-MB-231 and MCF-7R cells were independent of the PI3K/Akt pathway. As demonstrated in Fig. 11, MDA-MB-231 cells have elevated PLD activity, which was demonstrated to be required for the survival of MDA-MB-231 cells in the absence of serum (Zhong et al., 2003). PA, the metabolic product of PLD, activates mTOR (Fang et al., 2001), which has also been implicated in survival signaling (Schmelzie et al., 2000). mTOR is inhibited by rapamycin, which was reported to be competitive with PA for binding to mTOR (Fang et al., 2001). Consistent with this hypothesis, higher concentrations of rapamycin were required to inhibit mTOR in cells with elevated PLD activity (Chen et al., 2003). As shown in Fig. 13, rapamycin induced apoptosis in serum-deprived MCF-7R and MDA-MB-231 cells, but not in MDA-MB-435S cells as indicated by reduced cell viability (Fig. 13A) and PARP cleavage (Fig. 13B). Consistent with rapamycin being competitive with PA (Fang et al., 2001; Chen et al., 2003), higher concentrations of rapamycin were required to induce apoptosis in MDA-MB-231 cells. There are significant cleavages of caspase 3 and caspase 9 in rapamycin treated MCF-7R and MDA-MB-231 cells (Fig. 13C

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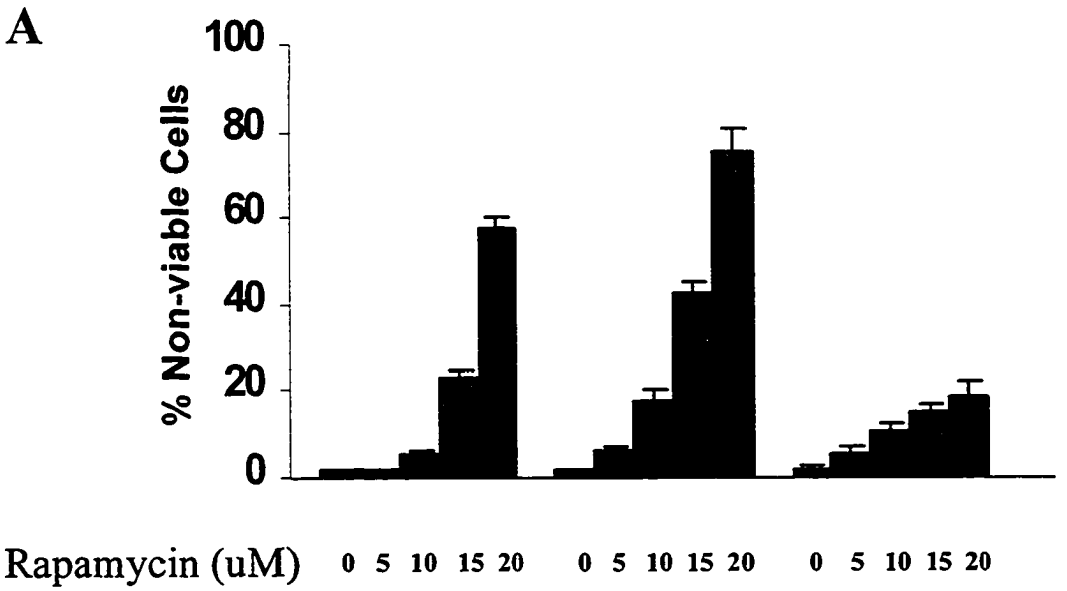
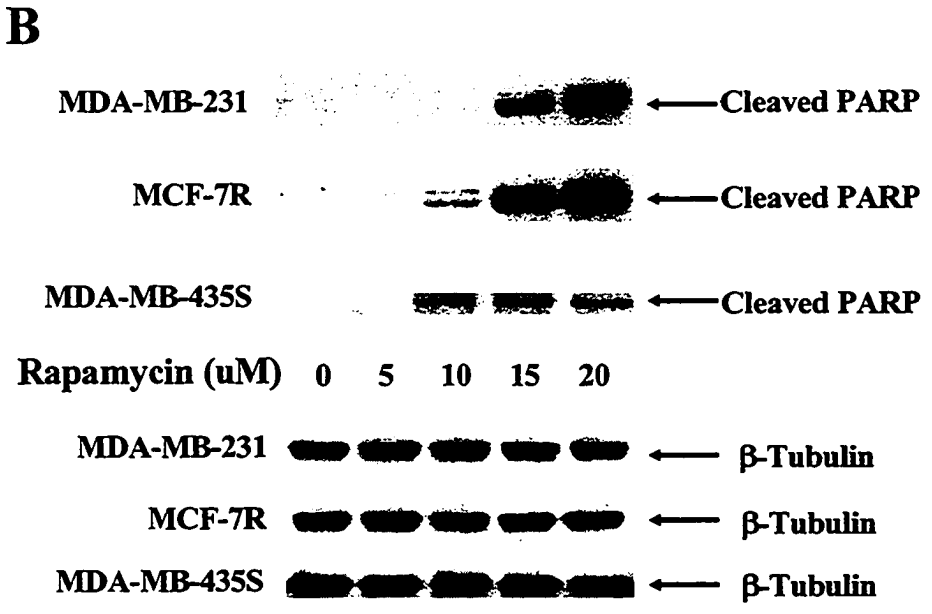
and D). These data indicated that the PI3K-independent survival signals generated in MDA-MB-231 and MCF-7R cells where there is elevated PLD activity are dependent upon mTOR.





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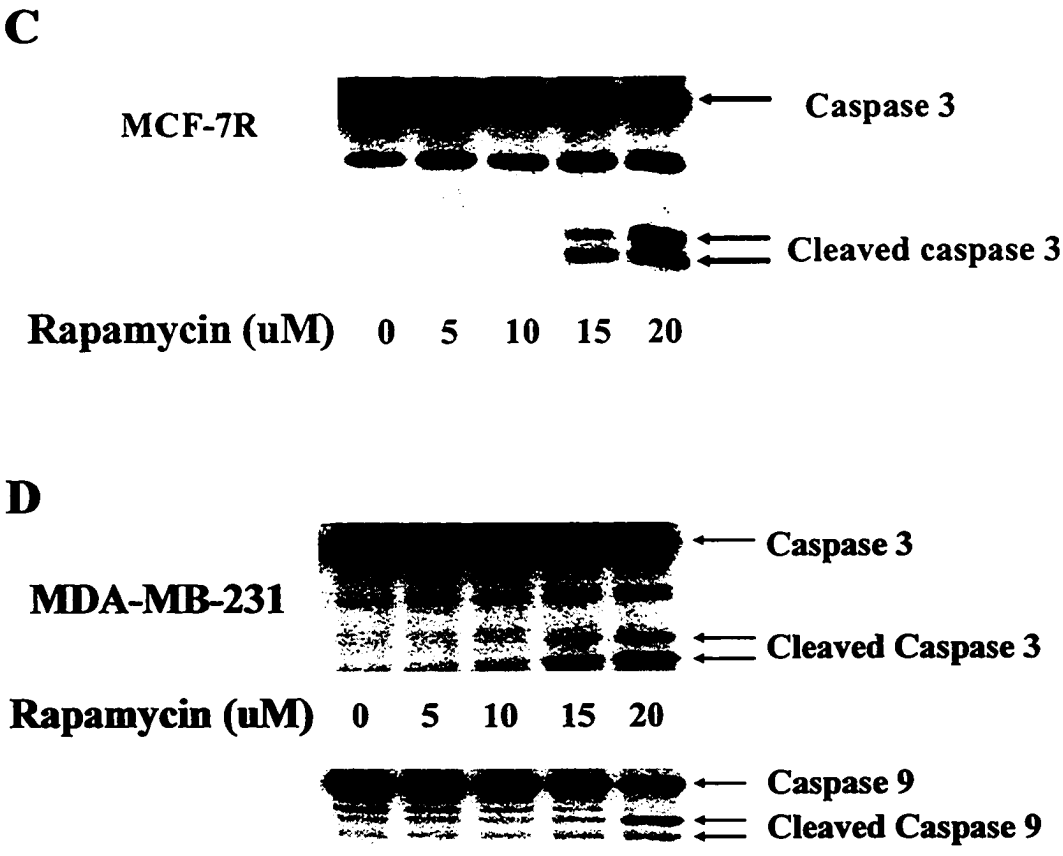

Fig. 13. mTOR dependent survival signal in breast cancer cells with elevated PLD activity

(A) MDA-MB-231, MCF-7R, and MDA-MB-435S cells were plated as described in Fig. 10. At the next day, the cells were shifted into media lacking serum in the presence of the indicated concentrations of rapamycin. 24 hr later the % of non-viable cells was determined as in Fig. 10. Error bars represent the standard deviation for 3 independent experiments. (B) MDA-MB-231, MCF-7R, and MDA-MB-435S cells were prepared as in A and the levels of cleaved PARP were determined by Western Blot analysis as in Fig. 10. Blots were stripped and reprobed with an antibody to β-tubulin to control for loading as in Fig. 10. (C) MCF-7R cells were prepared as in A, the levels of caspase 3, and cleaved caspase 3 were determined by Western Blot analysis. (D) MDA-MB-231 cells were prepared as in A, the levels of caspase 3, cleaved caspase 3 and caspas 9, cleaved caspas 9 were determined by Western Blot analysis. The experiment shown in B, C and D is representative of three independent experiments.

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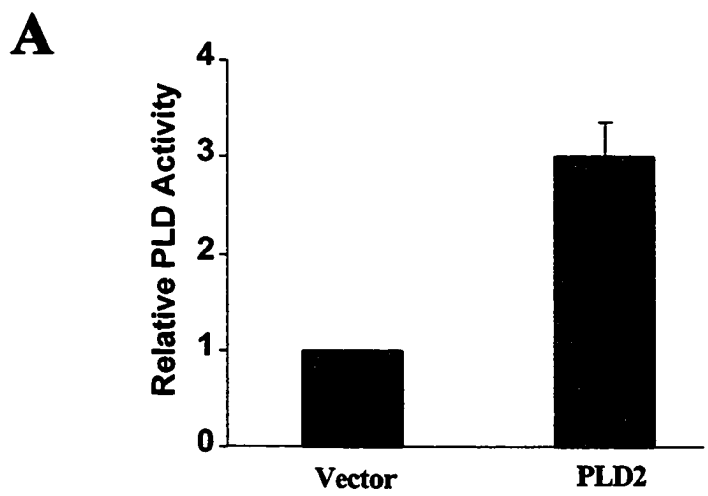
5. Elevated expression of PLD2 provides an mTOR dependent survival signal in MCF-7 cells

MCF-7R cells with elevated PLD activity compared to MCF-7 cells (Fig. 11B) are resistant to the apoptotic signal induced by serum withdrawal (Fig. 10); however the survival signal in MCF-7R cells is sensitive to rapmaycin treatment (Fig. 13). To test if elevating PLD activity can provide mTOR-dependent survival signal in MCF-7 cells, wild type PLD2 was transiently transfected into MCF-7 cells. As shown in Fig. 14A, the transiently transfection led to a 3-fold elevation of PLD activity. These transiently transfected MCF-7 cells were resistant to the serum starvation-induced apoptotic stress in the absence of rapamycin, but not in the presence of rapamycin (Fig. 14B and C). These data further suggest that PLD generates an mTOR dependent survival signal.



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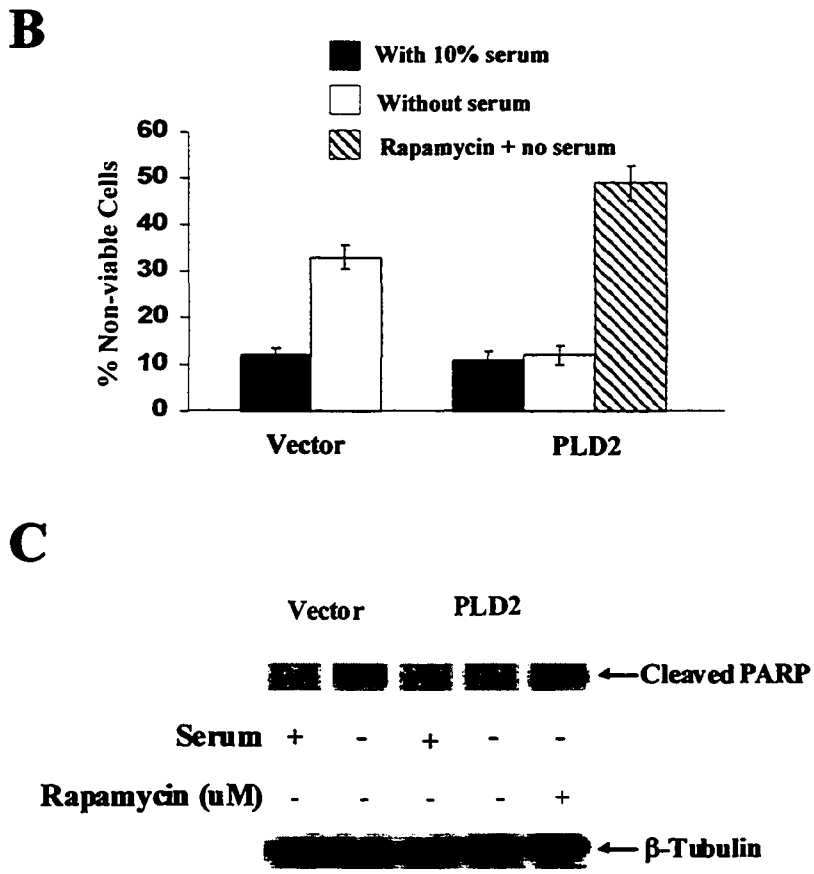

Fig. 14. Elevated expression of PLD2 provides an mTOR dependent survival signal in MCF-7 cells

(A) MCF-7 cells were plated in 60mm culture dish at 2 x 10^5 cells per dish. 24 hr later, the cells were transfected with either pCGN-mPLD2, which expresses wild type PLD2 (Colley et al., 1997) or the pCGN empty vector. 30 hr later, cells were placed into media containing 0.5% bovine calf serum for overnight and the relative PLD activity in the MCF-7 cells transfected with pCGN-mPLD2 relative to those transfected with the empty vector was determined as in Fig. 11B. The data were normalized to the PLD activity in MCF-7 cells transfected with the empty vector, which was given a value of 1. (B) MCF-7 cells were prepared and transfected as in A. 30 hr after transfection, the cells were placed in media containing either 0% or 10% serum and with or without rapamycin (5 μM) as indicated for 24hr. The % of non-viable cells was determined as in Fig. 10. Error bars represent the standard deviation for 3 independent experiments. (C) MCF-7 cells were prepared as A. The levels of cleaved PARP were determined as in Fig. 10. The experiment shown is representative of three independent experiments.

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6. **Blocking S6K activity is not sufficient for rapamycin to induce apoptosis. Rapamycin-induced apoptosis is p53 independent and not mediated by downregulating the protein level of c-myc**

S6 kinase is an important downstream target of mTOR and it has been reported that S6 kinase signals cell survival (Harada et al., 2001). These previous data suggest that S6K activity could be an important target for rapamycin inducing apoptosis in MDA-MB-231 and MCF-7R cells. However the data shown in Fig. 15A indicated that inhibition of S6K activity is not sufficient for rapamycin to induce apoptosis in human breast cancer cells. Consistent with the previous data that inhibition of S6K activity is not enough for the proliferation inhibition of rapamycin (Chen et al., 2003). The p53 tumor suppressor activates the expression of genes engaged in promoting growth arrest or cell death in response to multiple forms of cellular stress. p53 expression is elevated in apoptotic cells. Some reports show p53 is mutant in MDA-MB-231 cells (Shao et al., 1995), so it is not surprise that rapamycin induced apoptosis is independent on p53. Interestingly, rapamycin decreased the protein level of p53 instead of upregulating it (Fig. 15B). C-myc has critical roles in the regulation of apoptosis and cell cycle. C-myc gene expression alone is sufficient to confer resistance to anti-estrogen in human breast cancer cells (Venditti et al.,

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2002). However the data shown in Fig. 15C indicated there is no significant downregulation of the protein level of C-myc in rapamycin-induced apoptosis.

Discussion

The data provided suggest that survival signals generated by PLD are dependent upon mTOR and active in breast cancer cells. MDA-MB-231 cells, which have highly elevated levels of PLD activity, have relatively low levels of Akt phosphorylation, indicating that the PI3K/Akt survival pathway is not highly active in these cells. In contrast, MDA-MB-435S cells have high levels of Akt phosphorylation and low levels of PLD activity. Inhibition of mTOR with rapamycin sensitized MDA-MB-231, but not MDA-MB-435S cells, to the apoptotic stress of serum withdrawal. In contrast, inhibition of PI3K with LY294002 sensitized MDA-MB-435S, but not MDA-MB-231 cells to the apoptotic stress. These data demonstrate that there are distinguishable alternative survival signals active in different human breast cancer cell lines - the well-established PI3K/Akt pathway and a novel PLD/mTOR pathway.

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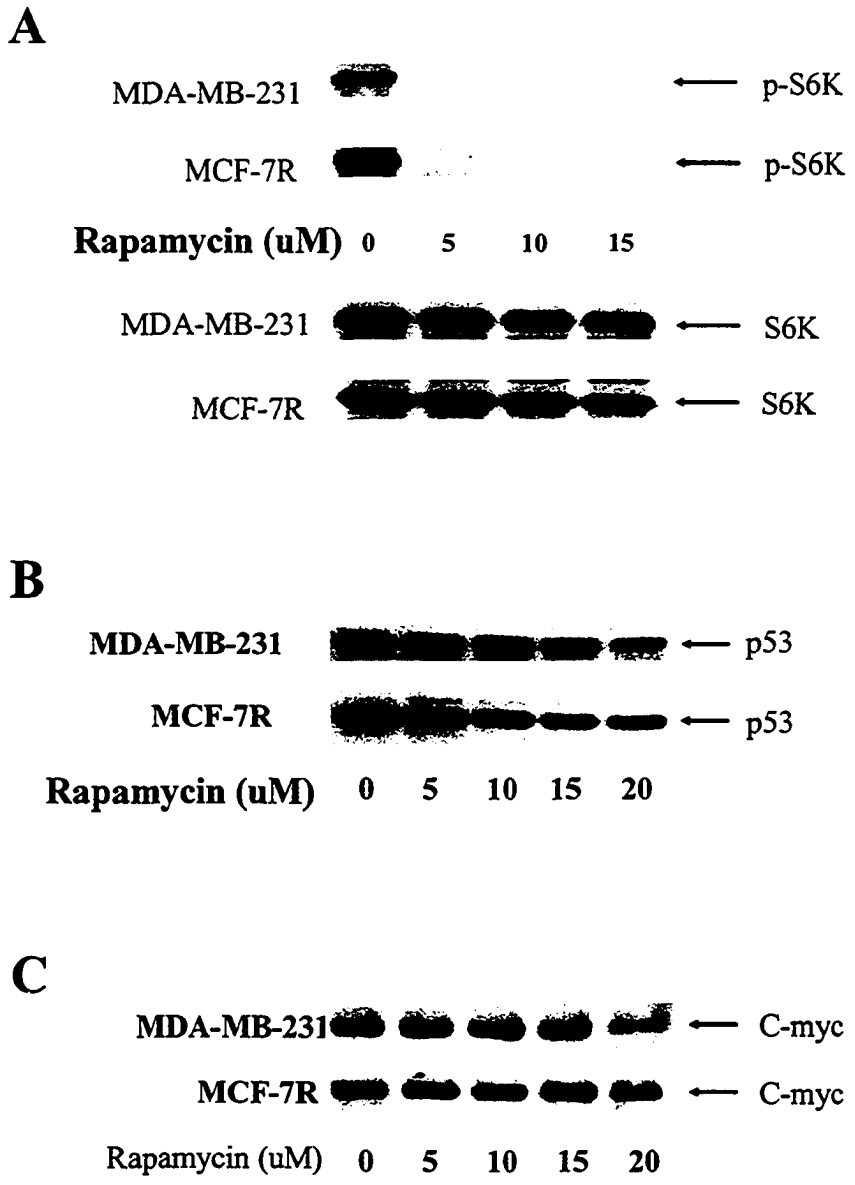

Fig. 15. Blocking S6K activity is not sufficient for rapamycin to induce apoptosis. Rapamycin-induced apoptosis is p53 independent and not mediated by downregulating the protein level of c-myc

MDA-MB-231, and MCF-7R cells were plated as described in Fig. 10. At the next day, the cells were shifted into media lacking serum in the presence of the indicated concentrations of rapamycin. 24 hr later, cells were collected as described in the experimental procedure. Cell lysate was subjected to Western blot analysis and the levels of phosphorylated S6K, S6K (A), p53 (B) and c-myc (C) were examined. The experiment shown is representative of three independent experiments.

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mTOR has also been implicated as one of several targets of the PI3K/Akt survival pathway (Nave et al., 1999; Sekulic et al., 2000). Akt phosphorylates and inactivates tuberous sclerosis complexes (TSC)-1 and -2, which negatively regulates mTOR (Manning et al., 2002; Inoki et al., 2002; Potter et al., 2002). Akt also phosphorylates and inactivates several other proteins that negatively regulate cell cycle progression and induce apoptosis. These include glycogen synthase kinase-3β (GSK3β), BAD, forkhead family transcription factors and MDM2 (Vivanco et al., 2002). The impact of Akt upon mTOR is apparently critical since cells with elevated Akt activity are highly sensitive to rapamycin (Aoki et al., 2001; Mills et al., 2001; Neshat et al., 2001; Podsypanina et al., 2001). It is therefore of interest that Akt phosphorylation is apparently low in breast cancer cells with elevated PLD activity. This suggests that in cells with elevated PLD activity survival signals are generated by PLD/mTOR signaling pathway, and therefore circumvent the need of the dysregulation of PTEN/PI3K/Akt pathway. It has been proposed that the dysregulation of the protein synthesis machinery through mTOR may generate cell cycle progression signals that contribute to cancer (Ruggero et al., 2003). Thus, the ability of PLD to stimulate mTOR (Fang et al., 2001) may provide the most significant component of PLD generated survival signals. A model for the

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alternative survival signals generated by the PLD/mTOR and PI3K/Akt pathways is presented in Fig. 16. It may also be of interest that since PLD activity is dependent upon PIP2 (Exton, 1999; Foster et al., 2003) and PIP2 is converted to PIP3 by PI3K and the reverse reaction is catalyzed by PTEN, that these pathways could alternatively be activated by regulation of PIP2 and PIP3 levels. Activation of PI3K could shut down the PLD pathway by reducing PIP2 levels (Fig. 16).

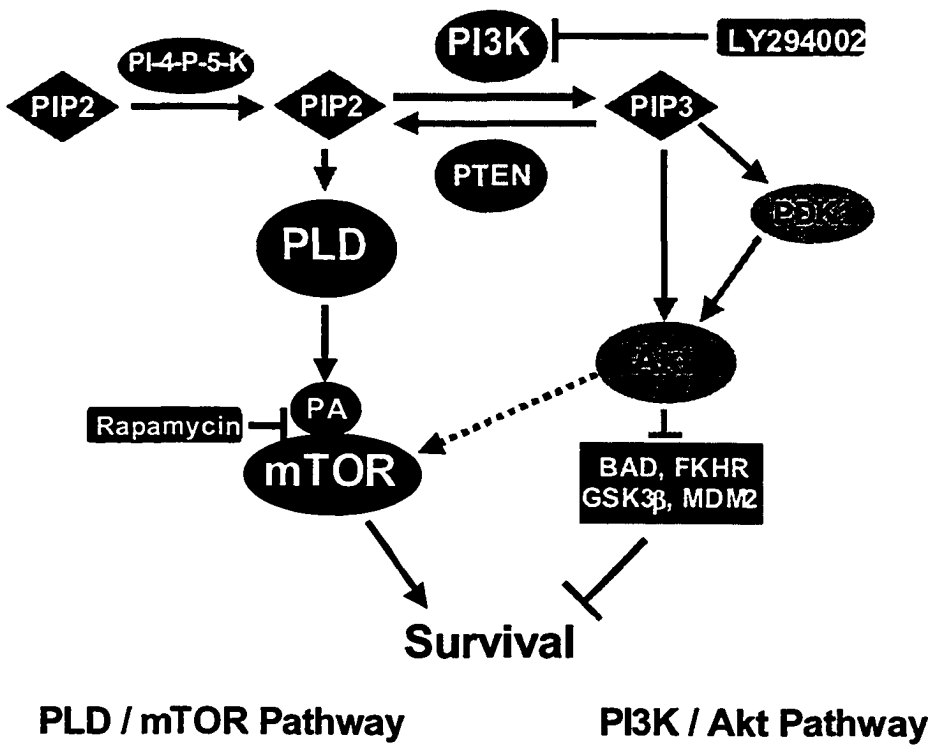

Fig. 16. Alternative survival pathways

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Rapamycin or the rapamycin analogue CCI-779 has been reported to block the growth of tumors that lack functional PTEN and are with the concomitant elevation of Akt (Neshat et al., 2001; Podsypanina et al., 2001). Similarly, rapamycin blocked the transformation of chicken embryo fibroblasts by Akt (Aoki et al., 2001). The effect of rapamycin on PTEN deficient cells was cytostatic and did not lead to apoptosis, nor was there any apoptosis reported in response to rapamycin in the Akt-transformed cells. However, these experiments were not performed under the low serum conditions used here. Therefore it is difficult to compare these studies here. However, the data presented in Fig. 13 clearly indicate that MDA-MB-435S cells, which have elevated Akt phosphorylation, are less sensitive to rapamycin than MDA-MB-231 cells, which do not have high levels of Akt phosphorylation. Moreover, MDA-MB-231 cells required higher levels of rapamycin to induce apoptosis (Chen et al., 2003) – presumably due to competition with the PA generated by PLD (Fang et al., 2001; Chen et al., 2002). And this high concentration of rapamycin did not induce marked apoptosis in MDA-MB-435S cells. Thus, while rapamycin is likely cytostatic in cells with elevated PI3K/Akt signals, this treatment does not appear to induce apoptosis.

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PLD activity has been reported to be elevated in response to most mitogenic and oncogenic signals. More recently, PLD activity has been shown to cooperate with overexpression of a tyrosine kinase to transform rat fibroblasts (Lu et al., 2000; Joseph et al., 2001) and to overcome cell cycle arrest and apoptosis in cells with high intensity Raf signals (Joseph et al., 2002). In addition, many proteins that regulate PLD activity have also been implicated in regulating cell cycle progression and cell transformation, including RalA (Jiang et al., 1995), Rho family GTPases (Exton, 1999), and PKCα (Hornia et al., 1999). Thus, evidence supporting a role for PLD in regulating both growth factor- and oncogene-induced cell proliferation is substantial. The elevation of PLD activity in a majority of breast (Noh et al., 2000) and renal (Zhao et al., 2000) cancer tissues examined is therefore likely to be a contributing factor in cancer progression. The ability of PLD to provide a survival signal in breast cancer cells and in cells transformed by v-Src (Zhong et al., 2003) suggests that a critical aspect of PLD signaling is to prevent apoptosis. The data presented here indicate that PLD/mTOR provides a novel survival signal in cells where the PI3K/Akt pathway is not activated.

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