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Phospholipase D Elevates the Level of MDM2 and Suppresses DNA Damage-Induced Increases in p53

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Phospholipase D (PLD) has been reported to generate survival signals that prevent apoptosis induced by serum withdrawal. We have now found that elevated expression of PLD also suppresses DNA damage-induced apoptosis. Since DNA damage-induced apoptosis is often mediated by p53, we examined the effect of elevated PLD expression on the regulation of p53 stabilization. We report here that PLD suppresses DNA damage-induced increases in p53 stabilization in cells where PLD has been shown to provide a survival signal. Elevated expression of PLD also led to increased expression of the p53 E3 ubiquitin ligase MDM2 and increased turnover of p53. PLD1-stimulated increases in MDM2 expression and suppression of p53 activation were blocked by inhibition of mTOR and the mitogen-activated protein kinase pathway. Although PLD did not activate the phosphatidylinositol 3-kinase (PI3K)/Akt survival pathway activate the basal levels of PI3K activity were partially required for PLD1-induced increases in MDM2. These data provide evidence that survival signals generated by PLD involve suppression of the p53 response pathway.

Cell proliferation is perhaps the most carefully regulated cellular activity. Protection from undesired proliferation prevents cancer and other proliferative disorders. The mechanisms through which cells overcome these protections have been aggressively investigated because they are frequently dysregulated in human cancer (18). Many of the protections against cell proliferation involve cell cycle checkpoints where several criteria have to be met in order for a cell to continue through the cycle and divide (20). It is at these checkpoints that decisions are made as to whether the cell should stop, proceed, or, when appropriate, undergo apoptosis. Mitogenic signaling involves the generation of signals that allow passage through cell cycle checkpoints. Since the default pathway for inappropriate cell proliferation signals is frequently apoptosis, some of the signals generated by mitogens have been termed "survival signals," since they prevent apoptosis.

The most studied survival signaling pathway involves the activation of phosphatidylinositol (PI) 3-kinase (PI3K), which generates PI-3,4,5-tris phosphate (PIP3). PIP3 production results in the recruitment and activation of Akt, a kinase that phosphorylates several key proteins that regulate apoptosis (46). Recently, another phospholipid-modifying enzyme has been implicated in the generation of survival signals. This enzyme is phospholipase D (PLD), which catalyzes the hydrolysis of phosphatidylcholine to phosphatidic acid (PA) and choline (10). There are two PLD isoforms, PLD1 and PLD2 (9, 17), and both have been implicated in mitogenic signaling (13). PLD activity is elevated in response to platelet-derived growth factor (36), fibroblast growth factor (32), epidermal growth factor (EGF) (42), insulin (26), insulin-like growth factor 1 (3), growth hormone (51), and sphingosine 1-phosphate (2). PLD activity is also elevated in cells transformed by a variety of transforming oncogenes including v-Src (41), v-Ras (21, 22), v-Fps (23), and v-Raf (14). PLD activity was also able to induce a transformed phenotype in cells with elevated expression of a tyrosine kinase (24, 29). Both PLD1 and PLD2 have been reported to induce anchorage-independent growth and enhance cell cycle progression of mouse fibroblasts (1, 31). PLD activity prevented cell cycle arrest and apoptosis in cells with overexpressed Raf (25). PLD also prevented apoptosis in cells transformed by v-Src and in the MDA-MB-231 human breast cancer cell line (50). PLD activity also overcame apoptosis induced by H_2O_2 (34) and glutamate (27). Thus, an emerging role for PLD in the control of cell proliferation is to provide a survival signal that allows cells to avoid apoptosis under conditions of stress or inappropriate mitogenic signals (13).

It was previously reported that PLD1 cooperated with c-Src to transform 3Y1 rat fibroblasts (29). PLD1 also protected 3Y1 cells overexpressing c-Src from apoptosis (50), indicating that PLD1 generates a survival signal in these cells. The ability of PLD1 to cooperate with a tyrosine kinase such as c-Src is similar to the ability of simian virus 40 T antigen to cooperate with Ras to transform human cells (16). T antigen contributes to transformation by down-regulating the cell cycle checkpoint proteins Rb and p53 (35). Thus, it is possible that PLD1 might similarly regulate control of cell cycle checkpoint proteins.

In this report, we describe studies of the effect of PLD activity upon p53 stabilization. We have taken advantage of the ability of 3Y1 cells overexpressing c-Src to tolerate elevated expression of PLD1 to demonstrate that PLD elevates basal MDM2 levels and suppresses DNA damage-induced increases in the stabilization of p53. The data provide evidence that the survival signals generated by PLD are mediated, at least in part, by suppression of p53 stabilization.

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MATERIALS AND METHODS

Cells, cell culture conditions, and plasmids. 3Y1 rat fibroblasts overexpressing c-Src (3Y1$^{c\text{-}Src}$ cells) were described previously (15, 19). The generation of 3Y1 and 3Y1$^{c\text{-}Src}$ cells that conditionally express PLD1 (3Y1-P1 and 3Y1$^{c\text{-}Src}$-P1 cells) was described previously (24, 49). PLD1 expression and activity were induced with 10 μM ponasterone A (PonA) (Stratagene). MCF-7 cells were obtained from the American Type Culture Collection. All cell lines were maintained in Dulbecco's modified Eagle medium (DMEM) (Gibco) supplemented with 10% bovine calf serum (HyClone). The MCF-7 cell lines stably expressing PLD2 were established by transfection with pcDNA3.1(−)-hPLD2 by using Lipofectamine Plus reagent (GIBCO) according to the manufacturer's instructions. pcDNA3.1(−)-hPLD2 was constructed as follows. The human PLD2 gene (9) was excised from pBluescript-SK-hPLD2 (9) with NotI and HindIII and was ligated into the polylinker region of the pcDNA3.1(−) expression plasmid (Stratagene) which was cut with NotI and HindIII. The plasmid was amplified in *Escherichia coli* (XL-1-Blue host strain; Stratagene). The plasmid was then stably transfected into MCF-7 cells under G418 selection.

Materials. Generation of the monoclonal antibodies raised against p53 (pAb240 and pAb421) was described previously (4). Antibodies against Akt, phosphorylated Akt (Ser473), S6 kinase, phosphorylated S6 kinase (Thr389), mitogen-activated protein (MAP) kinase, phosphorylated MAP kinase (Thr202/Tyr204), and poly(ADP ribose) polymerase (PARP) were from Cell Signaling Technology. Antibodies against MDM2, HDM2, p21, actin, and tubulin were from Santa Cruz Biotechnology. Antibodies to PLD1 and PLD2 were from Upstate Biotechnology. For nonimmune controls, we used ChromPure rabbit or mouse immunoglobulin G (IgG) from Jackson ImmunoResearch. Rapamycin and cycloheximide (CHX) were obtained from Sigma-Aldrich. U0126, PD98059, and LY294002 were obtained from Cell Signaling Technology. Camptothecin (CPT) and adriamycin (ADR) were obtained from Calbiochem.

Western blot analysis. Samples were adjusted into gel-loading buffer (50 mM Tris-HCl [pH 6.8], 100 mM dithiothreitol, 2% sodium dodecyl sulfate, 0.1% bromophenol blue, 10% glycerol) and then heated for 5 min at 100°C prior to separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After samples were transferred to nitrocellulose membranes (Osmonics), membrane filters were blocked with 5% nonfat dry milk in phosphate-buffered saline (PBS) with 0.05% Tween 20 and then incubated with the appropriate antibody diluted in 5% nonfat dry milk in PBS with 0.05% Tween 20. 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 by using an enhanced chemiluminescence detection system (Pierce).

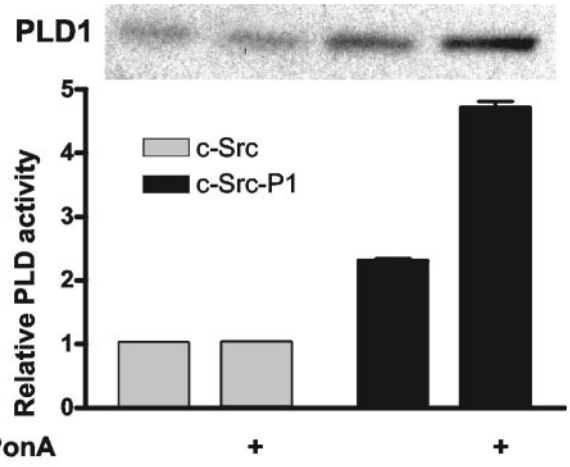

FIG. 1. Conditional expression of PLD1 in 3Y1$^{c\text{-}Src}$ cells. The construction of 3Y1$^{c\text{-}Src}$-P1 cells was described previously (24). PLD1 protein levels in the 3Y1$^{c\text{-}Src}$ and 3Y1$^{c\text{-}Src}$-P1 cells were determined in the presence and absence of PonA (10 μM, 20 h), as indicated, by Western blot analysis with an anti-PLD1 antibody (upper panel). Aliquots from these cells were also analyzed for PLD activity as described in Materials and Methods. The PLD activity values were normalized to untreated 3Y1$^{c\text{-}Src}$ cells. Error bars represent the standard deviation for triplicate samples from a representative experiment that was repeated two times.

Immunoprecipitation. Cells were washed twice with ice-cold PBS and scraped into the modified radioimmunoprecipitation assay (RIPA) buffer containing 50 mM Tris-HCl (pH 7.6), 1% Igepal CA-630, 0.25% sodium deoxycholate, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM NaF, and 1× protease inhibitor cocktail, consisting of 0.5 mM AEBSF [4-(2-aminoethyl)benzenesulfonyl fluoride], 1 μM leupeptin, 0.15 μM aprotinin, and 1 μM protease inhibitor E-64. The cells were then incubated at 4°C for 25 min by gentle rocking, sonicated for 20 s on ice, and centrifuged at 12,000 × *g* at 4°C for 10 min. The supernatant was precleared with protein G-Sepharose 4 Fast Flow beads (Amersham Pharmacia Biotech), and 500 μg of the precleared proteins was adjusted to 500 μl in the modified RIPA buffer and then incubated with the antibody for 1 h as described above. The immunocomplex was captured by incubation with 50 μl of protein G-Sepharose 4 Fast Flow bead slurry and collected by centrifugation at 12,000 × *g* for 20 s at 4°C. The beads were washed three times with the modified RIPA buffer and once with wash buffer (50 mM Tris [pH 7.6]), and subjected to Western blot analysis.

Assay of PLD activity. PLD activity was determined by a transphosphatidylation reaction in the presence of 0.8% butanol as described previously (29). Cells in 100-mm culture dishes were prelabeled with [^{3}H]myristate for 4 to 5 h in DMEM containing 0.5% bovine serum. Lipids were extracted and characterized by thin-layer chromatography. Relative levels of PLD activity were then determined by measuring the intensity of the corresponding phosphatidylbutanol band in the autoradiograph with a Molecular Dynamics scanning densitometer and Image-Quant software.

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

RESULTS

Elevated expression of PLD1 suppresses DNA damage-induced apoptosis. It was reported previously that elevated PLD activity suppressed apoptosis induced by the withdrawal of serum in 3Y1 cells with elevated expression of c-Src (50). We wished to extend these studies to the apoptosis induced by DNA damage. As described above, elevated PLD1 expression is not tolerated well and actually stimulates apoptosis in normal rat fibroblasts deprived of serum (49). However, in the 3Y1$^{c\text{-}Src}$ cells, PLD1 was not only tolerated, it caused transformation (24, 29). Therefore, to alleviate problems associated with high levels of PLD1 expression, we developed a 3Y1$^{c\text{-}Src}$-P1 cell line (24). These cells express elevated but tolerated levels of PLD1 in the absence of induction, and upon treatment with PonA, expression of PLD1 and PLD activity are both elevated by 16 h after treatment with PonA (Fig. 1). These data are consistent with the previously reported levels of PLD1 protein and activity levels in these cells (24).

Having established that the 3Y1$^{c\text{-}Src}$-P1 cells express elevated PLD1 and that expression could be further elevated by treatment with PonA, we subjected these and the parental 3Y1$^{c\text{-}Src}$ cells to the DNA-damaging agent CPT and examined cell viability. As shown in Fig. 2A, the 3Y1$^{c\text{-}Src}$-P1 cells were substantially more resistant to CPT than the parental 3Y1$^{c\text{-}Src}$ cells. To investigate whether the loss of cell viability was due to apoptosis, we examined PARP cleavage in response to DNA damage, and as with cell viability, elevated PLD1 expression suppressed CPT-induced PARP cleavage (Fig. 2B). These data suggest that elevated expression of PLD1 suppresses the apoptotic response to DNA damage.

Elevated expression of PLD suppresses DNA damage-induced increases in the level of p53. DNA damage stimulates the stabilization of p53, which in turn results in either cell cycle arrest or, if the damage is too extreme, apoptosis (12). We therefore examined p53 levels induced by either CPT (Fig. 3A)

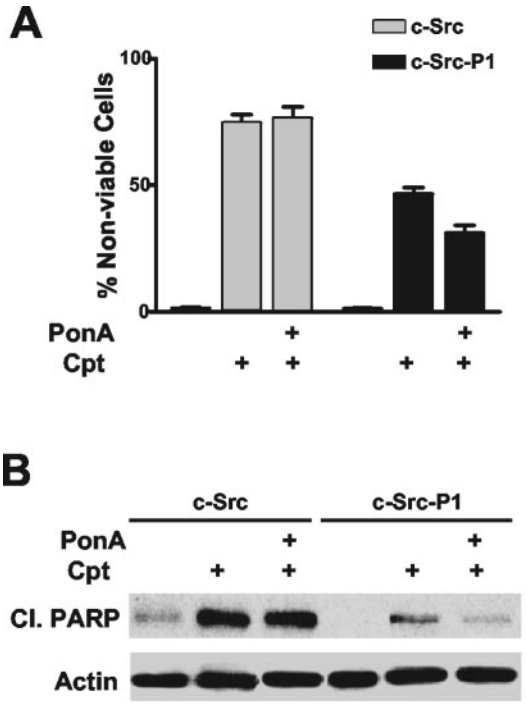

FIG. 2. Elevated expression of PLD1 suppresses DNA damage-induced apoptosis. (A) 3Y1$^{c\text{-}Src}$ and 3Y1$^{c\text{-}Src}$-P1 cells were pretreated with PonA for 16 h. CPT was then added where indicated, and cell viability was examined 20 h later. The percentage of nonviable cells was determined by the uptake of trypan blue. Error bars represent the standard deviation for the average of data from three independent experiments. (B) Cell lysates from 3Y1$^{c\text{-}Src}$ and 3Y1$^{c\text{-}Src}$-P1 cells prepared as described above were examined for the proteolytically cleaved PARP fragment (Cl. PARP) by Western blot analysis with an anti-PARP antibody. The data shown are representative of results obtained at least three times.

or ADR (Fig. 3B) in the 3Y1$^{c\text{-}Src}$ and 3Y1$^{c\text{-}Src}$-P1 cells in the presence and absence of PonA. The ability of the DNA-damaging agents to induce increased levels of p53 was substantially reduced in the 3Y1$^{c\text{-}Src}$-P1 cells, and the induction of p53 was reduced even further when the cells were pretreated with PonA to increase PLD1 expression. These data indicate that elevated PLD activity in the 3Y1$^{c\text{-}Src}$ cells, where PLD1 has been shown to provide a survival signal (50), suppresses DNA damage-induced increases in the level of p53.

While elevated expression of either PLD1 or PLD2 transforms 3Y1 cells with an overexpressed tyrosine kinase (24, 29), elevated expression of either PLD1 or PLD2 in the parental 3Y1 cells that did not have elevated expression of c-Src induced apoptosis (49). We therefore examined the effect of elevated PLD1 expression on DNA damage-induced increases in p53 in the parental 3Y1 cells. The PonA-inducible expression system for PLD1 in 3Y1 cells (3Y1-P1 cells) was characterized previously (49). These cells were treated with either CPT or ADR in the presence and absence of PonA, as shown in Fig. 3A. In contrast with the 3Y1$^{c\text{-}Src}$ cells, elevated PLD1 expression in the 3Y1 cells did not suppress the induction of p53 by either CPT or ADR (Fig. 3B). Thus, the ability of PLD1 to suppress the induction of p53 was apparently restricted to cells where elevated PLD activity was providing a survival signal.

It has recently been found that elevated PLD activity can provide a survival signal in MCF-7 human breast cancer cells (7). We therefore examined the effect of CPT and ADR on p53 levels in MCF-7 cells and in MCF-7 cells that stably express PLD2. PLD2 was used in these cells rather than PLD1 because, as described previously (7), PLD2 expression is tolerated by the MCF-7 cells better than PLD1. As shown in Fig. 3C, the ability of both CPT and ADR to increase the level of p53 was substantially reduced in MCF-7 cells that stably express PLD2 relative to the parental MCF-7 cells. The levels of PLD activity and PLD2 protein in the MCF-7 cells expressing PLD2 and MCF-7 cells transfected with the parental expression vector are shown in Fig. 3D. These data indicate that the effects of PLD upon p53 induction are not restricted to the 3Y1 cells with elevated expression of c-Src. The data also indicate that the ability of PLD to suppress induction of p53 may be restricted to cellular contexts where PLD is able to provide a survival signal.

We next examined whether the effect of PLD upon DNA damage-induced p53 was also reflected at the level of p53-targeted gene expression. A well-established transcriptional target of p53 is the cyclin kinase inhibitor p21 (12). As shown in Fig. 4, DNA damage-induced increases in p21 were similarly suppressed in the 3Y1$^{c\text{-}Src}$-P1 cells and further suppressed by the addition of PonA. These data further support the observation that elevated PLD activity suppresses DNA damage-induced increases in p53 levels and also argue that the p53 in these cells is wild type and capable of activating downstream p53 transcriptional targets (Fig. 3A).

Elevated expression of PLD1 accelerates degradation of p53. To investigate the mechanism of PLD1 inhibition of p53 induction, we first examined whether there was a difference in the half-life of p53 when PLD1 expression was elevated. 3Y1$^{c\text{-}Src}$, 3Y1$^{c\text{-}Src}$-P1, and PonA-treated 3Y1$^{c\text{-}Src}$-P1 cells were subjected to CPT treatment (5 μM for 1 h). CHX (80 μg/ml) was then added to inhibit new p53 synthesis, and p53 levels were examined at 30-min intervals for 1.5 h (Fig. 3). As shown in Fig. 5A, the relative increases of the level of p53 induced by CPT were similar to those shown in Fig. 3. The relative p53 levels normalized to the level of p53 in the cells not treated with CHX (zero time point) was determined by using densitometer quantification of the data (Fig. 5A). As shown in Fig. 5B, p53 levels dropped substantially faster in the PonA-treated 3Y1$^{c\text{-}Src}$-P1 cells. Similar data were obtained for the MCF-7 cells where the p53 turned over substantially faster in the MCF-7 cells overexpressing PLD2 (Fig. 5C and D). These data indicate that p53 is being degraded more rapidly in cells with elevated PLD activity and suggest that elevated PLD activity suppresses p53 expression, at least in part, by increasing the turnover of p53 protein.

Elevated expression of PLD1 increases basal MDM2 levels and inhibits DNA damage-induced decreases in MDM2. The stability of p53 is mediated by the E3 ubiquitin ligase MDM2, which facilitates ubiquitination of p53 and targets it for degradation by the proteasome (30). We therefore examined the effect of elevated PLD1 expression upon MDM2 expression in the 3Y1$^{c\text{-}Src}$ cells. As shown in Fig. 6A, basal levels of MDM2 were elevated in the 3Y1$^{c\text{-}Src}$-P1 cells relative to those of the

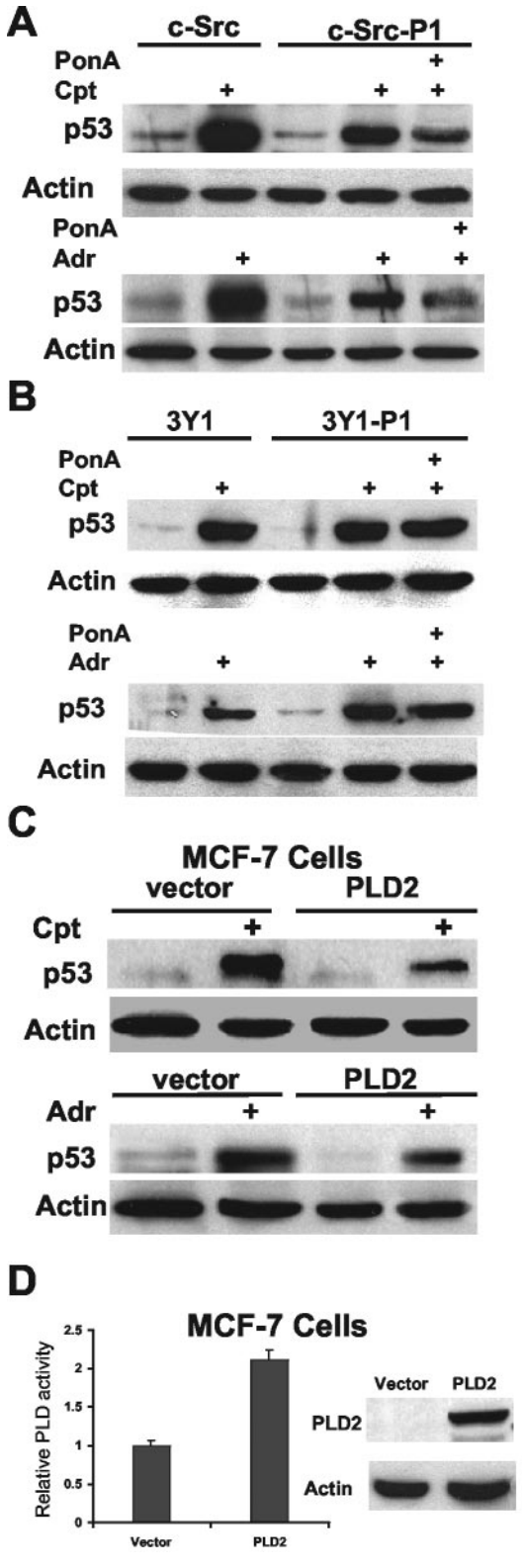

FIG. 3. Elevated expression of PLD1 suppresses DNA damage-induced increases in the level of p53 in cells where PLD provides survival signals. (A) $3Y1^{c\text{-}Src}$ and $3Y1^{c\text{-}Src}$-P1 cells grown in DMEM with 10% bovine calf serum were treated with 5 μM CPT or 0.3 μM ADR for 4 h. The cells were then collected, and lysates were analyzed for p53 protein levels by using Western blot analysis with an anti-p53 antibody. Where indicated, PonA (10 μM) was added for 16 h prior to the addition of CPT and ADR to increase the expression of PLD1 as described previously (24). (B) 3Y1 and 3Y1-P1 cells were treated with CPT, ADR, and PonA, and p53 levels were determined as described above. (C) MCF-7 cells stably transfected with pcDNA3.1(−)-PLD2 or the parental pcDNA3.1(−) vector were treated with either CPT or ADR, and p53 levels were determined as described above. (D) The relative levels of PLD2 protein determined by Western blot and PLD activity in the MCF-7 cells stably expressing PLD2 and transfected with the parental empty vector are shown. The data shown are representative of results obtained at least three times.

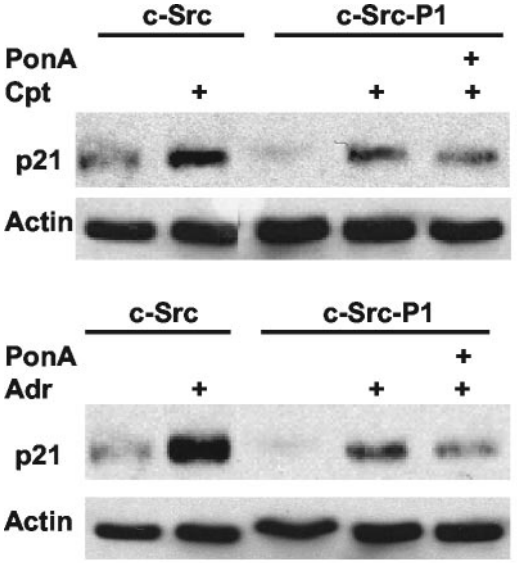

FIG. 4. Elevated expression of PLD1 suppresses DNA damage-induced increases of the level of p21. $3Y1^{c\text{-}Src}$ and $3Y1^{c\text{-}Src}$-P1 cells grown in DMEM with 10% bovine calf serum were treated with 5 μM CPT or 0.3 μM ADR for 4 h. The cells were then collected, and lysates were analyzed for p21 protein levels by using Western blot analysis with an anti-p21 antibody. Where indicated, PonA (10 μM) was added for 16 h prior to the addition of CPT and ADR to increase the expression of PLD1 as described previously (24). The data shown are representative of results obtained twice.

parental $3Y1^{c\text{-}Src}$ cells. PonA treatment to further elevate PLD1 expression resulted in a further increase in the level of MDM2 in the $3Y1^{c\text{-}Src}$-P1 cells. PonA had no effect upon MDM2 expression in the $3Y1^{c\text{-}Src}$ cells (Fig. 6A). Both CPT and ADR treatment of the $3Y1^{c\text{-}Src}$-P1 cells partially suppressed MDM2 expression (Fig. 6B). We also used transient transfection of PLD1 into the $3Y1^{c\text{-}Src}$ cells, and as shown in Fig. 6A, this transfection led to increased MDM2 levels relative to a vector control. Both CPT and ADR suppressed MDM2 levels in the uninduced $3Y1^{c\text{-}Src}$-P1 cells, and this suppression was reversed by the induction of PLD1 expression with PonA (Fig. 6B). Elevated expression of PLD2 in MCF-7 cells similarly led to increased basal human MDM2 (HDM2) expression (Fig. 6C). We also examined whether there was increased association between MDM2 and p53 in the $3Y1^{c\text{-}Src}$-P1 cells, and as shown in Fig. 6D, increased expression of PLD1 resulted in increased levels of MDM2 in p53 immunoprecipitates. These data show that elevated expression of PLD leads to increased expression of the p53 E3 ubiquitin ligase MDM2 and increased association of MDM2 with p53.

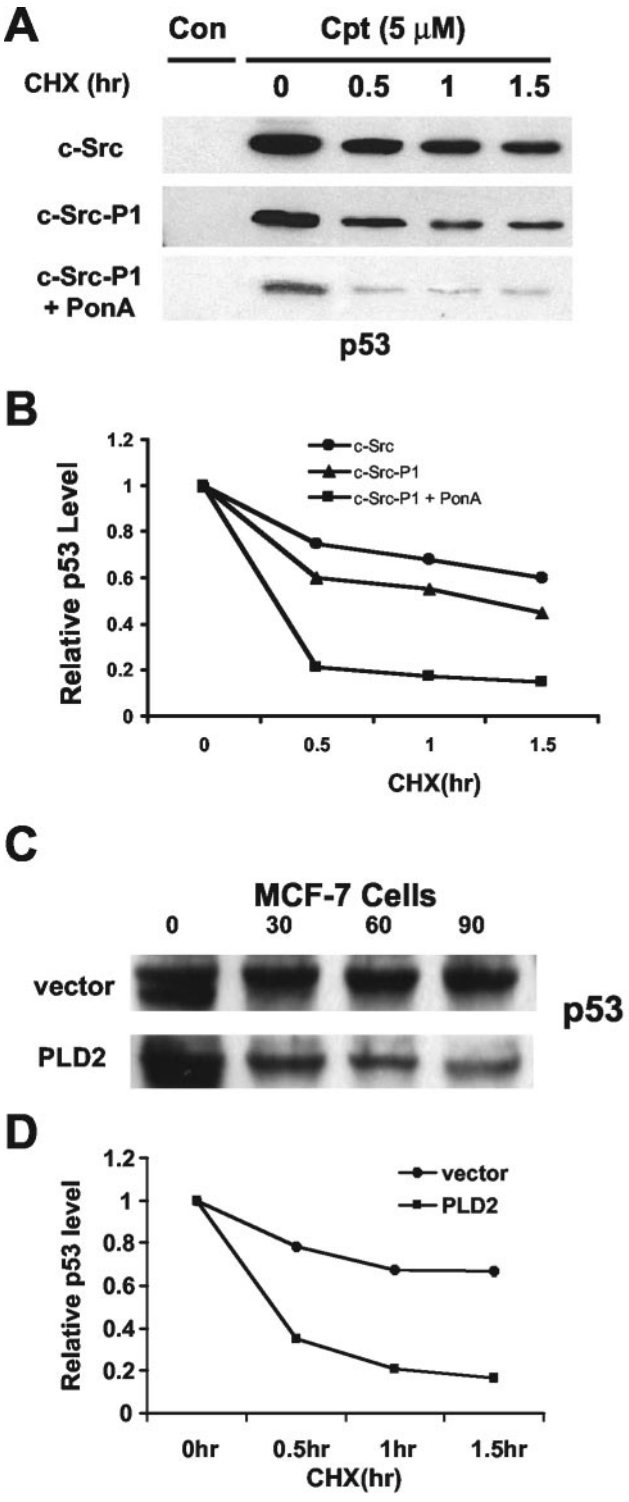

FIG. 5. Elevated PLD activity accelerates degradation of p53. (A) 3Y1$^{c\text{-}Src}$ and 3Y1$^{c\text{-}Src}$-P1 cells were treated with 5 μM CPT where indicated for 1 h. At this time, CHX (80 μg/ml) was added, and the cells were harvested at the indicated times. Where indicated, PonA (10 μM) was added for 16 h prior to the addition of CPT. Lysates were analyzed by Western blotting with anti-p53 antibody and, as a control, antitubulin antibody. (B) The results from the p53 data were analyzed by densitometer analysis, and the p53 levels normalized to the CPT-induced levels in the absence of CHX (zero time point) were determined. (C) MCF-7 cells and MCF-7 cells stably expressing PLD2 were treated with CHX as described above, and the levels of p53 were determined at the indicated times. (D) Densitometer analysis of the data in panel C is shown. The data shown are representative of results obtained three times.

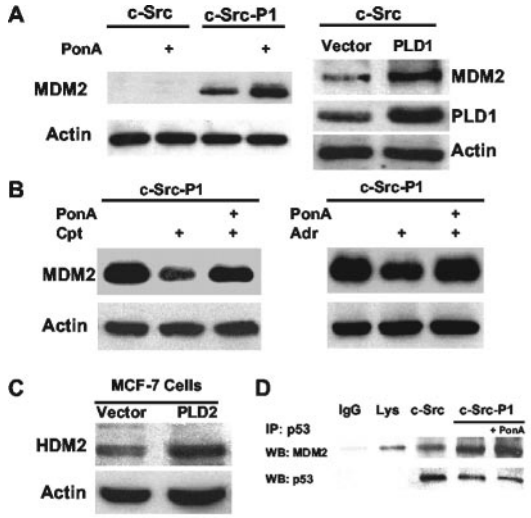

FIG. 6. Elevated expression of PLD1 increases basal MDM2 levels and inhibits DNA damage-induced decreases in MDM2. (A) 3Y1$^{c\text{-}Src}$ and 3Y1$^{c\text{-}Src}$-P1 cells were pretreated with PonA (10 μM) for 16 h where indicated, and the cells were then lysed and analyzed by Western blot analysis with an anti-MDM2 antibody. In the right panel, 3Y1$^{c\text{-}Src}$ cells were transiently transfected with pCGN-PLD1 and the parental pCGN vector as indicated. MDM2 and PLD1 protein levels were determined by Western blot analysis. (B) 3Y1$^{c\text{-}Src}$-P1 cells were pretreated with PonA where indicated, as described above. CPT (5 μM) or ADR (0.3 μM) was then added for 4 h where indicated, and lysates were examined for MDM2 expression as described above. (C) HDM2 expression levels were determined in MCF-7 cells and MCF-7 cells stably expressing PLD2 as described above. (D) Lysates from 3Y1$^{c\text{-}Src}$ and 3Y1$^{c\text{-}Src}$-P1 cells were immunoprecipitated (IP) by using a mouse monoclonal p53 antibody. The p53 immunoprecipitates were then subjected to Western blot (WB) analysis with antibodies raised against HDM2 and p53 as indicated. A nonimmune immunoglobulin control immunoprecipitate (IgG) is shown, as is a portion of whole-cell lysate (Lys) that was not subjected to immunoprecipitation. The amount run on the gel was 4% of that used in the immunoprecipitates. PonA, where indicated, was included as described above. The data shown are representative of results obtained at least three times.

The data also explain, at least in part, the PLD-induced increase in p53 turnover as shown in Fig. 5.

Like p53, MDM2 is usually regulated at the level of stabilization (30). We therefore compared the half-life of MDM2 in 3Y1$^{c\text{-}Src}$, 3Y1$^{c\text{-}Src}$-P1, and PonA-treated 3Y1$^{c\text{-}Src}$-P1 cells. The 3Y1$^{c\text{-}Src}$, 3Y1$^{c\text{-}Src}$-P1, and PonA-treated 3Y1$^{c\text{-}Src}$-P1 cells were treated with CHX (80 μg/ml) to inhibit new MDM2 synthesis, and MDM2 levels were examined at 30-min intervals as in Fig. 5 (Fig. 7A). The relative MDM2 levels normalized to the level of MDM2 in the cells not treated with CHX (zero time point) were determined by using densitometer quantification of the data shown in Fig. 7A. As shown in Fig. 7B, MDM2 levels dropped substantially faster in the 3Y1$^{c\text{-}Src}$ cells than did those of the 3Y1$^{c\text{-}Src}$-P1 or PonA-treated 3Y1$^{c\text{-}Src}$-P1 cells. These data indicate MDM2 is being degraded more slowly in cells with elevated PLD activity and suggest that PLD increases

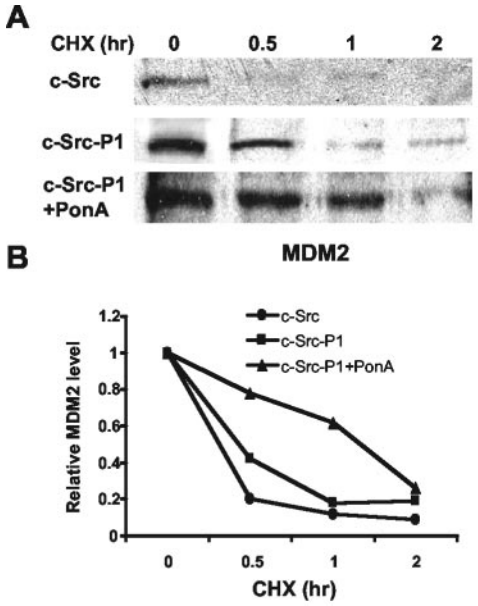

FIG. 7. Elevated expression of PLD1 increases the half-life of MDM2. (A) 3Y1$^{c\text{-}Src}$ and 3Y1$^{c\text{-}Src}$-P1 cells were treated with CHX (80 μg/ml), and the cells were harvested at the indicated times. Where indicated, PonA (10 μM) was added for 16 h prior to the addition of CHX. Lysates were analyzed by Western blotting with anti-MDM2 antibody and, as a control, antitubulin antibody. (B) The levels of MDM2 were determined by densitometer analysis and normalized to the level of MDM2 in the absence of CHX (zero time point). The data shown are representative of results obtained twice.

MDM2 expression, at least in part, by increasing the half-life of MDM2 protein.

PLD1-induced increases in MDM2, suppression of p53, and apoptosis are dependent upon MAP kinase and mTOR. It was previously reported that the activation of MAP kinase by EGF was dependent upon PLD (40). mTOR (mammalian target of rapamycin) has also been reported to be dependent upon PLD-generated PA (6, 7, 11). We therefore examined the effect of inhibitors of MEK, the kinase that phosphorylates MAP kinase (U0126), and mTOR (rapamycin) on the PLD-stimulated effects on p53 stabilization. Surprisingly, we found that both U0126 and rapamycin inhibited the PLD-suppressed induction of p53 by CPT (Fig. 8A). When U0126 and rapamycin were added to the induced 3Y1$^{c\text{-}Src}$-P1 cells together, the CPT-induced increase in p53 was restored almost to the level seen in the parental c-Src cells. Similarly, both U0126 and rapamycin inhibited the PLD-induced increases in MDM2, and treatment with U0126 and rapamycin together resulted in an even stronger reduction in MDM2 (Fig. 8B). We also found that another MEK inhibitor (PD98059) also prevented the effect of PLD upon p53 and MDM2 expression (data not shown). Fig 7C and D show that elevated PLD1 expression stimulated increases in the phosphorylation of mTOR substrate p70S6 kinase and MAP kinase and that these increases were sensitive to rapamycin and U0126, respectively. These data suggest that the PLD-induced effects upon PLD expression are dependent upon both mTOR and MAP kinase.

We next examined the effect of the MEK and mTOR inhibitors upon the suppression of DNA damage-induced apoptosis.

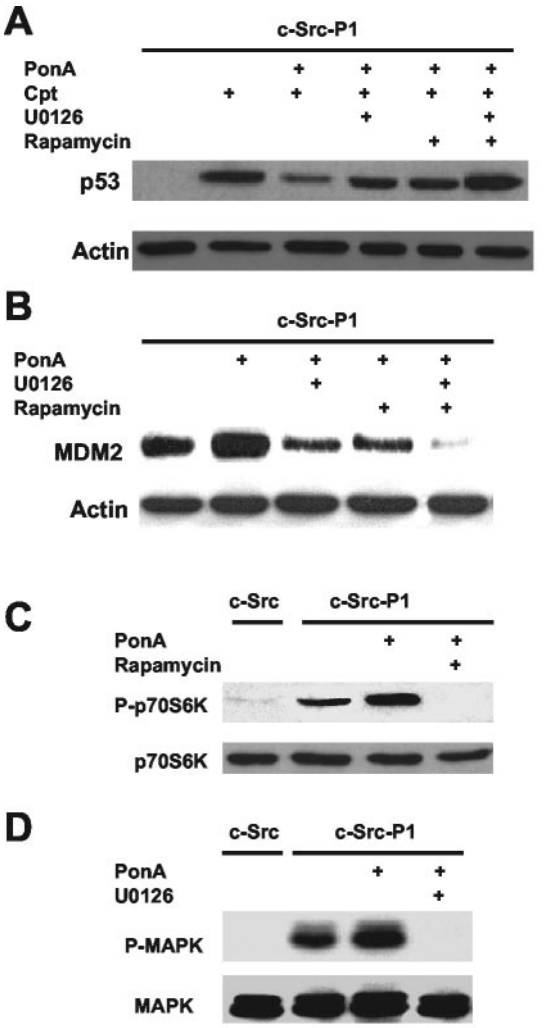

FIG. 8. PLD1-induced suppression of p53 and increases in MDM2 are reversed by inhibitors of MAP kinase and mTOR. (A) 3Y1$^{c\text{-}Src}$-P1 cells were treated with CPT and PonA (Fig. 2A). Where indicated, U0126 (20 μM) and rapamycin (300 nM) were added 2 h prior to PonA treatment. Cells were then lysed and analyzed for the levels of p53 and actin by using Western blot analysis. (B) 3Y1$^{c\text{-}Src}$-P1 cells were treated with PonA as described above. Where indicated, U0126 (20 μM) and rapamycin (300 nM) were added 2 h prior to PonA treatment. Cells were then lysed and analyzed for the levels of MDM2 and actin by using Western blot analysis. (C) 3Y1$^{c\text{-}Src}$ and 3Y1$^{c\text{-}Src}$-P1 cells were treated with PonA and rapamycin as indicated, as described above. Cell lysates were prepared, and the levels of S6 kinase (p70S6K) and phosphorylated S6 kinase (P-p70S6K) were determined by Western blot analysis with antibodies raised against p70S6K and p70S6K phosphorylated at Thr389. (D) 3Y1$^{c\text{-}Src}$ and 3Y1$^{c\text{-}Src}$-P1 cells were treated with PonA and U0126 as described above. Cell lysates were prepared, and the levels of MAP kinase (MAPK) and phosphorylated MAPK (P-MAPK) were determined by Western blot analysis with antibodies raised against MAPK and MAPK phosphorylated at Thr202/Tyr204. All of the data shown are representative of results obtained at least three times.

The 3Y1$^{c\text{-}Src}$ cells were subjected to CPT and PonA, and cell viability and PARP cleavage were determined (Fig. 2). The experiment was extended to investigate the effects of U0126 and rapamycin, and as shown in Fig. 9, cell viability was reduced with both U0126 and rapamycin. The effect of both U0126 and rapamycin together was slightly greater than that with U0126 alone. When we examined PARP cleavage, the

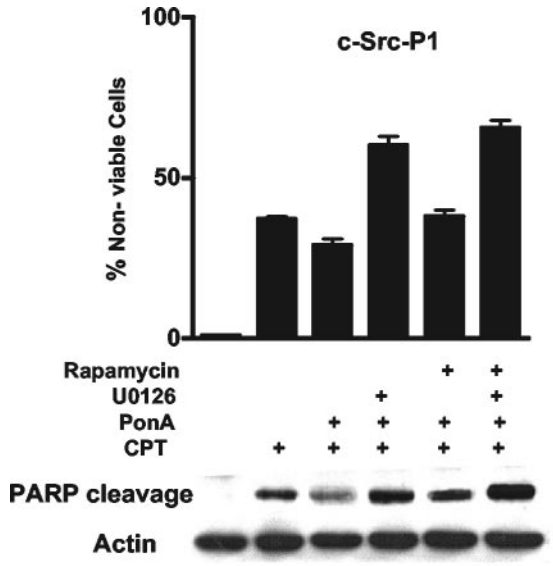

FIG. 9. PLD1 suppression of DNA damage-induced apoptosis is reversed by inhibitors of MAP kinase and mTOR. (A) 3Y1$^{c\text{-}Src}$-P1 cells were pretreated as indicated with PonA (16 h), and CPT was then added where indicated. U0126 (20 μM) and rapamycin (300 nM) were added 2 h prior to PonA treatment where indicated. Cell viability was then examined 20 h later. The percentage of nonviable cells and PARP cleavage were determined (Fig. 2). Error bars represent the standard deviation for the average of data from three independent experiments. The PARP cleavage data are representative of results obtained three times.

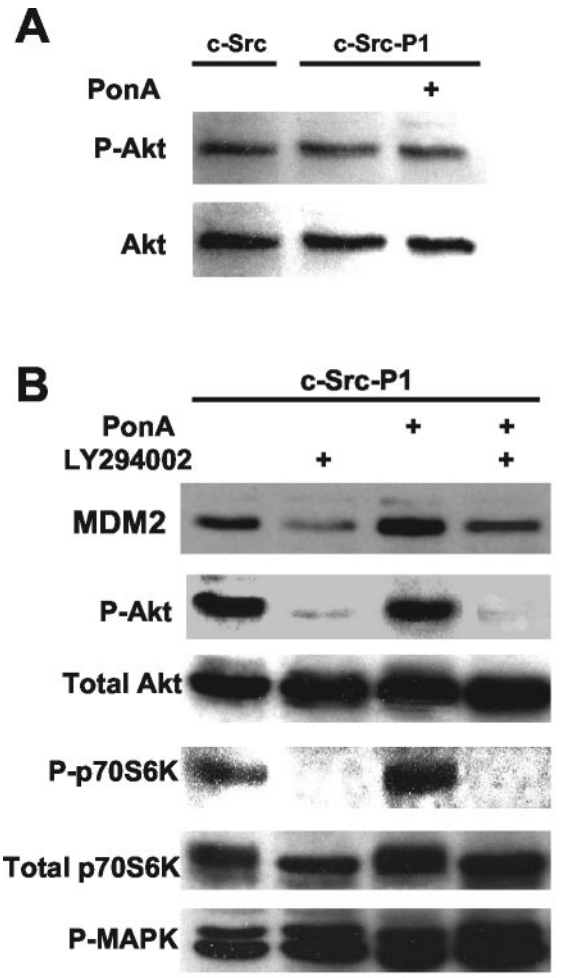

FIG. 10. Elevated PLD activity does not stimulate the PI3K/Akt survival pathway, but basal PI3K activity is required for maximum stimulation of MDM2. (A) 3Y1$^{c\text{-}Src}$ and 3Y1$^{c\text{-}Src}$-P1 cells were treated with PonA (Fig. 1). Cell lysates were prepared, and the levels of Akt and phosphorylated Akt (P-Akt) were determined by Western blot analysis with antibodies raised against Akt and Akt phosphorylated at Ser473. (B) 3Y1$^{c\text{-}Src}$-P1 cells were treated with PonA and LY294002 (20 μM) as indicated. The LY294002 was added at the same time as the PonA 20 h prior to the preparation of cell lysates. The cell lysates were then subjected to Western blot analysis with the indicated antibodies (Fig. 7). The data shown are representative of results obtained at least two times.

results resembled the effects of the drugs on MDM2 and p53 levels. Both U0126 and rapamycin substantially reversed the protective effect of PLD1 on PARP cleavage, and the two drugs together led to an apparent synergistic suppression of the inhibitory effect of PLD1 on PARP cleavage (Fig. 9). These data further support a role for both MAP kinase and mTOR in the PLD1 suppression of the p53 response pathway.

Elevated PLD activity does not stimulate the PI3K/Akt survival pathway, but basal PI3K activity is required for maximum stimulation of MDM2. The above data indicate that elevated PLD activity stimulates mTOR activity, as indicated by the increased phosphorylation of S6 kinase. It has been shown by many that survival signals generated by the PI3K/Akt pathway also go through mTOR (46). We therefore examined the effect of PLD activity upon Akt phosphorylation. As shown in Fig. 10A, elevated PLD1 expression had no effect on the phosphorylation state of Akt. However, the inhibition of basal Akt phosphorylation with the PI3K inhibitor LY294002 partially inhibited the PLD-stimulated increases in MDM2 (Fig. 10B). LY294002 completely inhibited both Akt and p70S6 kinase phosphorylation while having no inhibitory effect upon MAP kinase phosphorylation (Fig. 10B). These data indicate that while PLD1 does not stimulate PI3K activity and Akt phosphorylation, basal levels of PI3K and Akt are still important for the ability of PLD to increase MDM2 levels.

DISCUSSION

In this paper, we have provided evidence that elevated PLD activity increased basal levels of MDM2 and suppressed p53 stabilization. This effect was restricted to cell contexts where PLD activity is able to provide a survival signal. The ability of PLD to suppress p53 stabilization was blocked by inhibitors of both MAP kinase and mTOR. Although PLD activity did not activate the PI3K/Akt pathway, inhibition of PI3K also inhibited PLD1-induced increases in MDM2 expression, indicating that basal PI3K/Akt activation contributes to the PLD-induced suppression of p53 stabilization. A model for the suppression of p53 stabilization through elevated basal expression of MDM2 is illustrated in Fig. 11. In this model, it is proposed that PLD1-induced increases in MAP kinase and mTOR synergistically elevate MDM2 levels and increase the turnover of p53. The activation of mTOR was also dependent upon basal levels of PI3K activity, suggesting a link between the PLD and PI3K survival pathways. Although much remains to be learned

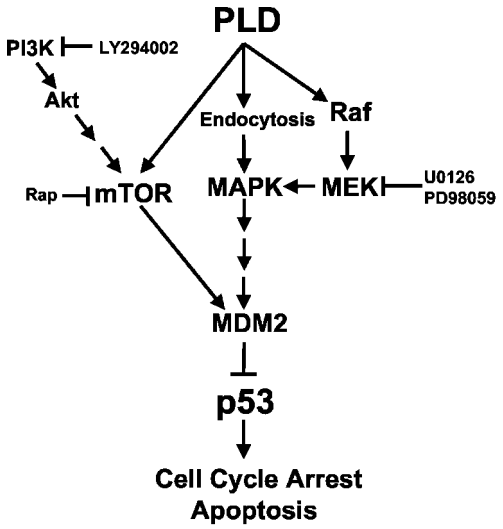

FIG. 11. Schematic model for suppression of p53 stabilization by PLD. It is proposed that the ability of PLD to suppress p53 expression is mediated by increasing the expression of the E3 ubiquitin ligase MDM2. The increased expression of MDM2 is dependent upon PLD1-induced increases in both mTOR and the Raf/MEK/MAP kinase cascade. Both mTOR (7, 11) and Raf (15, 38) have been reported to directly interact with and be affected by PA. PLD has also been reported to be required for endocytosis of the EGF receptor (40), and endocytosis of activated MEK has been reported to be required for the activation of MAP kinase (28, 40). Thus, it is proposed that PLD is required for the activation of both MAP kinase and mTOR, which work synergistically to elevate MDM2 and suppress p53 stabilization. Basal levels of PI3K activity and Akt are also partially required for elevated expression of MDM2, although PLD activity does not lead to increased Akt phosphorylation.

about the survival signaling pathways generated by PLD, the ability of PLD to suppress p53 stabilization provides a plausible mechanism for the reported survival signals generated by PLD (13, 25, 50).

The present study focused mostly on PLD1. PLD1 expression was reported to be elevated in breast cancer, and it was reported that there is elevated expression of PLD1 in the MDA-MB-231 cells, which have high levels of PLD activity (50). However, PLD2 has also been implicated in mitogenic and survival signals (13). Moreover, PLD2 expression was reported to be elevated in renal cancer (48). As reported here, PLD2 also elevates MDM2 and suppresses DNA damage-induced increases of p53 in MCF-7 cells. It has been hypothesized that PLD2 is activated in response to elevated PLD1 expression (13). Consistent with this hypothesis, we have reported previously that both PLD1 and PLD2 can cooperate with elevated expression of a tyrosine kinase to transform cells (24, 29). Thus, both PLD1 and PLD2 can apparently provide survival signals that suppress the activation of the p53 response.

A role for MAP kinase in the suppression of p53 stabilization is consistent with a previous report by Ries et al. (37), who showed that oncogenic Ras suppressed p53 levels via MAP kinase. As shown here, elevated PLD activity led to increased phosphorylation of MAP kinase, and the PLD suppression of p53 stabilization was dependent upon MAP kinase. The activation of MAP kinase by PLD is likely to be indirect through the enhancement of receptor endocytosis. It was previously demonstrated that the activation of MAP kinase in response to EGF requires receptor endocytosis (28, 45), and endocytosis of the EGF receptor and MAP kinase activation were both dependent upon PLD activity (40). Importantly, elevated PLD activity increased basal levels of receptor endocytosis in the absence of EGF (40). The proposed complex stimulation of MAP kinase by PLD is also reflected in Fig. 11.

It was previously proposed that survival signals generated by either PLD or PI3K represented alternative survival pathways that were related by a dependence upon PI-4,5-bisphosphate, a cofactor for PLD and a substrate for PI3K (13). The two pathways are also related in that they both target mTOR (6, 39). The evidence presented here, that elevated PLD activity does not elevate Akt phosphorylation, is consistent with this hypothesis in that PLD activity does not activate the PI3K/Akt signaling pathway. However, interestingly, the elevation of MDM2 by PLD was partially inhibited by inhibiting basal PI3K activity, indicating a linkage between the PLD and PI3K survival pathways. PLD-induced suppression of p53 stabilization was sensitive to rapamycin, implicating mTOR as a target of PLD. Although PLD can apparently stimulate mTOR without increasing Akt phosphorylation (6, 7, 11), there appears to be a requirement for a basal level of Akt activity since the inhibition of PI3K led to a partial inhibition of the PLD-induced increases in the level of MDM2. Inhibition of PI3K completely inhibited the phosphorylation of p70S6 kinase. Thus, the effect of inhibiting PI3K is likely due to an Akt requirement for the activation of mTOR. These data further suggest a link between the PLD and PI3K/Akt survival pathways in that both appear to target mTOR. There appears to be an Akt requirement for PLD survival signals, and there is also likely a PLD requirement for PI3K/Akt signals through mTOR since mTOR has a requirement for PLD-generated PA (6, 11).

The ability of PLD to suppress p53 stabilization can explain, at least in part, the ability of PLD to suppress apoptosis (25, 50) and to cooperate with tyrosine kinases to transform cells (24, 29). Tyrosine kinase activity is commonly elevated in a variety of human cancers, especially breast cancer, where there is elevated expression of tyrosine kinases such as the EGF receptor, Her2/Neu, and c-Src (5). Interestingly, elevated expression of PLD1 has been reported to be common in breast cancer tissues (33, 43). PLD activity has also been reported to be elevated in renal and gastric cancers (44, 48), and a polymorphism of the PLD2 gene was recently reported to be associated with the prevalence of colorectal cancer (47). Those reports suggest that elevated PLD activity may be providing a survival signal in these cancers. The data provided here reveal that PLD can suppress the level of a tumor suppressor that has been implicated in as much as 50% of human cancers. Elevated levels of MDM2 have also been reported in a substantial number of human cancers (reviewed by Chene [8]). Data presented here indicate that elevated PLD activity may be responsible for the presence of high levels of MDM2 in some of these cancers. The finding of elevated PLD activity in virtually all cancer types where it has been investigated, combined with the observation that PLD is able to provide survival signals in cancerous or transformed cells (50), suggests that elevated PLD activity in cancer cells is important for their survival. The ability of

PLD to increase MDM2 and suppress p53 stabilization provides strong support for this hypothesis.

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