Subject Review

Phospholipase D in Cell Proliferation and Cancer

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Abstract

Phospholipase D (PLD) has emerged as a regulator of several critical aspects of cell physiology. PLD, which catalyzes the hydrolysis of phosphatidylcholine (PC) to phosphatidic acid (PA) and choline, is activated in response to stimulators of vesicle transport, endocytosis, exocytosis, cell migration, and mitosis. Dysregulation of these cell biological processes occurs in the development of a variety of human tumors. It has now been observed that there are abnormalities in PLD expression and activity in many human cancers. In this review, evidence is summarized implicating PLD as a critical regulator of cell proliferation, survival signaling, cell transformation, and tumor progression.

Introduction

Phospholipid-metabolizing enzymes have been widely implicated in the transduction of intracellular signals in higher eukaryotic organisms that rely heavily on communication between cells (1). The generation of "lipid second messengers" at the site of ligand-receptor interactions has apparently evolved as a means to generating varied, rapid, and complex responses to extracellular signals. The most widely studied class of lipid metabolizing enzymes implicated in receptor-mediated signal transduction are enzymes that modify phosphatidylinositol (PI) and include PI kinases and phospholipase C (PLC; 2). Over the last decade, another phospholipase that uses phosphatidylcholine (PC) as a substrate has emerged as a critical component of intracellular signal transduction. Phospholipase D (PLD) catalyzes the hydrolysis of phosphatidylcholine to phosphatidic acid (PA) and choline. While the generation of choline by PLD may have some second messenger function, it is widely believed that PA is the most critical metabolite generated by PLD. However, inhibitors of choline kinase, which converts choline to phospho-choline has been reported to negatively regulate cell growth (3). PA can also be metabolically converted to diacylglycerol (DAG) and to lyso-PA, both of which have second messenger function that could contribute to the effects of PLD (4, 5). PLD has been implicated in membrane trafficking, cytoskeletal reorganization, receptor endocytosis, exocytosis, and cell migration (4, 5). A role for PLD in cell proliferation is indicated from reports showing that PLD activity is elevated in response to platelet-derived growth factor (PDGF; 6), fibroblast growth factor (7, 8), epidermal growth factor (EGF; 9), insulin (10), insulin-like growth factor 1 (11), growth hormone (12), and sphingosine 1-phosphate (13). PLD activity is also elevated in cells transformed by a variety of transforming oncogenes including v-Src (14), v-Ras (15, 16), v-Fps (17), and v-Raf (18). Thus, there is a growing body of evidence linking PLD activity with mitogenic signaling. While PLD has been associated with many aspects of cell physiology such as membrane trafficking and cytoskeletal organization (4, 5), this review will focus on the PLD activity initiated by mitogenic and oncogenic stimuli, which we will refer to as "mitogenic PLD activity." Evidence implicating PLD as a critical regulator of cell proliferation and survival are summarized with a discussion of a potential role for PLD in human cancer.

PLD Isoforms

Two mammalian PLD genes (*PLD1* and *PLD2*), both with splice variants, have been reported (19–22). There is considerable homology between the two genes; however, there are significant differences in the regulation and subcellular distribution of PLD1 and PLD2. PLD1 is activated by the ADP ribosylation factor (ARF)-, Ral-, and Rho-family GTPases, as well as by protein kinase C α (PKC-α; 5). Both PLD1 and PLD2 have an absolute requirement for PI-4,5-bis-phosphate (PIP2; 19, 21). PLD2 activity is elevated by fatty acids (21). PLD2 is constitutively active *in vitro* and this activity is unaffected by GTPases or PKC-α (21, 23, 24); however, an amino-terminal mutation to PLD2 results in the loss of constitutive activity and responsiveness to ARF (25). PLD2 may also be affected by PKC-α *in vivo* (26, 27). Studies on the subcellular distribution of PLD1 and PLD2 have been inconclusive; however, it is clear that there are differences in the distribution of the two isoforms. Both PLD1 and PLD2 are palmitoylated on conserved Cys residues (28–32), and this fatty acylation likely contributes to membrane association. PLD1 is found throughout the cell, but particularly in perinuclear, Golgi, and heavy membrane fractions (33–35). In contrast, PLD2 is localized almost exclusively on the plasma membrane in light membrane "lipid raft" fractions that co-fractionate with caveolin (33, 34, 36). Although PLD1 is primarily associated with intracellular membranes, it can also be found in the lipid rafts (32, 34, 37). Very little is known about differences between the splice variants of PLD1 and PLD2. Distinguishing characteristics of the PLD isoforms are summarized in Table 1.

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Table 1. Characteristics of PLD Isoforms

	PLD1	PLD2
PIP2-dependent	+	+
RalA-associated	+	–
Activated by:		
ARF-family GTPases	+	?
Rho-family GTPases	+	–
PKC-α	+	?
Fatty acids	–	+
Molecular weight	120,000	96,000
Predominant cellular location:	Peri-nuclear, Golgi	Plasma membrane, rafts

Regulation of Mitogenic PLD

GTPases

Several members of the small GTPase family have been implicated in the activation of PLD1, including members of the ARF-, Rho-, and Ral-family GTPases. Arf and Rho families of GTPases have been shown to activate PLD1 directly *in vitro* (20, 38–40) and possibly PLD2 *in vivo* (41, 42). Both Arf- and Rho-family GTPases have also been implicated in the regulation of PLD activity *in vivo* as reviewed recently (4, 5, 43, 44). There have been reports implicating Rho-family GTPases in mitogenic signaling through PLD (18, 45); however, more commonly, Rho-family GTPases have been more convincingly demonstrated to mediate PLD responses through agonists that stimulate secretion (46) or activate heptahelical receptors and heterotrimeric GTPases not usually implicated in mitogenic signaling (47). It is likely that the major role for the PLD activity stimulated by Rho family GTPases is the formation of actin stress fibers and regulation of cell motility since PLD activity has been implicated in the formation of stress fibers (48, 49). This would be consistent with many studies on Rho-family GTPases where it has been clearly established that the Rho-family GTPases regulate membrane traffic and actin dynamics (50). Thus, while available evidence does not indicate a major role for Rho-family GTPases in mitogenic signal transduction, the Rho-family GTPases may play a critical role in the regulation of PLD activity in cell migration. This process is critical for progression to a malignant metastatic cancer and a role for PLD in this process is described below. In this regard, it may be of interest that transforming mutants of the Rho-family GTPase Cdc42 are dependent on a "Rho insert" portion of Cdc42 (51). Mutations to the Rho-insert region of Cdc42 prevented both transformation and PLD1 activation (52, 53). Thus, while Rho family GTPases may not play a role in conventional mitogenic signal transduction, the transformed phenotypes induced by mutants of Rho-family GTPases may be dependent on PLD activity.

In contrast with the Rho-family GTPases, ARF-family GTPases have been strongly implicated in mitogenic signaling. ARF proteins have been reported to be required for the activation of PLD activity by PDGF (54, 55), EGF (56), insulin (56), phorbol esters (54, 57), and H-Ras (35, 58). All of the ARF-family GTPases can activate PLD activity *in vitro* (4, 20, 59); however, ARF6 has emerged as the likely regulator of mitogenic PLD activity. There are three classes of ARF family of GTPases: class I (ARF1–3), class II (ARF4 and 5), and class 3 (ARF6; 60). The class I ARFs have been linked most closely with vesicle transport in the ER and Golgi (60). Little is known about the functions of the class II ARF family, although ARF4 was recently implicated in the activation of PLD2 (42). This is of interest because PLD2 is the predominant PLD isoform in the light membrane lipid raft fractions where mitogenic PLD activity is localized (34, 35). The class III ARF6 co-localizes with PLD1 (46, 61) and ARF6 is localized in the lipid raft fractions. In contrast, ARF1 cannot be detected in light membrane fractions (35). Thus, there is a strong correlation between ARF proteins and mitogenic signaling, with ARF6, and possibly ARF4 being the key ARF proteins in regulating mitogenic PLD activity.

While there is good evidence for an ARF6 requirement for activation of mitogenic PLD activity, it is not clear how ARF6 gets activated in response to mitogenic signals. The elevated PLD activity in H-Ras-transformed cells was reported to be sensitive to brefeldin A (BFA; 58), a fungal metabolite that inhibits ARF-guanine nucleotide dissociation stimulator (GDS) proteins. PLD activity stimulated by PDGF and insulin was also reported to be sensitive to BFA (54–56, 62). However, the known GDS proteins for ARF6—ARNO, EFA6, and ARF-GEP100 have been reported to be insensitive to BFA (63–65). These reports suggest that the GDS proteins for ARF6 may display differential sensitivities to BFA in different cellular contexts, or alternatively, that there is another exchange factor for ARF6, yet to be identified, that is sensitive to BFA. In support of the first hypothesis, PMA-induced PLD activity has been reported to be both BFA-sensitive (54) and BFA-insensitive (66). Thus, while ARF6 is clearly implicated in mitogenic PLD activity, it remains to be determined how ARF6 is activated in response to mitogenic signals.

Another GTPase implicated in mitogenic PLD signaling is RalA (67), a Ras-family GTPase that interacts directly with PLD1, but does not activate it (68, 69). While RalA is not sufficient to activate PLD1 either *in vitro* or *in vivo* (68), RalA is required for the activation of PLD activity by EGF (70, 71), PDGF (70), insulin (70), Src (68), Ras (68, 72), Raf (18), and phorbol esters (70, 73). Thus, it is clear that RalA plays a critical role in the activation of PLD activity in response to mitogenic and oncogenic signals. Ras activates RalA through recruitment of GTP/GDP exchange factor for RalA, Ral-GDS (67, 74), and importantly, the Ral pathway emanating from H-Ras was recently reported to be the most critical for the transformation of human cells (75). Although activated RalA is not sufficient to activate PLD1, RalA can be co-immunoprecipitated with ARF proteins (58). Moreover, it was demonstrated that RalA is able to work synergistically with ARF1 to elevate PLD1 activity *in vitro* (76). More recently it was shown that a combination of activated RalA and activated ARF6 was sufficient to elevate PLD activity *in vivo* (35). Activated ARF1 could not work synergistically with activated RalA in this *in vivo* study—most likely because ARF1 and RalA do not co-localize in the cell, whereas both ARF6 and RalA do co-localize to lipid rafts where mitogenic PLD activity is localized (34, 35). A model for PLD activation by Ras was proposed whereby mitogenic signals mediated by Ras activate two parallel pathways leading to the activation of GDS proteins for both RalA and ARF6. This results in the activation of RalA,

which is already in a complex with PLD1. The activated form of RalA is proposed to recruit activated ARF6 into the RalA-PLD1 complex and the activated ARF6, now in a RalA/PLD1/ARF6 complex, then activates PLD1 through a direct interaction. This model for the synergistic activation of PLD by RalA and ARF6 is shown in Fig. 1A.

PKC-α

PKC-α has been implicated in the regulation of PLD1 activity in response to mitogenic signaling (77–79). How PKC-α regulates PLD1 has generated some confusion. *In vitro* studies have shown that PKC-α activates PLD1 in a kinase-independent manner (80). However, *in vivo*, PLD1 is phosphorylated by PKC-α (32, 81, 82), and inhibiting the kinase activity of PKC-α blocks the PLD activity induced by EGF (77, 79). This leaves us with an apparent contradiction whereby *in vitro* data suggest a PKC-α kinase-independent mechanism for PLD1 activation, and *in vivo* data suggesting a kinase-dependent mechanism. Interestingly, PLD1 phosphorylated by PKC-α localizes exclusively to light membrane lipid raft fractions (32), which is where mitogenic PLD activity is largely restricted (34). It has been proposed that the activation of PLD1 by PKC-α is regulated by both phosphorylation and protein-protein interactions (83). Thus, it could be speculated that the *in vivo* kinase requirement for PKC-α may reflect a phosphorylation requirement for proper localization into lipid rafts and the assembly of an active PLD1 complex consisting of a combination of factors including GTPases and PKC-α. In this regard, it may be of significance that the PLD activity induced by the growth factors PDGF and EGF is dependent on both PKC-α (77, 79, 84, 85) and Ras (70, 71, 86), whereas the oncogenic activation of PLD by H-Ras and Src is independent of PKC-α (16, 85, 87, 88). It has also been proposed that phosphorylation of PLD1 by PKC-α leads to the down-regulation of PLD1 activity (89). Therefore, it could be proposed that the PKC-α requirement in mitogenic PLD activity may be involved uniquely in the activation of PLD1 by tyrosine kinase growth factor receptors where the physiological activation is subsequently down-regulated as a negative feedback. In contrast, the oncogenic activation by H-Ras and v-Src avoids this negative feedback and maintains a sustained higher level of PLD activity that could contribute to

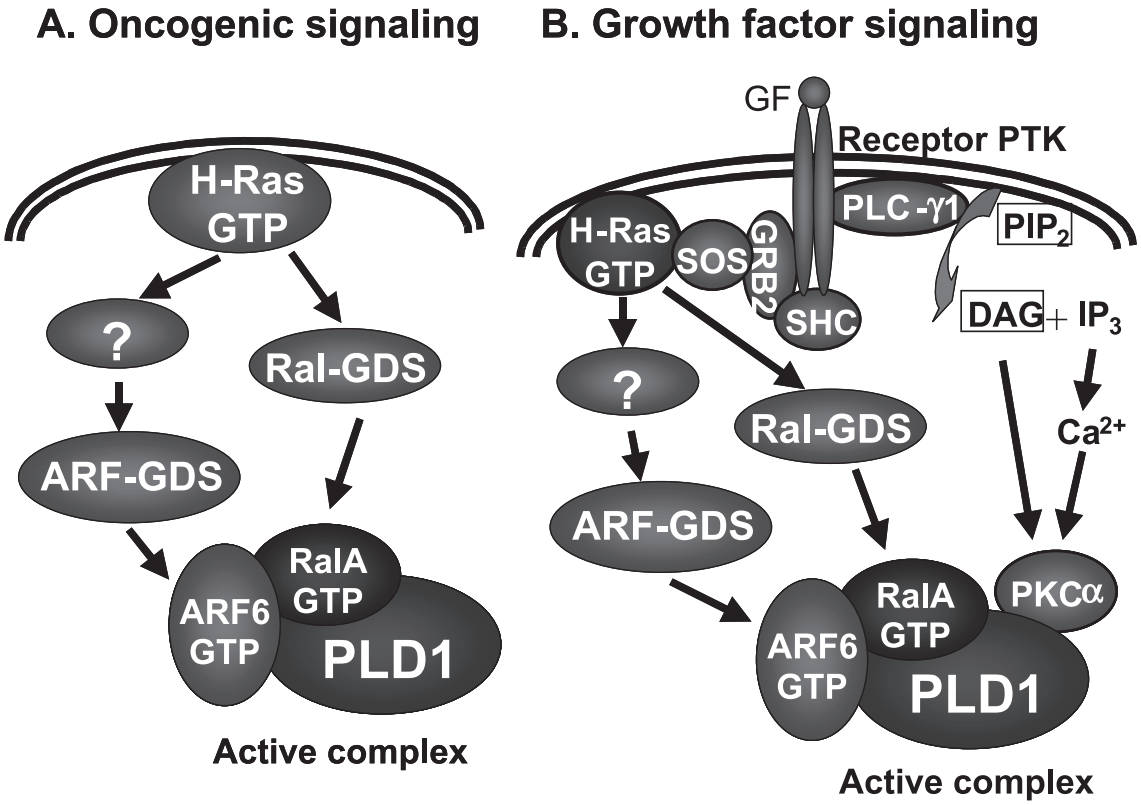

FIGURE 1. Activation of PLD1 by oncogene and growth factor signaling. **A.** Oncogenic activation of PLD1 through the synergistic action of RalA and ARF6. It is proposed that parallel pathways are activated, in this case by H-Ras, leading to the activation of Ral-GDS and an as yet unspecified ARF-GDS. Activation of Ral-GDS activates RalA, which is already in a complex with PLD1. The activation of RalA then recruits activated ARF6 into the RalA-PLD1 complex. The activated ARF6 then activates PLD1. **B.** Activation of PLD1 by tyrosine kinase growth factor receptors. In response to growth factor, the receptor dimerizes and there is transphosphorylation of tyrosine residues. This leads to the recruitment of the adaptor molecules Shc and Grb2, which is in complex with the Ras-GDS, Sos. Sos then activates H-Ras. H-Ras then activates Ral-GDS and ARF-GDS as in **A**. PLCγ can also be recruited and lead to the generation of diacylglycerol (*DAG*) and the activation of PKC-α. PKC-α then contributes to the activation and perhaps subsequent down-regulation of PLD1 in ways that are poorly understood. The activation of PLD1 by growth factors is dependent on PKC-α, whereas the activation of PLD1 by oncogenic H-Ras is PKC-α independent.

oncogenicity. It is clear, however, that PKC-α regulation of mitogenic PLD activity is very complex and that there is still much to be determined as to how PKC-α contributes. This is summarized in Fig. 1B.

PIP2

Both PLD1 and PLD2 have a stringent requirement for PIP2, indicating a requirement for the PI kinases that generate PIP2. In this regard, it is of interest that PI-4-P 5-kinase, which generates PIP2 from PIP, is a downstream target of PLD signaling (90, 91) and may therefore represent a positive feedback loop. Whether the formation of PIP2 regulates PLD activity in response to mitogenic stimuli remains to be determined, but it does provide an attractive hypothesis for regulating PLD2, which is constitutively active *in vitro*. Disruption of the PH domain of PLD2 results in relocalization of the protein from the plasma membrane to endosomes, and renders PLD2 unresponsive to activation *in vivo* (92). Moreover, elevating PIP2 levels with PI-4-P 5-kinase elevated PLD2 activity *in vivo* (93). This suggests that the generation of PIP2 could be critical for regulating PLD2 *in vivo*. This hypothesis is discussed below in the context of which PLD isoforms are mediating mitogenic responses.

PLD Isoforms in Mitogenic Signaling

Characterizing the role of the two PLD isoforms in mitogenic signaling has been problematic. The PLD activity stimulated in response to mitogenic signals is dependent on both ARF and RalA (35, 54–56, 62, 68, 71), both of which implicate PLD1 (19–21, 69). PKC-α has also been implicated in mitogenic signaling through growth factors (77–79), and this also implicates PLD1. However, mitogenic PLD activity has been shown to take place primarily in light membrane lipid raft fractions (34) where PLD2 is the predominant PLD isoform. PLD1 also localizes to these light membrane fractions (32, 34, 37), but a substantial majority of PLD1 has a peri-nuclear heavy membrane localization (34, 36). Thus, genetic evidence links mitogenic signaling with PLD1 and circumstantial evidence implicates PLD2. An intriguing resolution to this apparent paradox is that PLD1 could be regulating PLD2. A recent report by Mwanjewe *et al.* (94) suggested that the activation of PLD2 was dependent on the activation of PLD1. Han *et al.* (32) reported that phosphorylation of PLD1 by PKC-α could target PLD1 to the light membrane fractions since phosphorylated PLD1 was localized to the light membrane fraction. Endocytosis of the EGF receptor was blocked by catalytically inactive mutants of either PLD1 or PLD2 (95), indicating that both PLD1 and PLD2 are required for the process. Moreover, both PLD1 and PLD2 complimented tyrosine kinase overexpression to transform cells (96), to suppress cell cycle arrest (97), to prevent apoptosis (97, 98), and to enhance cell cycle progression (99) and proliferation (100). Thus, the current data support the involvement of both PLD1 and PLD2 in mitogenic signaling through the activation of PLD1 via RalA and ARF6, which leads to PLD2 activation in the light membrane fractions where both PLD1 and PLD2 are present, but where PLD2 is the majority of PLD protein in this membrane microdomain (33, 34, 36, 37). It is not yet clear how PLD1 might lead to the activation of PLD2; however, it could be speculated that PLD1 can elevate PLD2 activity via PI-4-P 5-kinase since PA activates PI-4-P 5-kinase (90, 91), which generates PIP2, an essential co-factor for PLD2. PI-4-P 5-kinase has also been reported to interact with both PLD1 and PLD2 and was sufficient to elevate PLD2 activity *in vivo* (93), indicating a functional link between PLD2 activity and PIP2 production. A model for a PLD1-dependent activation of PLD2 through the activation of PI-4-P 5-kinase is shown in Fig. 2.

Downstream Targets

Raf

While much has been established on the upstream regulation of PLD, relevant downstream PLD targets have been difficult to establish. PA is required for the activation of PI-4-P 5-kinase (90, 91), 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. Raf has a PA binding site in its COOH terminus (101) 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 mitogen-activated protein (MAP) kinase pathway (62, 101, 102). Activation of the MAP kinase pathway has been implicated in most mitogenic signaling schemes and therefore contributing to Raf activation almost certainly contributes to the mitogenic properties of PLD.

mTOR

An interesting new target for mitogenic PLD signaling is mTOR, the mammalian target of rapamycin, which is a target of

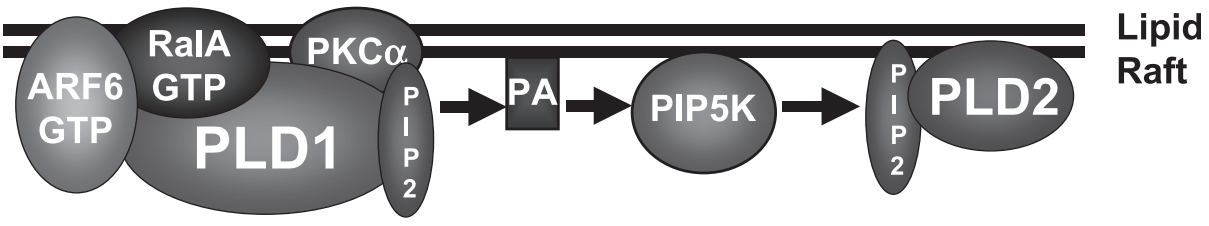

FIGURE 2. Mitogenic signaling through PLD1-dependent activation of PLD2. In this model, it is proposed that PLD1 is activated through the synergistic actions of ARF6 and RalA in lipid rafts as described in Fig. 1. PIP2 is required for both PLD1 and PLD2 activity. On activation of PLD1, all available PIP2 is used by PLD1, which is present in low levels in the lipid rafts. PLD2, which is present at high levels in lipid rafts, is inactive in the absence of sufficient PIP2. The generation of PA by PLD1 would activate PI-4-P 5-kinase (*PIP5K*), which would then generate sufficient PIP2 to activate the high levels of PLD2.

survival signals generated by the PI-3-kinase/Akt pathway (103, 104). It was recently reported that PA is required for activation of mTOR (105, 106). PA was shown to interact with mTOR competitively with rapamycin (105), and consistent with this hypothesis, elevated PLD activity confers resistance to rapamycin (107), further implicating mTOR as a downstream target of PLD. mTOR is a protein kinase that regulates both cell cycle progression and cell growth (108, 109). mTOR controls these processes by regulating translation, transcription, membrane traffic, and protein degradation (109). Since both PLD and mTOR have been implicated in survival signaling, and because there is a PLD requirement for mTOR activation, mTOR is a strong candidate for a critical downstream target of mitogenic PLD activity. mTOR is a particularly attractive target for mitogenic signaling through PLD, since it apparently integrates signals for both cell division and cell growth (109).

Vesicle Transport and Signaling

There is increasing evidence supporting a role for endocytosis and retrograde vesicle movement in signal transduction. Internalization of nerve growth factor and its receptor TrkA has been demonstrated to be required for transmission of NGF-mediated signals from distal axons to the cell body (110). Endocytosis of the EGF receptor is required for EGF-induced MAP kinase activation (111, 112). PLD has previously been implicated in vesicle budding and trafficking in Golgi membranes (reviewed in Refs. 113, 114). PLD1 is activated by ARF family of GTPases (19), which have likewise been implicated in vesicle budding in the Golgi (60) and receptor endocytosis (115). Thus, it was speculated previously that the role that PLD plays in the transduction of intracellular signals may be similar to the proposed role for ARF and PLD in vesicle transport from Golgi membranes—that being the stimulation of vesicle formation for receptor endocytosis (58, 95, 102). The activation of PLD by EGF is dependent on RalA (70, 71) and RalA was reported to be required for EGF-induced receptor endocytosis (95, 116). Importantly, blocking PLD activity prevented the activation of MAP kinase, but not MEK, the kinase that phosphorylates MAP kinase (95), indicating that receptor endocytosis was required for transduction of the mitogenic signals that activate MAP kinase. This observation was consistent with previous reports indicating that endocytosis is required for signals generated by EGF (111, 112). Similarly, BFA, which prevents insulin-induced PLD1 activation, inhibited MAP kinase activation and receptor internalization in response to insulin (62), suggesting that PLD activity also regulates endocytosis of the insulin receptor. PLD1 has been reported to be present in endosomes (117), and it was recently reported that the EGF receptor present in endosomes is still active (118), further supporting a role for vesicles in mitogenic signal transduction.

How PLD and its product PA might influence membrane topology and vesicle formation is not clear. A model was proposed recently whereby Ca^{2+} would aggregate the phosphate head group of PA to generate a curvature of the membrane that could initiate invagination of a vesicle (102). The generation of PA by PLD results in a significant change in both charge and pH at the membrane. The lower pH could result in the protonation of proteins so that they might be attracted to the negative charge of the phosphate on PA. The association of proteins with the PA-enriched membrane regions could initiate the changes in membrane topology that could ultimately result in the generation of an endocytic vesicle. Collectively, there is increasing evidence that receptor-mediated endocytosis is an important aspect of intracellular signal transduction and it has become clear that PLD plays a critical role in this process. The endocytosis requirement for MAP kinase activation coupled with the PA requirement for Raf recruitment suggests that PLD plays more than one role in the activation of MAP kinase—activation of MEK by Raf, and then endocytosis of the receptor to form an active "signaling endosome" where MAP kinase can be phosphorylated. This is represented schematically in Fig. 3.

Proliferation and Survival Signals Generated by PLD

Cooperation With Tyrosine Kinases in Cell Transformation

While a role for PLD in cell proliferation is indicated by several reports showing that PLD activity is elevated in response to mitogens and oncogenes, these studies by themselves did not demonstrate a PLD requirement for cell proliferation and/or transformation. However, elevated PLD has been shown to contribute to cell transformation and survival. v-Src, with its activated kinase, transforms fibroblasts in culture and elevates PLD activity (14). In contrast, elevated expression of c-Src does not, by itself, lead to transformation, nor does it elevate PLD activity (119). However, elevated expression of c-Src, in combination with elevated expression of either PLD1 or PLD2, transforms rat fibroblasts (96) and enhances cellular proliferation (100). Similarly, elevated

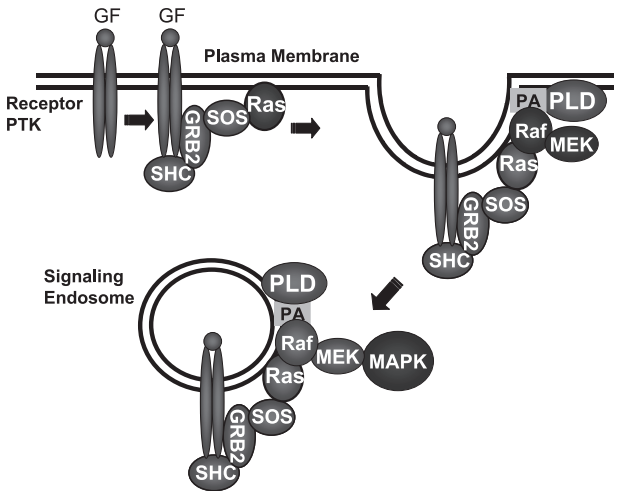

FIGURE 3. Growth factor stimulation of PLD-dependent Raf recruitment, receptor endocytosis, and MAP kinase activation. In response to growth factors, there is receptor dimerization and recruitment of the Ras activation molecules and the activation of PLD1 as shown in Fig. 1. Another downstream target of Ras is Raf kinase, which also needs PA for targeting to lipid rafts. This leads to the phosphorylation of MEK. MEK phosphorylates MAP kinase, but this phosphorylation requires endocytosis of the receptor, which is also dependent on PLD activity. Thus, endocytosis results in the formation of an active signaling endosome as shown.

expression of either PLD1 or PLD2 transformed rat fibroblasts overexpressing the EGF receptor (71, 96). Both PLD1 and PLD2 have also been reported to induce anchorage-independent growth and enhance cell cycle progression of mouse fibroblasts (99). Thus, while circumstantial evidence correlates PLD activity with cell proliferation signals, these studies indicate that PLD activity can actually contribute to proliferation and transformation.

PLD Prevents Cell Cycle Arrest and Apoptosis

In addition to enhancing cell proliferation, elevated PLD expression was also shown to be able to prevent cell cycle arrest and apoptosis. High-intensity Raf signaling induces cell senescence (120, 121) or apoptosis in the absence of serum (97). If expression of either PLD1 or PLD2 is elevated in these cells, they continue to proliferate and tolerate the high-intensity Raf signal (97). Similarly, fibroblasts overexpressing c-Src undergo apoptosis in response to growth factor deprivation, and both PLD1 and PLD2 were able to provide survival signals that prevented apoptosis (98). v-Src-transformed cells did not undergo apoptosis in the absence of serum; however, inhibiting PLD activity in these cells led to apoptosis (98). PLD activity has also been reported to overcome H_2O_2- (122) and glutamate (123)-induced apoptosis. These data indicate that in addition to enhancing cell proliferation, PLD provides a survival signal(s) that overcomes cell cycle arrest and default apoptotic programs.

PLD and Cell Cycle Regulation

The ability of PLD to cooperate with elevated expression of a tyrosine kinase is reminiscent of a model for transformation primary cells postulated by Weinberg and colleagues (124–127). In their model, a signaling oncogene such as Ras or Src will cooperate with Myc or large T antigen to transform primary cells (124, 126). It also resembles the model for cell cycle progression postulated by Stiles *et al.* (128) whereby two different factors were required for cell division, one that made cells "competent" and another that was required for "progression." In Fig. 4, complementing transforming genes are designated either Group 1 or Group 2 oncogenes, whereby the Group I signaling genes stimulate "competence" and passage through an early G_1 growth factor-dependent restriction point (129), sometimes referred to as G_1-pm for post-mitotic (130). The Group 1 genes also stimulate exit from a quiescent G_0 state induced by the absence of growth factors (131, 132). The absence of growth factors, by definition, stimulates exit to G_0 in the G_1-pm portion of G_1 before the restriction point, which is about 3–4 h in virtually all cells tested in culture (130). Group 1 genes such as an activated Ras gene provide growth factor independence and avoid cell cycle exit to G_0 and quiescence (131). Group 2 genes facilitate "progression" through cell cycle checkpoints and most importantly through the second part of G_1, sometimes referred to as G_1-ps for pre-S (130), and require cyclins that along with their partner kinases prevent the inhibitory function of Rb on cell cycle progression (133). In this oversimplified context, the ability of PLD to complement elevated expression of a tyrosine kinase (71, 96), which activates Ras proteins, would suggest that PLD genes could be class 2 (progression) genes that facilitate progression through cell cycle checkpoints. This would indicate that elevated PLD activity is able to provide what T antigen accomplishes. T antigen sequesters and inactivates p53 and Rb (134), both of which block passage through cell cycle checkpoints. Consistent with a role for PLD in progression through cell cycle checkpoints, preliminary studies indicate that elevated PLD activity is able to suppress p53 expression.[1] p53 also stimulates apoptosis when there is excessive damage to the cell, and PLD has been shown to prevent apoptosis in cells with high-intensity Raf signals (97) and in MDA-MB-231 breast cancer cells (98). Thus, the ability to suppress apoptosis may be due in part to the ability to suppress p53 expression. It is also of interest that Myc has also been reported to complement signaling oncogenes such as Ras in the transformation of primary rodent cells (124), since elevated PLD activity leads to elevated Myc expression (107).

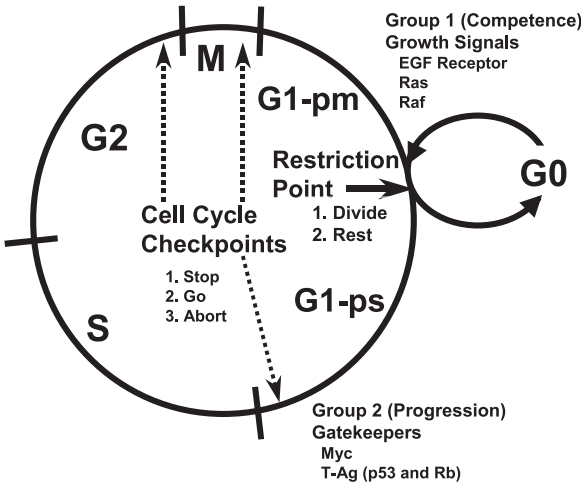

FIGURE 4. Cell cycle regulation by PLD. Two complementation groups for cell transformation based on a model of Weinberg and colleagues (124, 126, 127) and Stiles *et al.* (128) are proposed to regulate progression through different stages of the cell cycle. As shown, there are signals that facilitate passage through the restriction point or exit from a quiescent G_0 state (*Group 1*; *competence*), and there are signals necessary for passage through cell cycle checkpoints (*Group 2*; *progression*). PLD, like Myc and SV40 large T antigen (which inhibits the gatekeeper function of p53 and Rb), cooperates with Group 1 signals to transform rat fibroblasts. This places PLD in Group 2 which provides an override of gatekeeper function and facilitates passage through cell cycle checkpoints. The model is clearly an oversimplification since there is overlap in function between Group 1 and Group 2 genes including PLD, but it does reflect the ability of Group 1 genes to complement Group 2 genes to generate transformed rodent cells. Transformation of human cells requires additional alterations that provide immortality, usually achieved by the expression of the telomerase gene (126).

In addition to acting as a progression factor to allow passage through cell cycle checkpoints, PLD may also contribute to Group 1 competence signaling. The recruitment of Raf to PLD-generated PA (62) is likely important for activation of the MAP kinase pathway. Activation of the MAP kinase pathway is thought to be critical for exit from G_0 or passage through the restriction point. Similarly, receptor endocytosis is required for

[1] Hui and Foster, manuscript in preparation.

the activation of MAP kinase (95, 111, 112), and PLD is required for ligand-induced endocytosis of the EGF receptor (95). Thus, while PLD likely plays a critical role in facilitating progression through cell cycle checkpoints, PLD may also be required for the competence of cells to pass the restriction point and exit from G_0.

PLD and Cancer

PLD as an Alternative Survival Signal in Cancer Cells

The ability of PLD to suppress apoptosis in cells with elevated tyrosine kinase activity (98) and cooperate with tyrosine kinases to transform cells (71, 96) would make PLD a good candidate oncogene in cancers where elevated tyrosine kinase expression is common. Such a cancer is breast cancer where there is frequently elevated expression of tyrosine kinases such as the EGF receptor, Her2/Neu, and c-Src (135). Consistent with this possibility, elevated expression of PLD1 and elevated PLD activity was reported in breast cancer tissues (136, 137). PLD activity has also been reported to be elevated in gastric (138) and renal cancers (139) and a polymorphism of the *PLD2* gene was recently reported to be associated with the prevalence of colorectal cancer (140). We have examined the level of PLD activity in several breast cancer cell lines and find that PLD activity is similarly elevated in many of these cell lines (98, 107).[2] Interestingly, there is a strong, although not complete, correlation between elevated PLD activity and a loss of the estrogen receptor. This observation suggests that elevated PLD activity could be providing a survival signal that is normally provided by estrogen in a developing tumor. Consistent with this hypothesis, inhibiting PLD activity in MDA-MB-231 cells, a breast cancer cell line with highly elevated PLD activity, resulted in apoptosis (98). This was not observed for MCF-7 cells that are estrogen receptor positive and do not have highly elevated levels of PLD activity (98). Elevated PLD activity in the MDA-MB-231 cells also lead to rapamycin resistance (107), indicating that mTOR is affected by the elevated PLD activity in these cells. And more recent data indicate that a PLD/mTOR survival pathway can be distinguished from the PI-3-kinase/Akt pathway in breast cancer cell lines.[3] Thus, PLD-generated increases in PA that activate mTOR may generate a PI-3-kinase/Akt-independent survival signal or potentiate a weak PI-3-kinase/Akt signal. Since both mTOR and PLD can stimulate Myc expression (107, 141), it is possible that the PLD-generated survival signals are mediated in part by stimulating Myc expression, which like PLD, can cooperate with signaling oncogenes to transform primary cells (71, 96, 124, 126). Estrogen also stimulates Myc expression in the estrogen receptor-positive MCF-7 breast cancer cell line (142), and the induction of Myc by estrogen has been proposed to contribute to the mitogenic effects of estrogen in breast epithelia and in breast cancer progression (143). Thus, it could be hypothesized that the elevated PLD activity, which like estrogen, stimulates Myc expression in breast cancer cells (107), could contribute to loss of estrogen-dependent proliferation in tumor progression. This hypothesis, whereby PLD generates an mTOR-dependent survival signal that can lead to independence from estrogen or PI-3-kinase signaling, is represented schematically in Fig. 5. Interestingly, the two putative survival pathways, PLD/mTOR and PI-3-kinase/Akt, are connected by their dependence on PIP2 and PIP3—the levels of which are controlled in part by PI-3-kinase and PTEN (144). Thus, the control of these two survival pathways may be coordinately regulated through the level of phosphorylated phosphoinositides as indicated in Fig. 5. mTOR has been implicated as a key regulator of protein synthesis and cell growth and it has been proposed that regulators of the protein synthesis machinery may contribute to cancer (145).

Regulation of Metastasis

Tumor invasion is a compulsory step in metastasis and it has been proposed that invasion is actually dysregulated cell motility (146). Cell motility or migration is essential for many physiological processes such as embryogenesis, neurite outgrowth, and wound healing; however, inappropriate cell motility can cause calamities such as the spreading of cancers. Cytoskeleton networks, which include actin microfilaments, microtubules, and intermediate filaments, play a central role in

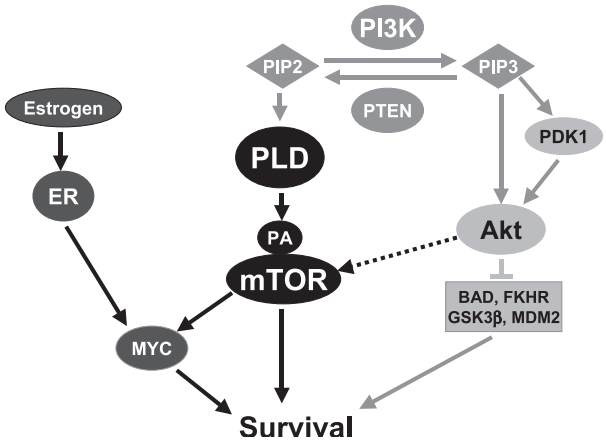

FIGURE 5. Proposed model for the generation of alternative survival signals in breast cancer cells. The PI-3-kinase/Akt survival pathway is shown on the right. PI3K generates PIP3, which recruits and activates Akt via phosphorylation by PDK1. Akt then phosphorylates and inactivates several substrate proteins that negatively regulate cell proliferation or stimulate apoptosis including: GSK3β, BAD, forkhead family transcription factors (*FKHR*), and MDM2 [reviewed by Vivanco and Sawyers (144)]. Akt also activates mTOR indirectly, which indicates that there is overlap in the two pathways. The PLD/mTOR pathway involves the generation of PA, which leads to the activation of mTOR. mTOR then phosphorylates several substrate proteins that regulate protein synthesis. It has been proposed that regulators of protein synthesis provide survival signals (145). The two pathways are also connected by their different lipid requirements. Akt requires PIP3 and PLD requires PIP2, thus the generation of PIP3 from PIP2 by PI3K would deplete PIP2, whereas PTEN would generate PIP2 from PIP3. Since both mTOR and PLD have been shown to induce Myc expression (107, 141), it is suggested that PLD-generated survival signals are mediated in part by elevated Myc expression. Of interest, is that estrogen also stimulates Myc expression in the estrogen receptor-positive breast cancer cells, which has been proposed to contribute to the mitogenic effects of estrogen. Thus, elevated PLD activity could contribute to loss of estrogen-dependent proliferation in tumor progression.

[2]Xu and Foster, unpublished data.
[3]Chen and Foster, Novel phospholipase D/mTOR survival signal in MDA-MB-231 human breast cancer cells, submitted for publication.

cell motility and have been subject to intense scrutiny in studies on tumor invasion (146). PLD activity has been shown to provoke the reorganization of actin cytoskeleton (21, 48, 49, 147, 148). PLD activity has also been implicated in cell motility (148) and PLD2 was shown to stimulate cell protrusions in v-Src-transformed cells (149), which is also consistent with a role for PLD in cell migration. Rho family GTPases, which activate PLD1 (5), have also been shown to regulate cell motility, and have been implicated in metastasis (50). PLD activity has also been implicated in tumor invasion (150, 151). Interestingly, MDA-MB-231 human breast cancer cells, which have very high levels of PLD activity (98), migrate and invade matrigel in culture, whereas MCF-7 breast cancer cells, with relatively low PLD activity (98), do not (152).

Protease secretion is also a property of invasive cancer cells (153) and PLD activity has also been correlated with elevated protease secretion (154–157). A dominant-negative mutant of RalA, which blocks PLD activity in v-Src- and v-Ras-transformed cells (68), also blocked protease secretion and tumor formation in the lungs of nude mice (154). Thus, the link of PLD with cytoskeletal organization and protease secretion suggest the likelihood that PLD contributes to cell motility and invasiveness and therefore may play an important role in metastasis of cancer cells.

Summary

Hanahan and Weinberg (158) described several hallmarks that a tumor must attain for a normal cell to become a malignant cancer cell. These include: (*a*) an active growth signal such as Ras to avoid quiescence; (*b*) insensitivity or suppression of anti-growth signals such as p53 and Rb to allow progression through cell cycle checkpoints; (*c*) suppression of default apoptosis programs by activated PI-3-kinase or suppression of PTEN; (*d*) immortality, which usually involves the activation of the telomerase gene, which avoids the destruction of the telomeres and cellular senescence; (*e*) the ability to invade the circulatory system, which usually involves increased protease secretion and cell motility; and (*f*) the ability to stimulate angiogenesis once a tumor gets to a critical size where added circulation is required for nutrition. It is therefore of interest that PLD contributes to four of these six hallmarks, that being: (*a*) growth signaling through Raf recruitment and receptor endocytosis (62, 95); (*b*) gatekeeper override, possibly through increased Myc expression (71, 96, 107) and suppression of p53 expression[4]; (*c*) suppression of apoptosis (97, 98) possibly through mTOR (105, 107) and suppression of p53; and (*e*) the ability to invade the circulatory system, which usually involves increased protease secretion and cell motility (148, 154, 157).

While much work remains to establish the molecular details as to how PLD contributes to transformation and tumor progression, the existing data strongly suggest that PLD plays a critical role in many aspects of cell proliferation, survival, and metastasis. The involvement of PLD in so many aspects of cell proliferation suggests that PLD or targets of PLD signaling could prove to be valuable targets for therapeutic intervention in cancers such as breast cancer, where a substantial percentage of tumors apparently have elevated PLD activity. The observation that blocking PLD activity in the MDA-MB-231 cells, where there is a very high level of PLD activity, leads to apoptosis (98) reinforces the notion that PLD is a promising target. The pharmaceutical industry has also taken notice (159).

[4]Chen, Hui, and Foster, unpublished results.

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