mTOR-dependent Suppression of Protein Phosphatase 2A Is Critical for Phospholipase D Survival Signals in Human Breast Cancer Cells*

Received for publication, April 18, 2005, and in revised form, July 13, 2005. Published, JBC Papers in Press, August 18, 2005, DOI 10.1074/jbc.M504192200

Li Hui, Vanessa Rodrik, Rafal M. Pielak, Stefan Knirr, Yang Zheng, and David A. Foster

From the Department of Biological Sciences, Hunter College of the City University of New York, New York, New York 10021

A critical aspect of tumor progression is the generation of survival signals that overcome default apoptotic programs. Recent studies have revealed that elevated phospholipase D activity generates survival signals in breast and perhaps other human cancers. We report here that the elevated phospholipase D activity in the human breast cancer cell line MDA-MB-231 suppresses the activity of the putative tumor suppressor protein phosphatase 2A in a mammalian target of rapamycin (mTOR)-dependent manner. Increasing the phospholipase D activity in MCF7 cells also suppressed protein phosphatase 2A activity. Elevated phospholipase D activity suppressed association of protein phosphatase 2A with both ribosomal subunit S6-kinase and eukaryotic initiation factor 4E-binding protein 1. Suppression of protein phosphatase 2A by SV40 small t-antigen has been reported to be critical for the transformation of human cells with SV40 early region genes. Consistent with a critical role for protein phosphatase 2A in phospholipase D survival signals, either SV40 small t-antigen or pharmacological suppression of protein phosphatase 2A restored survival signals lost by the suppression of either phospholipase D or mTOR. Blocking phospholipase D signals also led to reduced phosphorylation of the pro-apoptotic protein BAD at Ser-112. The ability of phospholipase D to suppress protein phosphatase 2A identifies a critical target of an emerging phospholipase D/mTOR survival pathway in the transformation of human cells.

Mitogenic signaling involves the generation of signals that allow passage through cell cycle checkpoints. Because the default pathway for inappropriate cell proliferation signals is frequently apoptosis, some mitogenic signals have been termed “survival signals,” because they prevent default apoptotic programs (1–3). Survival signals are frequently dysregulated in human cancer (3, 4). Phospholipase D (PLD), which is elevated in several human cancers (5), generates a survival signal that suppresses apoptosis in human breast cancer cells (6, 7). Interestingly, PLD targets mTOR, the mammalian target of rapamycin (8), which has a requirement for the PLD metabolite phosphatidic acid (9). PLD stimulates increased mTOR activity and the phosphorylation of mTOR target genes, such as ribosomal subunit S6-kinase (6, 10, 11). Survival signals generated by phosphatidylinositol 3-kinase also target mTOR indirectly through activation of Akt kinase (12). Thus, mTOR appears to be a common target of both PLD- and phosphatidylinositol 3-kinase-generated survival signals.

The critical targets of mTOR for survival signals have not been fully established, although the translational machinery activated by mTOR has been suggested (13, 14). mTOR phosphorylates S6-kinase and 4E-BP1 (15) to enhance the translation of select mRNA transcripts (8). Another target of mTOR implicated in the regulation of protein translation is PP2A (16). PP2A dephosphorylates the mTOR substrates S6-kinase and 4E-BP1 (17). Suppression of PP2A could be highly significant, because the transformation of human cells by the combination of H-Ras and SV40 early region genes (18) was shown to require the SV40 small t-antigen (19, 20), which interacts with and suppresses PP2A (21–23). Based on these studies, it has been proposed that PP2A is a tumor suppressor gene (24). In this regard, it is of interest that PLD, similar to SV40 early region genes, cooperates with signaling oncogenes to transform rat fibroblasts in culture (25, 26). The transformation of human cells with SV40 early region genes also requires the large t-antigen, which interacts with and suppresses p53 (18), and in this regard, it may be of significance that elevated PLD activity stimulates an mTOR-dependent increase in the expression of MDM2, which suppresses the induction of p53 (11).

The ability of PLD to cooperate with a signaling oncogene to transform cells (25, 26) and to suppress p53 expression (11) suggests that PLD is able to achieve much of what SV40 early region genes accomplish in cell transformation and tumorigenesis. We therefore asked whether elevated PLD activity, similar to SV40 small t-antigen, suppresses PP2A. We report here that the elevated PLD activity in the human breast cancer cell line MDA-MB-231 causes an mTOR-dependent suppression of PP2A that is critical for the survival signals generated by PLD.

EXPERIMENTAL PROCEDURES

Cells, Cell Culture Conditions, and Transfection—MCF7 and MDA-MB-231 cells were obtained from the American Type Culture Collection and were maintained in Dulbecco’s modified Eagle’s medium with 10% bovine calf serum. Transfections were performed using Lipofectamine 2000™ reagent (Invitrogen) according to the vendor’s instructions. Transient transfection studies were performed 48 h after transfection as described previously (7, 31). Transfection efficiency was determined by transfection of pEGFP-C1 (Clontech), which expresses green fluorescent protein. The percentage of green cells was determined microscopically and was routinely in excess of 70%. The MCF7-P2 and the 231-P2DN cells are pooled clones of stable transfectants. Cell viability was determined by trypan blue exclusion as described previously (27).

*This work was supported by Grant CA64677 from the NCI, National Institutes of Health, Support of Continuous Research Excellence Grant GM60654 from the National Institutes of Health, and Research Centers in Minority Institutions Award RR-03037 from the National Center for Research Resources of the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom correspondence should be addressed: Dept. of Biological Sciences, Hunter College of the City University of New York, 695 Park Ave., New York, NY 10021. Tel.: 212-772-4075; Fax: 212-772-5227; E-mail: foster@genect.hunter.cuny.edu.

2 The abbreviations used are: PLD, phospholipase D; 4E-BP1, 4E binding protein 1; elf4E, eukaryotic initiation factor 4E; mTOR, mammalian target of rapamycin; PARP, poly(ADP-ribose) polymerase; PP2A, protein phosphatase 2A; siRNA, small interfering RNA; FOS, fosfotericin.
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Materials—mTOR siRNA and antibodies against S6-kinase, phosphorylated S6-kinase (Thr-389), 4E-BP1, mTOR, and PARP were from Cell Signaling Technology. The antibody against actin was from Sigma. Antibodies raised against PP2A and unmethylated PP2A were from Santa Cruz Biotechnology, as was the polyclonal antibody raised against the Flu tag (Y11), used to evaluate Flu-tagged PLD2. The antibody for small t-antigen was from Calbiochem. The antibody against BAD and phosphorylated BAD (Ser-112) was from Cell Signaling. Rapamycin was obtained from LC Laboratories, and fostriecin and cyclosporin A were obtained from Calbiochem. The plasmid expression vectors for PLD1 and PLD2 (pCGN-mpPLD2 and pCGN-mpPLD1) and the dominant negative PLD1 and PLD2 mutants (pCGN-mpPLD2-K758R and pCGN-mpPLD1-K898R) (28) were a gift from Dr. Michael Frohman (State University of New York at Stony Brook, NY). The SV40 small t-antigen expression vectors pCEP4/Smt and pCEP4/Smt (mut 3) (29, 30) were obtained from Estelle Sontag (Southwestern Medical School, Dallas, TX). The rapamycin-resistant mTOR expression vector pCDNA3-mTOR-RR (S2035I) was provided by Dr. Bob Abraham (Burnham Institute, La Jolla, CA).

Western Blot Analysis—Samples were adjusted into gel-loading buffer (50 mM Tris-HCl, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromphenol blue, 10% glycerol) and then heated for 5 min at 100 °C prior to separation by SDS-polyacrylamide gel electrophoresis. After transferring to nitrocellulose membranes (Osmonics), membrane filters were blocked with 5% nonfat dry milk in phosphate-buffered saline with 0.05% Tween 20 (PBS-T) and then incubated with the appropriate antibody diluted in 5% nonfat dry milk in PBS-T. Depending upon the origin of the primary antibodies, either anti-mouse or anti-rabbit IgG conjugated with horseradish peroxidase was used, and the bands were visualized using the enhanced chemiluminescence detection system (Pierce).

PLD Activity—PLD activity was determined by the transphosphatidylation reaction in the presence of 0.8% butanol as described previously (26, 31). Relative levels of PLD activity were then determined by measuring the intensity of the corresponding phosphatidylbutanol band in the autoradiograph using a Molecular Dynamics scanning densitometer and ImageQuant software.

Immunoprecipitation—Cells were washed twice with ice-cold phosphate-buffered saline and scraped into the modified radioimmune precipitation assay buffer (50 mM Tris-HCl, pH 7.6, 1% Igepal CA-630, 0.25% sodium deoxycholate, 150 mM NaCl, 10 mM MgCl2, 1 mM EDTA, 1 mM Na3VO4, 1 mM NaF, and 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 Biosciences), and 1000 μg of the precleared proteins was adjusted to a volume of 500 μl in the modified radioimmune precipitation assay buffer and then incubated with the antibody for overnight as described above. The immunocomplex was captured by incubation with 50 μl of protein G-Sepharose 4 Fast Flow bead slurry collected by centrifugation at 12,000 × g for 20 s at 4 °C. The beads were washed three times with the modified radioimmune precipitation assay buffer and once with wash buffer (50 mM Tris, pH 7.6) and then subjected to Western blot analysis.

PP2A Activity—Cells were lysed on the plates with phosphate lysis buffer containing 20 mM HEPES (pH 7.4), 10% glycerol, 0.1% Igepal CA-630, 1 mM EGTA, 30 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 0.15 μM aprotinin. PP2A-C immunoprecipitation complexes were prepared as described above, except for the different lysis buffer used. Agarose beads were washed twice with phosphatase lysis buffer and once with phosphatase assay buffer (Promega), and the activity of PP2A was determined using a malachite green phosphatase assay protocol with a phosphopeptide (R-RA-pt-VA) as the substrate (Promega, Madison, WI) followed by the measurement of absorbance at 620 nm. PP2A activity is given in pmol of phosphate/min/μg of protein.

siRNA—The mTOR siRNA duplexes were obtained from Cell Signaling Technology. A non-targeted negative control duplex siRNA was used as a negative control. Cells were plated on 6-well plates in medium containing 10% serum at 20% confluence. After 1 day, transfection of siRNA was performed using transfection reagent (Mirus) with 100 nM siRNA, and 3 days later, the cells were lysed and analyzed by Western blot analysis with mTOR antibodies.

RESULTS

PP2A Activity in Human Breast Cancer Cells Inversely Correlates with the Level of PLD Activity—The human breast cancer cell lines MCF7 and MDA-MB-231 have been widely used as breast cancer cells with less aggressive and more malignant phenotypes, respectively (32). We have reported previously (7) that there is elevated PLD activity in the MDA-MB-231 cells relative to the MCF7 cells and that this PLD activity provides a survival signal that suppresses apoptosis in cells deprived of serum. Because the suppression of PP2A has been implicated in the transformation of human cells (2, 21), we examined the level of PP2A activity in the MCF7 and MDA-MB-231 cells. As shown in Fig. 1A, there was an inverse correlation between the level of PLD activity and PP2A activity in these cells. MDA-MB-231 cells, with ∼8-fold higher PLD activity than the MCF7 cells, had less than half the level of PP2A activity observed in the MCF7 cells. PP2A is (reversibly) methylated throughout the cell cycle and is demethylated at the G1/S cell cycle boundary (33), where PLD has been speculated to promote cell cycle progression (5). It has also been reported that methylated PP2A has increased phosphatase activity (34). We therefore examined the methylation state of PP2A using an antibody that recognizes unmethylated PP2A. As shown in the lower panel of Fig. 1A, there was almost 4-fold more unmethylated PP2A in the MDA-MB-231 cells relative to the MCF7 cells, further indicating that PP2A is down-regulated in the MDA-MB-231 cells. Thus, the level of both PP2A activity and PP2A methylation status correlates inversely with the level of PLD activity in the MDA-MB-231 and MCF-7 cells.

The data in Fig. 1A suggest the possibility that PLD activity in the MDA-MB-231 cells suppresses PP2A activity. To test this more directly, we examined the PLD and PP2A activity in MDA-MB-231 cells and in MDA-MB-231 cells stably expressing a catalytically inactive dominant negative K758R mutant of PLD2 (231-P2DN) (28), which we have used previously to suppress the survival signals in MDA-MB-231 cells (7, 27). As shown in Fig. 1B, the dominant negative PLD2 reduced PLD activity to ∼20% that observed in the vector control MDA-MB-231 cells. The dominant negative PLD also increased the level of PP2A activity and decreased the level of unmethylated PP2A 4-fold (Fig. 1B). These data indicate that suppression of PP2A activity in the MDA-MB-231 cells is dependent upon the elevated PLD activity in these cells.

We next investigated whether increasing PLD activity in MCF7 cells would suppress PP2A activity. We generated MCF7 cells stably expressing elevated PLD2 (MCF7-P2 cells). As shown in Fig. 1C, cells with elevated PLD2 had ∼7-fold higher levels of PLD activity than empty vector control MCF7 cells (MCF7- v). The elevated PLD activity also suppressed PP2A activity and increased the level of unmethylated PP2A (Fig. 1C).
We previously demonstrated that both PLD1 and PLD2 can provide survival signals in MDA-MB-231 cells (7). We therefore also examined the effect of PLD1 on PP2A activity in MDA-MB-231 and MCF7 cells. Because stable expression of PLD1 and PLD1 mutants is generally not tolerated by cells as well as PLD2, we used transient, rather than stable, expression of PLD1 and a catalytically inactive dominant negative K898R mutant of PLD1. As shown in Fig. 1D, the dominant negative PLD1 mutant, similar to PLD2, suppressed PLD activity, elevated PP2A activity, and reduced the level of unmethylated PP2A normalized to the total PP2A as shown in parentheses. Similarly, the levels of PLD activity, PP2A activity, and unmethylated PP2A were determined as in A in MDA-MB-231 cells that were stably transfected with either an empty vector (231-v) as a control or a vector expressing a catalytically inactive dominant negative mutant of PLD2 (P2DN). C, the levels of PLD activity, PP2A activity, and unmethylated PP2A were determined in MCF7 cells that were stably transfected with either an empty vector control (MCF7-v) or a vector expressing wild type PLD2 (MCF7-P2). D, MDA-MB-231 cells were transiently transfected with either an empty vector control or with a vector expressing dominant negative PLD1. PLD activity, PP2A activity, and unmethylated PP2A levels were determined 48 h later as described for B. E, MCF7 cells were transiently transfected with either an empty vector control or with a vector expressing wild type PLD1. PLD activity, PP2A activity, and unmethylated PP2A levels were determined 48 h later as described for C. All experiments shown are representative of those repeated three times.

We previously demonstrated that both PLD1 and PLD2 can provide survival signals in MDA-MB-231 cells (7). We therefore also examined the effect of PLD1 on PP2A activity in MDA-MB-231 and MCF7 cells. Because stable expression of PLD1 and PLD1 mutants is generally not tolerated by cells as well as PLD2, we used transient, rather than stable, expression of PLD1 and a catalytically inactive dominant negative K898R mutant of PLD1. As shown in Fig. 1D, the dominant negative PLD1 mutant, similar to PLD2, suppressed PLD activity, elevated PP2A activity, and reduced the level of unmethylated PP2A in MDA-MB-231 cells. And as shown in Fig. 1E, transient expression of wild type PLD1 in MCF7 cells suppressed PP2A activity and increased the level of unmethylated PP2A. Thus, both PLD1 and PLD2 can suppress PP2A activity. Collectively, the data in Fig. 1 show a strong correlation between elevated PLD activity and the suppression of PP2A activity.

**Suppression of PP2A by PLD Is Dependent upon mTOR**—We previously reported that PLD2 could provide a rapamycin-sensitive survival signal in MCF7 cells, implicating mTOR as a mediator of the PLD-generated survival signals (6). Rapamycin has been reported to block the dephosphorylation of S6-kinase and to increase PP2A activity (17), possibly by preventing phosphorylation of a PP2A regulatory subunit α4 (mammalian homologue of yeast TAP42) (16). We therefore examined the effect of suppressing mTOR on the PLD-dependent suppression of PP2A activity in MDA-MB-231 and the MCF-P2 cells with elevated PLD2 expression. Two approaches were employed, the first being the effect of rapamycin on PP2A activity in the two cell types. As shown in Fig. 2A, rapamycin increased PP2A activity in both the MDA-MB-231 and MCF7-P2 cells. The lower panel of Fig. 2A shows that the rapamycin was working, because phosphorylation of the mTOR substrate S6-kinase was blocked by rapamycin. As reported previously (27), elevated PLD activity increases that amount of rapamycin required to suppress mTOR, and MDA-MB-231 cells have very high levels of PLD activity that requires high concentrations of rapamycin to suppress mTOR (6). To establish that the high concentration of rapamycin (20 μM) used here was because of an effect on mTOR and not another cellular target, we introduced the rapamycin-resistant mTOR mutant used previously to demonstrate the specificity of rapamycin (6) into the MDA-MB-231 cells and examined the effect of rapamycin on PP2A activity. As shown in Fig. 2B, the rapamycin-resistant mTOR mutant reversed the effect of the high concentration of rapamycin used, indicating that the effect of rapamycin was on mTOR and not another
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PP2A-C has also been implicated in the dephosphorylation of 4E-BP1 (17). We therefore examined the association between the PP2A and S6-kinase and 4E-BP1 in MCF-v and MCF-P2 cells. Lysates from these cells were immunoprecipitated with antibodies raised against either S6-kinase or 4E-BP1 followed by Western blot analysis using antibodies raised against the catalytic subunit of PP2A and either S6-kinase (upper panel) or 4E-BP1 (lower panel). As shown in Fig. 3A, high levels of PP2A could be detected in both the S6-kinase and 4E-BP1 immunoprecipitates from the MCF7 cells. In contrast, very little PP2A could be detected in the immunoprecipitates from the MCF7-P2 cells. Treating the MCF7-P2 cells with rapamycin restored the association between PP2A and both S6-kinase and 4E-BP1 (Fig. 3A). We next examined the association between PP2A and both S6-kinase and 4E-BP1 in MDA-MB-231 cells and the MDA-MB-231 cells expressing the dominant negative PLD2 mutant. As shown in Fig. 3B, there was very little co-precipitation of PP2A with either S6-kinase or 4E-BP1 from MDA-MB-231 cell lysates. However, in the MDA-MB-231 cells expressing the dominant negative PLD2, association between PP2A and both S6-kinase and 4E-BP1 was restored (Fig. 3B). These data further suggest that PLD suppresses PP2A.

SV40 Small t-Antigen or Suppression of PP2A Activity Restores Survival of MDA-MB-231 Cells Lost through Inhibition of Either PLD or mTOR—The data presented above indicate that PLD suppresses PP2A in an mTOR-dependent manor. We demonstrated previously that PLD suppresses apoptosis in MDA-MB-231 cells subjected to serum withdrawal (7), and this suppression is dependent on mTOR (6). This raises the question as to whether the mTOR-dependent suppression of PP2A is required for the survival signals generated by PLD. To address this question, we examined the effect of the PP2A inhibitor fostriecin (FOS) on MDA-MB-231 cells, where the survival signals generated by PLD have been blocked. We first examined the effect of FOS on PP2A activity in the MDA-MB-231 cells, and as shown in Fig. 4A, FOS suppressed PP2A activity, whereas the PP2B inhibitor cyclosporine A had no effect. We then examined the effect of FOS on the survival of MDA-MB-231 cells subjected to serum withdrawal in the presence of rapamycin. Rapa-

FIGURE 2. Suppression of PP2A by PLD is dependent upon mTOR. A, MDA-MB-231 and MCF7-P2 cells were treated with rapamycin (20 μM) for 1 h, at which time PP2A activity was determined. The effect of rapamycin treatment on the phosphorylation state of the mTOR substrate S6-kinase was determined by Western blot using antibodies against phosphorylated S6-kinase (P-p70S6K) and S6-kinase (p70S6K).

FIGURE 3. PLD suppresses the association between the PP2A catalytic subunit and both S6-kinase and 4E-BP1. A, lysates from MCF7-v and MCF7-P2 cells were immunoprecipitated with antibodies to either S6-kinase (upper panel) or 4E-BP1 (lower panel), immunoprecipitates were subjected to Western blot analysis using antibodies raised against PP2A-C and either S6-kinase (upper panel) or 4E-BP1 (lower panel). Where indicated, rapamycin (20 μM) was added to the MCF-P2 cells 1 h prior to the preparation of cell lysates. B, lysates from 231-v and 231-P2DN cells were to immunoprecipitated with either anti-S6-kinase antibody (upper panel) or with anti-4E-BP1 antibody (lower panel), and the immunoprecipitates were subjected to Western blot analysis using antibodies raised against the catalytic subunit of PP2A and either S6-kinase (upper panel) or 4E-BP1 (lower panel) as described for A.
mycin blocks PLD-generated survival signals and induces apoptosis in MDA-MB-231 cells subjected to serum withdrawal (6). As shown in Fig. 4B, these cells were highly sensitive to rapamycin in the absence of serum, as indicated by the loss of cell viability and increased cleavage of the caspase 3 substrate PARP. Thus, blocking the mTOR-dependent survival signal generated by PLD in these cells in the absence of serum results in apoptosis. However, when FOS was present, the effect of rapamycin was substantially reduced (Fig. 4B). The PP2B inhibitor was not able to suppress apoptosis under these conditions. We also examined the effect of FOS on the survival of MDA-MB-231 cells expressing the dominant negative PLD2. As shown in Fig. 4C, these cells, with suppressed PLD activity, were highly sensitive to the withdrawal of serum, as indicated by the loss of cell viability and increased PARP cleavage relative to vector control cells. Thus, expression of the dominant negative PLD2 blocked the survival signal generated by PLD in these cells, and the removal of serum then resulted in apoptosis. However, when FOS was present, the MDA-MB-231 cells expressing the dominant negative PLD2 cells survived as well as the vector control MDA-MB-231 cells when the serum was withdrawn. The PP2B inhibitor did not suppress apoptosis under these conditions. These data provide evidence that the suppression of PP2A by elevated PLD activity in MDA-MB-231 cells is critical for the survival signals generated by PLD in these cells.

Transformation of human cells with SV40 early region genes required small t-antigen (20), which interacts with and suppresses PP2A (24). We therefore investigated whether SV40 small t-antigen could rescue survival of serum-starved MDA-MB-231 cells expressing the dominant negative PLD2. This experiment was performed in two ways, where MDA-MB-231 cells were transiently transfected with a vector that expresses the dominant negative PLD2 (P2DN) or where the dominant negative PLD2 in combination with vectors expressing SV40 small t-antigen or a small t-antigen mutant does not interact with PP2A (29, 30). As shown in Fig. 5A, the dominant negative PLD2 increased cell death and PARP cleavage as in Fig. 4 and as described previously (6). Co-transfection with the SV40 small t-antigen vector reduced cell death and PARP cleavage, whereas the vector expressing the SV40 small t-mutant did not. A similar experiment was performed on MDA-MB-231 cells stably expressing dominant negative PLD2, and as shown in Fig. 5B, SV40 small t-antigen (but not the small t-antigen mutant) reduced cell death and PARP cleavage in these cells. These data further indicate that PP2A is a critical target of PLD survival signals in MDA-MB-231 cells.

**Suppression of mTOR or PLD Inhibits Phosphorylation of BAD at Ser-112**—BAD, a pro-apoptotic molecule of the Bcl2 family of apoptosis regulators, is regulated by reversible phosphorylation (36) and is a target of survival signals (1). Dephosphorylation of BAD at Ser-112 by PP2A has been shown to be critical for the pro-apoptotic effects of BAD (37, 38). When PLD is suppressing PP2A in an mTOR-dependent manner, then suppression of PLD signaling should suppress phosphorylation of BAD at Ser-112. MDA-MB-231 cells were treated with either rapamycin or dominant negative PLD2. As shown in Fig. 6A, rapamycin suppressed the phosphorylation of BAD at Ser-112. The suppression of BAD phosphorylation was reversed by the PP2A inhibitor FOS, indicating that the reduced phosphorylation of BAD in the presence of rapamycin was due to PP2A. We also examined the effect of the dominant negative PLD2 on BAD phosphorylation. The vector expressing the dominant negative PLD2 mutant was transiently transfected into MDA-MB-231 cells, and BAD phosphorylation was investigated 24 h later. As shown in Fig. 6B, the dominant negative PLD2 also suppressed BAD phosphorylation at Ser-112. When the small t-antigen-expressing vector was co-transfected, the effects of the dominant negative PLD2 were reversed. The reversal was not observed when the small t-mutant

**FIGURE 4.** Inhibition of PP2A restores the survival signals in MDA-MB-231 cells lost upon inhibiting either mTOR or PLD. A, PP2A activity in MDA-MB-231 cells was determined as Fig. 1. Fostriecin (FOS) (5 μM) and Cyclosporin A (CsA) (1 μM) were added where indicated 16 h prior to assessing PP2A activity. The PP2A activity was normalized to the PP2A activity in the Me2SO (DMSO) vehicle control, which was given a value of 1. Error bars represent the standard deviation for triplicate samples from a representative experiment repeated three times. B, MDA-MB-231 cells were placed in serum-free medium along with rapamycin (Rap) (20 μM), FOS (5 μM), CsA (1 μM), or the Me2SO vehicle, as indicated. 16 h later, cell viability was determined by trypan blue exclusion, as described under “Experimental Procedures.”PARP cleavage was determined by Western blot analysis using an antibody that recognizes cleaved PARP (Cl PARP). Actin-loading controls were performed by Western blot. The experiment is representative of one repeated three times. C, 231-v and 231-P2DN cells were placed in serum-free medium along with FOS (5 μM), CsA (1 μM), or the control (Con) Me2SO vehicle, as indicated. 24 h later, cell viability and PARP cleavage was determined as described for B. The experiment is representative of one repeated three times.
that does not bind PP2A was used. These data further indicate that the mTOR-dependent signals generated by PLD involve PP2A. These data also suggest that the survival signals generated by PLD involve suppression of BAD dephosphorylation by PP2A.

**DISCUSSION**

The generation of survival signals in an emerging tumor is critical if cells are to escape from the normal constraints built in to prevent unwanted proliferation (39). During the past several years, it has become apparent that mTOR is a critical target of survival signals and for progression through cell cycle checkpoints (2, 14, 40). The demonstration of a phosphatidic acid requirement for mTOR (9) implicated PLD activity in the generation of mTOR-mediated survival signals.

Consistent with a role for PLD in mTOR-mediated survival signals, elevated PLD activity in the human breast cancer cell line MDA-MB-231 provided a rapamycin-sensitive survival signal (6). Moreover, elevated PLD activity has been implicated in breast, kidney, gastric, and colon cancer (see Ref. 5 for review), indicating that PLD plays a role in human cancer. In this report, we have shown that the elevated PLD activity in the human breast cancer cell line MDA-MB-231 leads to an
mTOR-dependent suppression of PP2A that is critical for suppressing apoptosis.

The requirement of SV40 small t-antigen for the transformation of human cells with SV40 early region genes (20) underscores the importance of targeting PP2A in human cancer. We recently reported that PLD activity suppresses the induction of p53 (11), which is also targeted by SV40 early region genes in the transformation of human cells (18, 20). The data presented here are consistent with a model where the elevation of PLD activity suppresses apoptosis by facilitating progression through the same cell cycle checkpoints overcome by SV40 early region genes. Consistent with this hypothesis, PLD, similar to SV40 early region genes, cooperates with signaling oncogenes to transform rat fibroblasts in culture (18, 20, 25, 26).

Recent studies have implicated dysregulation of translational control in a number of human cancers including breast cancer (14, 41, 42). Increased expression of 4E-BP1, which inhibits eIF4E, was shown to revert the malignant phenotype of transformed rodent fibroblasts (42). Moreover, elevated expression of eIF4E partially rescued rapamycin-inhibited G1-phase progression (43), indicating that mTOR effects on cell cycle progression are mediated by a 4E-BP1 suppression of eIF4E. We demonstrated here that PP2A is associated with 4E-BP1 in MCF7 cells and that this association is disrupted in a rapamycin-dependent manner with elevated PLD activity. Similarly, association between PP2A and 4E-BP1 in MDA-MB-231 cells was stimulated by the suppression of PLD activity. Because hyperphosphorylated 4E-BP1 has a decreased affinity for eIF4E, the association between PP2A and 4E-BP1 would likely result in the dephosphorylation of 4E-BP1 and the sequestering of eIF4E. Thus, the dissociation of PP2A from 4E-BP1 induced by PLD activity should enhance the release of eIF4E from the inhibitory constraints of 4E-BP1 and stimulate the initiation of translation. The ability of PLD to stimulate translation is consistent with the emerging paradigm that translation contributes to the survival signals.

PP2A has also been shown to interact with and dephosphorylate the pro-apoptotic Bcl family protein BAD at Ser-112 (37, 38). This dephosphorylation is necessary for the pro-apoptotic effects of BAD (38). Data presented here indicate that the suppression of PP2A by PLD also reduces phosphorylation of BAD at Ser-112. The ability of PLD to prevent the dephosphorylation of BAD likely contributes to the ability of PLD to suppress apoptosis and underscores the critical importance of targeting PP2A in survival signals.

The data presented here provide further evidence of the ability of elevated PLD activity in human cancer cells to interfere with signals that regulate cell cycle progression and apoptosis. The data reported here reveal an mTOR-dependent suppression of PP2A activity, which is apparently critical for the transformation of human cells (21). PLD also stimulates the dissociation of PP2A from the mTOR substrates S6-kinase and 4E-BP1 and suppressed the dephosphorylation of BAD at a PP2A site. Because PLD activity is elevated in a large number of human cancers (5) and the suppression of PP2A is apparently critical for the transformation of human cells (19, 21), it is likely that the elevated PLD activity in human cancer is a critical component of tumor progression. A model for the mTOR-dependent targeting of PP2A by PLD-generated survival signals is shown in Fig. 7.

Acknowledgments—We thank Drs. Mike Frohman, Estelle Sontag, and Bob Abraham for plasmid expression vectors used in this study.
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J. Biol. Chem. 2005, 280:35829-35835.
doi: 10.1074/jbc.M504192200 originally published online August 18, 2005

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