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Phospholipase D Meets Wnt Signaling: A New Target for Cancer Therapy

Dong Woo Kang[1], Kang-Yell Choi[2], and Do Sik Min[1,2]

Abstract

Phospholipase D (PLD) has been increasingly recognized as a critical regulator of cell proliferation and tumorigenesis. PLD regulates downstream effectors by generating phosphatidic acid (PA), and the expression and activity of PLD are elevated in many different types of human cancer. Aberrant activation of Wnt/β-catenin signaling, followed by hyper-activation of target genes, is linked to a wide range of cancers. New studies reveal a direct connection between the PLD and the Wnt signaling pathways; PLD is a transcriptional target of β-catenin/T-cell factor (TCF) and reinforces Wnt/β-catenin signaling related with cellular transformation. In this review, we discuss the emerging importance of PLD and PA in the Wnt/β-catenin signaling network, which is associated with tumorigenesis, and suggest that the PLD/PA signaling pathway is a potential therapeutic target for the treatment of cancer. *Cancer Res; 71(2); 293–7. ©2011 AACR.*

Background

The Wnt signaling pathway plays an important role in cell proliferation and differentiation, and its constitutive activation has been implicated in tumorigenesis of different cancer types (1). Wnt signaling leads to the stabilization of β-catenin, which activates T-cell factor (TCF)/LEF–dependent transcription of target genes. Mutations in genes of the Wnt/β-catenin pathway, particularly β-catenin and *APC*, are critical in human cancers (2). Downstream target genes of Wnt/β-catenin signaling are also frequently mutated in cancers, indicating the importance of investigating the target genes of the Wnt/β-catenin pathway to understand the development and progression of cancer.

Phospholipase D (PLD) catalyzes the hydrolysis of phosphatidylcholine (PC) to generate phosphatidic acid (PA), which consequently activates a signaling cascade. Two mammalian isoforms of PC-specific PLD, PLD1 and PLD2, have been identified and characterized (3). Although PLD1 and PLD2 show structural similarities, modes of activation and functional roles of these proteins are distinct. Our recent studies show that both PLD1 and PLD2 are novel target genes of β-catenin/TCF (4, 5). PLD and PA have been implicated in a diverse range of pathophysiologic processes, such as inflam-

mation and phagocytosis, diabetes, oncogenesis, and metastasis (3). In cancer research, PLD in particular has received special attention in the last several years. As a tumor-promoting second messenger, PLD-generated PA is at the center of critical signaling networks implicated in cancer. Exploration of the potential roles of PLD isoforms in tumor biology has only just begun, and our understanding of the mechanism responsible for the development of the disease is currently limited. New studies connecting PLD and Wnt/β-catenin/TCF will help shed light on our understanding of cancers associated with PLD signaling.

Phospholipase D and Cancer

A role for PLD in growth and survival of cells has been implicated by the observation that PLD activity is increased in response to mitogenic signals. Previous studies give evidence of PLD as the potential oncogene product in tumorigenesis. PLD is upregulated at the protein and/or activity levels in various cancers, including breast, colon, gastric, kidney, and thyroid cancer (6). Overexpression of PLD isozymes has been reported to induce anchorage-independent growth, tumor cell invasion, and the formation of metastases in syngeneic mice (7, 8). Significant correlation of PLD2 expression levels with tumor size and patient survival in colorectal carcinoma has been reported by recent clinical studies (6). Moreover, a polymorphism in the *PLD2* gene is associated with the increased risk of colorectal cancer (6). PLD2 point mutations have also been identified in breast cancer cells (6), and change of glutamine to alanine in PLD2 (Q163A) resulted in higher enzymatic activity and invasiveness in breast cancer cells, compared with those of wild-type of PLD2 (Y. H. Jang and D. S. Min, unpublished observations). PLD1 tends to be overexpressed in tumors expressing high levels of cytokeratins 5/17, markers of basal-like tumors, which are frequently associated

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with poor prognosis (9). These studies provide compelling evidence that elevated activity and expression of PLD observed in cancer are functionally linked with oncogenic signals and tumorigenesis.

Multiple protein targets, both upstream and downstream of PLD, have been linked to the propagation of survival signals and metastasis in the progression of cancer. PA can function as a membrane anchor for recruitment and/or activation of proteins that encode specific PA-binding domains, and can be converted to other bioactive molecules, such as diacylglycerol or lysophosphatidic acid. PA generated by growth factor–induced PLD activation interacts with the pleckstrin homology domain of SOS, a Ras guanine nucleotide exchange factor, and thus recruits SOS to the plasma membrane, involving Ras activation, which leads to cell transformation (10). Additionally, the ability of PA to bind to Raf may permit differential signaling through the Raf-MAP/ERK kinase (MEK)–extracellular signal regulated kinase (ERK) pathway rather than through other Ras effector pathways (11). One of the critical targets of PA is mTOR, known as a key regulator of cell growth. PA interacts with mTOR in a manner that is competitive with rapamycin, and as a consequence, elevated PLD activity confers rapamycin resistance, which could have important unintended clinical consequences (12). The apparent mechanism for the suppression of mTOR by rapamycin is to prevent the interaction between mTOR and PA, which facilitates the formation and/or stability of mTOR complexes (12). Reducing the level of PA could be the strategy for repression of the survival signal that suppresses apoptosis. The potential for PLD to impact the targeting mTOR in anticancer therapies argues strongly that serious attention be given to the role of PLD and PA in the regulation of mTOR (12).

In addition to its roles in modulation of signaling proteins, PA also affects the expression of genes, such as the suppression of proapoptotic or tumor suppressor genes (*p53*, *p21*, *Egr-1*, and *PTEN*) and the upregulation of antiapoptotic or survival genes (*Bcl2*, *cIAP*, *survivin*, *HIF-1α*, *MMP-2*, *-9*, *COX-2*, and *NOS-2*; refs. 4, 13–18). Considering the role for PLD in tumor progression, PLD inhibitors have emerged as potential anticancer drugs. Isoform-selective or dual-specific small molecular PLD inhibitors have recently been developed and characterized (19, 20). PLD inhibitors reduced invasiveness and anchorage-independent growth in metastatic breast and colorectal cancer models (4, 19). Therefore, PLD inhibitors have potential for use in the treatment of cancers directly or indirectly associated with PLD.

Modulation of Phospholipase D Expression in Cancer

Although aberrant PA signaling is observed in various disease states and increased expression of PLD plays an important role in cell proliferation and oncogenesis, the molecular mechanisms mediating PLD expression in cancer cells have just begun to emerge. Our group and other investigators have recently shown that PLD is subject to regulation at the level of transcription (21–24). Despite abundant infor-

mation about involvement of PLD activity in cell growth, its precise role and regulatory mechanisms for its target genes are poorly understood. PA activates NF-κB, which mediates activation of PLD1 transcription by mitogenic signals [phorbol myristate, platelet derived growth factor (PDGF), or epidermal growth factor] via its binding on the PLD1 promoter (Fig. 1A; refs. 21, 22). Thus, a positive feedback loop dependent on enzymatic activity of PLD isozymes selectively enhances expression of PLD1, but not PLD2.

The conversion of transient signaling to long-term effects involving cellular behavior is accomplished by altering the expression of target genes.

We have identified 2 functional PA-responsible NF-κB–binding elements on the PLD1 promoter (17), but not on the PLD2 promoter. PLD-dependent PA is at the center of critical signaling networks implicated in cancer. Ectopic expression of NF-κB increased PLD1 expression, but not PLD2 expression (17). Moreover, PDGF, a physiologic PLD activator, also enhances the binding of NF-κB to the identical NF-κB–binding sites of PLD1 promoter as PA, and the mutation of these sites abolished PDGF-induced PLD1 expression (Fig. 1A; ref. 22). PDGF-stimulated PLD1 induction increased matrix metalloproteinase (MMP)-2 or -9 and enhanced invasion of breast cancer cells (Fig. 1A; ref. 22). Therefore, it is suggested that PDGF-mediated induction of PLD1 might promote cancer progression by increasing MMP upregulation and that the compounds suppressing PLD expression might be useful for cancer therapy.

Expression of PLD1, but not PLD2, was induced by mutated *Ras* through binding of the Sp1 transcription factor (23), whereas Ewing's sarcoma fusion protein EWS/Fli induced selective PLD2 expression by binding to the ETS site of the PLD2 promoter (Fig. 1A; ref. 24). However, our new study shows that both PLD1 and PLD2 are transcriptionally induced by Wnt/β-catenin signaling via binding of TCF-4/β-catenin in their promoters (Fig. 1B; refs. 4, 5). Thus, differential expression of PLD isozymes might be mediated by usage of different transcription factors dependent upon the physiologic status of cells.

Because PLD inhibitors suppress growth factor–induced MMP upregulation and invasion via inhibition of PLD1 expression, we propose that autoregulation of PLD activity might be coupled to induction of PLD1 expression, and thereby play a role in tumorigenesis. Thus, PLD inhibitors may inhibit cancerous cell growth via inhibition of both expression and activity of PLD.

Phospholipase D Isozymes Are Direct Transcriptional Targets of Wnt/β-Catenin Signaling

The Wnt signaling pathway is constitutively activated in the majority of colorectal tumors as well as in other tumor types, leading to the transcriptional activation of target genes involved in cancer pathogenesis. Thus, identifying target genes for Wnt/β-catenin signaling could aid in the diagnosis and therapy of the disease.
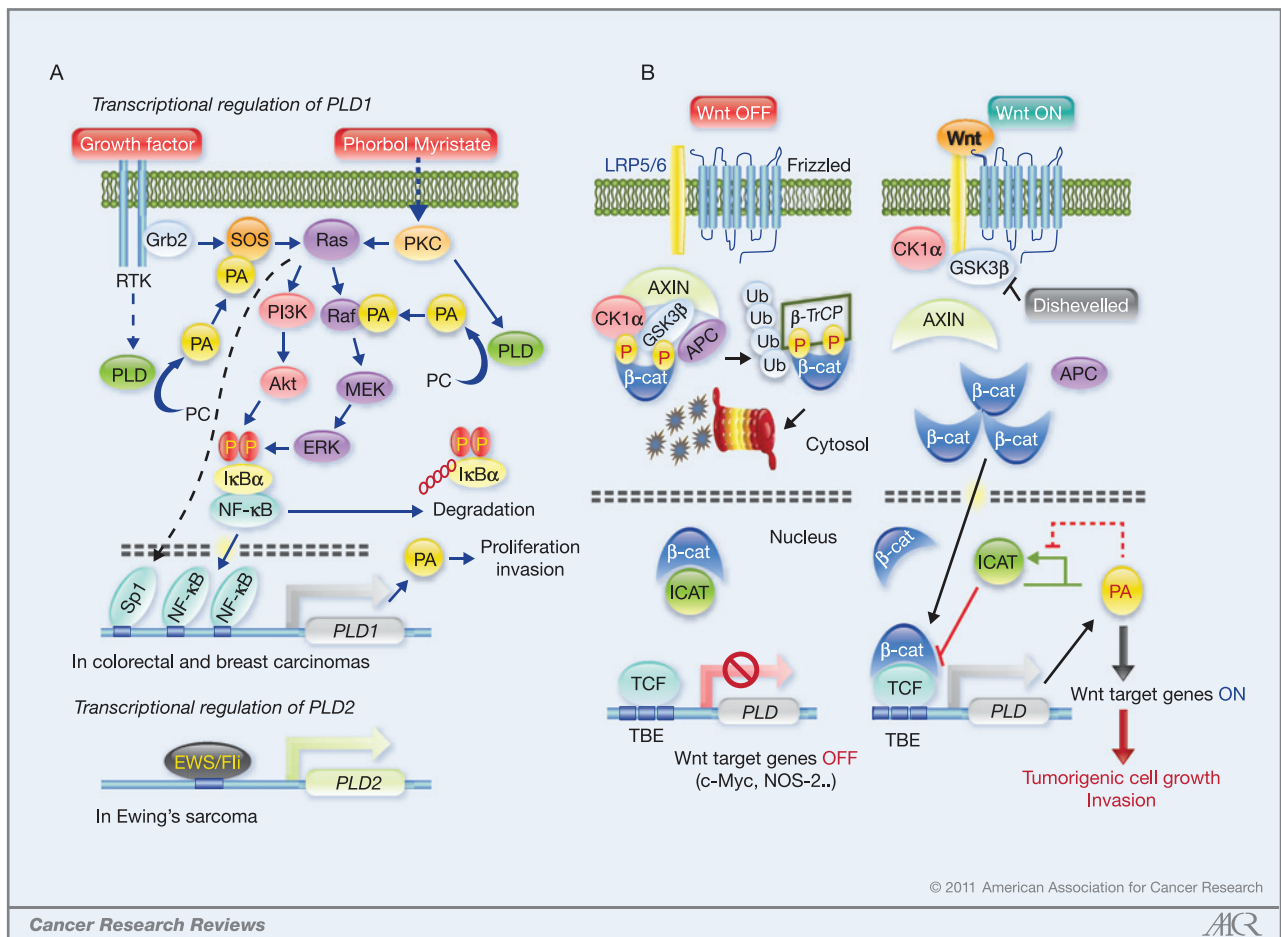
Figure 1. Growth factor– or Wnt signaling–induced PLD expression and role of PA in cancer. A, growth factor– or mitogen-induced PLD activation induces Ras/Raf/ERK/NF-κB or Ras/phosphoinositide 3-kinase (PI3K)/Akt/NF-κB signaling cascades and enhances binding of NF-κB to the PLD1 promoter. Active oncogenic Ras induces expression of PLD1 through binding of the Sp1 to the upstream PLD1 promoter. Increased PLD1 expression and subsequent PA production promote invasion of cancer cells. Ewing's sarcoma fusion protein EWS/Fli selectively induces PLD2 expression through binding to PLD2 promoter. B, in the absence of Wnt, cytoplasmic β-catenin is phosphorylated and then subsequently degraded. When Wnt binds to the frizzled receptors, formation of the "destruction complex" is inhibited and accumulated β-catenin is translocated into the nucleus, and forms the β-catenin/TCF complex. *PLD1* and *PLD2* genes are transcriptionally activated via binding of β-catenin/TCF to the TBE of its promoters. PLD-generated PA suppresses expression of ICAT, which inhibits the interaction of β-catenin/TCF-4, followed by promotion of Wnt/β-catenin/TCF signaling, and upregulates transcription of the target genes and ultimately stimulates tumorigenic cell growth.

More than 100 transcriptional target genes of Wnt/β-catenin signaling have been identified so far (see The Wnt Homepage, http://www.stanford.edu/~rnusse/wntwindow.html), and around 40 Wnt/β-catenin–signaling target genes are known to be associated with proliferation and cancer. Findings from our recent study show that PLD isozymes are transcriptional targets of Wnt/β-catenin signaling involving cellular transformation (Fig. 1B; refs. 4, 5). Ectopic expression of β-catenin and/or TCF-4 promoted the expression of PLDs, whereas the transfection of dominant negative TCF-4 or short hairpin RNA (shRNA) for β-catenin inhibited β-catenin/TCF-4–induced PLD upregulation. PLD1 and PLD2 promoters contain 3 and 2 functional TCF-4 binding elements (TBE), respectively; and that binding of β-catenin/TCF-4 to PLD promoters is enhanced by Wnt3a treatment. Moreover, expression of PLD was enhanced in various tissues including intestinal tissues of mice injected with the GSK3β inhibitor LiCl (5), indicating physiologic relevance of PLD expression by Wnt/β-catenin signaling *in vivo*.

Phospholipase D Activates Wnt/β-Catenin Signaling via a Positive Feedback Loop

Several Wnt target genes (*Gastrin*, *FGF*, *c-Met*, *Tiam1*, and *Pygopus*) are known to activate the Wnt/β-catenin signaling pathway (25–29). Gastrin, which plays a central role in the pathophysiology of gastrointestinal malignancies, enhances

β-catenin/TCF−dependent transcription activity through stabilization of β-catenin in colorectal cancer cells (25). FGF stabilizes β-catenin by downregulating GSK3β activity (26). The receptor for hepatocyte growth factor, c-MET induces tyrosine phosphorylation and nuclear accumulation of β-catenin (27). Rac1-specific activator, Tiam1, enhances β-catenin/TCF−dependent transcription activity (28). Pygopus induces activation of β-catenin/TCF target genes via association with β-catenin (29).

Using 2 RNA interference (RNAi)–based loss-of-function screens, Firestein and colleagues (30) identified genes that both modulate β-catenin activity and are essential for colon cancer cell proliferation; *PLD1* was one of the genes identified. We recently identified transcriptional activation of PLD by Wnt3a, and the second messenger PA produced by PLD further induced activation of the Wnt/β-catenin–signaling target genes via promotion of the β-catenin/TCF-4 transcription complex (4, 5). We found that the expression levels of PLD and β-catenin are significantly correlated in colorectal cancer tissues, which supports the presence of a strong link between the Wnt/β-catenin pathway and PLD *in vivo*.

Signaling pathways must be stringently controlled to ensure appropriate intensity and duration of signal required for the regulation of biological processes. Various signaling pathways have, therefore, evolved positive and negative feedback mechanisms. Several proteins are known to inhibit binding of β-catenin to TCF and subsequent target gene *trans*-activation (31). We found that PLD1 enhanced Wnt/β-catenin signaling via selective downregulation of ICAT (an inhibitor of β-catenin–interacting protein), a negative regulator of Wnt/β-catenin signaling (Fig. 1B; D.W. Kang and D.S. Min, unpublished observations). PLD activity is required for the downregulation of ICAT expression in several cancer cells (D.W. Kang and D.S. Min, unpublished observations). The PLD1 inhibitor suppressed association of β-catenin with TCF-4, and selectively enhanced expression of ICAT, but not Chibby, PPARγ, FOXO1, FOXO3, or plakoglobin (D.W. Kang and D.S. Min, unpublished observations), which are also known to interactively inhibit binding of β-catenin to TCF as negative regulators of Wnt signaling (32–35). Moreover, the PLD1

inhibitor selectively enhanced interaction of ICAT with β-catenin, but did not affect the binding of other negative regulators of Wnt signaling with β-catenin (D.W. Kang and D.S. Min, unpublished observations). Thus, it is suggested that ICAT is a selective molecular target of the PLD1-mediated Wnt/β- catenin signaling pathway. Expression of ICAT was abolished by PLD1, and *vice versa*, and PLD1-driven invasion of cancer cells is negatively regulated by ICAT, suggesting the presence of a PLD1-ICAT negative feedback loop. Thus, we propose that the PLD/PA signaling pathway promotes Wnt/β-catenin/TCF signaling via negative regulation of ICAT antagonizing Wnt signaling and could, therefore, emerge as a promising new target for cancer therapy (Fig. 1B).

Implications and Future Directions

Overall, we identified a bidirectional cross-talk between PLD and Wnt/β-catenin signaling pathways involving enhancement of cellular proliferation and transformation. The critical increments of PLD activities in various cancers strongly indicate the importance of cross-talk in tumorigenesis. Growing evidence showing involvement of PLD in development of cancer further strengthens the tumorigenic contributions of Wnt/β-catenin signaling. *In vivo* studies testing the anticancer efficacy of PLD inhibitors in animal models and subsequent clinical investigations will lead to the development of new potent anticancer drugs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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