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Phospholipase D2 stimulates cell protrusion in v-Src-transformed cells☆

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Abstract

Phospholipase D (PLD) activity has been implicated in several aspects of cell physiology including vesicle transport, signal transduction, cell proliferation, cytoskeletal structure, and oncogenic transformation. Two PLD isoforms (PLD1 and PLD2) have been identified and characterized. We have expressed both wild-type and catalytically inactive forms of PLD1 and PLD2 in 3Y1 rat fibroblasts and in 3Y1 cells transformed by v-Src, a tyrosine kinase that elevates PLD activity. The v-Src-transformed 3Y1 cells have small, but distinct cell protrusions, implicated in cell migration and metastasis. We report here that elevated expression of PLD2 substantially increased the length of the cell protrusions and that a catalytically inactive PLD2 mutant abolished the cell protrusions. The extended protrusions in the PLD2-overexpressing cells were dependent upon microtubule assembly. These data suggest a role for PLD2 in the v-Src-mediated formation of cell protrusions that may be critical for the invasive properties of v-Src-transformed cells.

Phospholipase D (PLD) has been reported to be overexpressed and hyperactivated in human cancers [1,2]. Consistent with these findings, it has been shown that PLD can facilitate oncogenic transformation [3,4] and cell survival [5]. PLD activity has also been implicated in tumor invasion [6–8]. Tumor invasion is a compulsory step in metastasis and has been proposed to be of dysregulated cell motility [9]. 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 cell motility. To achieve well-organized locomotion, it requires a precise coordination among these distinct networks [10–16]. Actin filaments are pivotal in cell polarity, contractile, and migration processes, and have been subject to intense scrutiny in studies on tumor invasion [9,12,17]. Microtubules are vital for intracellular trafficking of vesicles, organelles, and proteins; function in the dynamics of chromosome alignment and segregation during mitosis; and have also been found critical for cell polarity and the direction of cell migration [13,16]. It is thought that the actin cytoskeleton provides the driving force for the motile cells, while microtubules steer the cells and guide their movement in a directed manner [10–16]. There have been numerous reports of microtubule disrupting drugs arresting tumor invasion both in tissue culture and in tumor-transplanted animal studies [18–22].

Extracellular signals such as growth factors, cytokines, cell–cell, and cell–matrix interactions regulate cell motility through modulations on cytoskeleton components and their associated proteins and motors. Many of these signals are mediated by the non-receptor tyrosine kinase Src [23]. Src was found to localize along actin filaments and microtubules, as well as at focal adhesions and adherens junctions where both of these cytoskeletal networks interface [24]. At these sites, Src interacts with a broad range of downstream effectors that in turn control the processes of adhesion, migration, and invasion [23,25]. Src kinase activity and sometimes Src protein levels were found elevated in many types of cancer, with a correlation often observed between the increase in Src kinase activity and the degree of malignancy/invasiveness [24].

☆ *Abbreviations:* BtOH, butanol; PLD, phospholipase D.

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v-Src, a constitutively activated form of Src, efficiently transforms cells [24], and this is usually accompanied by an induction of cellular PLD activity [26,27]. Thus it is rational to speculate that PLD may mediate some of the Src effects in developing malignant phenotypes. PLD activity has been shown to provoke the reorganization of actin cytoskeleton [28–33]. However, it has not yet been determined whether microtubule dynamics is influenced by PLD. Data presented here demonstrate in cells transformed by v-Src, PLD2 stimulated microtubule extension, and cell protrusion.

Materials and methods

Materials. 1-Butanol (1-BtOH), *t*-BtOH, cytochalisins B and D, and colchicine were obtained from Sigma. [^{3}H]Myristate was from New England Nuclear. The secondary antibodies to rabbit or mouse immunoglobulin conjugated with horseradish peroxidase were from Bio-Rad. The anti-mouse immunoglobin conjugated with Rhodamine Red-X and the anti-rabbit immunoglobin conjugated with cyanine were from Jackson ImmunoResearch.

Cell culture conditions. Parental and 3Y1 rat fibroblasts transformed by v-Src (3Y1$^{v\text{-}Src}$ cells) have been described previously [34]. These cells and the stable or transient transfectants derived from them were maintained at 37 °C in a humidified 5% CO_2 atmosphere, in Dulbecco's modified Eagle's medium (DMEM) supplemented with 50 units/ml penicillin, 50 μg/ml streptomycin, and 10% (v/v) bovine calf serum. Cells were grown to 50% confluency and then made quiescent by replacing fresh DMEM containing 0.5% serum one day before the experiment.

Transfection and cell line establishment. 3Y1 and 3Y1$^{v\text{-}Src}$ cells that overexpress recombinant PLD proteins were obtained by transfection of plasmids encoding human PLD1, mouse PLD2 or the catalytically inactive mutants human PLD1-K898R or mouse PLD2-K758R, using LipofectAMINE Plus reagent (Life Technologies) according to vendor's instructions. Transient transfectants were made quiescent 24 h after transfection and used for experiments 48 h after transfection. Stable transfectants were obtained by cotransfection with the hygromycin B selection vector pCEP4 (Invitrogen). Transfected cultures were selected with hygromycin B (200 mg/ml) 24 h after transfection for 10–14 days. At that time antibiotic-resistant colonies were picked, amplified under selective conditions, and used for experiments. All of the PLD proteins expressed were Flu-tagged and could be detected using anti-HA antibody raised against the Flu epitope. Immunofluorescence assay of Flu-tagged PLD2 was performed as described previously [35].

PLD assays. Cells were grown in 60 mm culture dishes to confluency and made quiescent as described above. These quiescent cells were labeled for 4 h with [^{3}H]myristate (40 Ci/mmol) at a final concentration of 1 μCi/ml, followed by 15 min of incubation with 1% (v/v) 1-BtOH. Afterwards, cells were put on ice, washed twice with cold phosphate buffered saline, and collected in 0.5 ml of methanol/6 M HCl (50:1, v/v). Lipids were extracted by adding 0.5 ml of chloroform. Phase separation was achieved by the addition of 155 μl of 1 M NaCl and the organic phase was recovered after centrifugation. This was followed by reextraction through the addition of 350 μl H_2O, 115 μl 1 M NaCl, and 115 μl methanol. An aliquot of thus obtained organic phase was then counted in a liquid scintillation counter for normalization. Lipids of equal amount of radioactivity were dried under a stream of nitrogen and redissolved in 50 μl of chloroform/methanol (9:1, v/v). Samples were then spotted on a precoated silica (60 A) plates and separated by thin layer chromatography with a solvent system of ethylacetate/trimethylpentane/acetic acid/H_2O (9:5:2:10). The product of metabolically labeled PBt was visualized by autoradiography of the thin layer chromatography plates and the films were scanned on a densitometer for quantification.

Results

PLD2 mediates v-Src-induced cell protrusions in 3Y1 rat fibroblasts

Cultured 3Y1 rat fibroblasts have a well-spread and flat morphology, whereas 3Y1$^{v\text{-}Src}$ cells take on a more refractile rounded morphology with moderate filopodia-like protrusions or pseudopods (Fig. 1A). 3Y1 and 3Y1$^{v\text{-}Src}$ cell lines overexpressing either PLD2 (3Y1$^{v\text{-}Src}$-P2) or a catalytically inactive mutant of PLD2 (3Y1$^{v\text{-}Src}$-P2-K758R) were established as described in Materials and methods. As shown in Fig. 1A, overexpression of PLD2 strongly potentiated the protrusions in the v-Src-transformed cells. In contrast, catalytically inactive PLD2 abolished the protrusions in 3Y1$^{v\text{-}Src}$ cells (Fig. 1A). Neither wild-type nor catalytically inactive PLD2 had any observable effect upon cell protrusion in the non-transformed parental 3Y1 cells. Expression levels of the flu-tagged PLD2 proteins are shown in Fig. 1B, where it can be observed that very different levels of expression of PLD2 were tolerated in the different cell lines. The same differential expression level of PLD proteins was obtained in two additional sets of independently derived clones. Although there were differential levels of PLD2 expression in the 3Y1 and 3Y1$^{v\text{-}Src}$ cells, the highest levels of PLD2 expression were observed in the parental 3Y1 cells where no cell protrusion was observed. Thus, the effect of PLD2 expression upon cell protrusion was dependent upon the presence of v-Src.

To further establish that the effect of PLD2 overexpression upon the cell protrusions in the 3Y1$^{v\text{-}Src}$ cells was due to PLD activity, we examined the level of PLD activity in the 3Y1$^{v\text{-}Src}$, 3Y1$^{v\text{-}Src}$-P2, and 3Y1$^{v\text{-}Src}$-P2-K758R cells. As shown in Fig. 2A, the level of PLD activity in the cells was consistent with the extent of cell protrusion observed in Fig. 1A. The inhibitory effect of the catalytically inactive PLD2 on cell protrusion suggested that PLD2 was required for the protrusions in the 3Y1$^{v\text{-}Src}$ cells. We next employed the "alcohol trap assay" where primary alcohols are used to generate a phosphatidyl-alcohol rather than phosphatidic acid [35,36]. Secondary and tertiary alcohols with their bulky side groups do not insert into membrane properly and are therefore poor substrates in this assay and serve as negative controls. This assay has become to be increasingly used to establish a PLD requirement. As shown in Fig. 2B, the primary alcohol 1-BtOH abolished the protrusions in both the 3Y1$^{v\text{-}Src}$ and 3Y1$^{v\text{-}Src}$-P2 cells, whereas the tertiary alcohol *t*-BtOH had no effect on cell protrusions in these cells. These data indicate that the

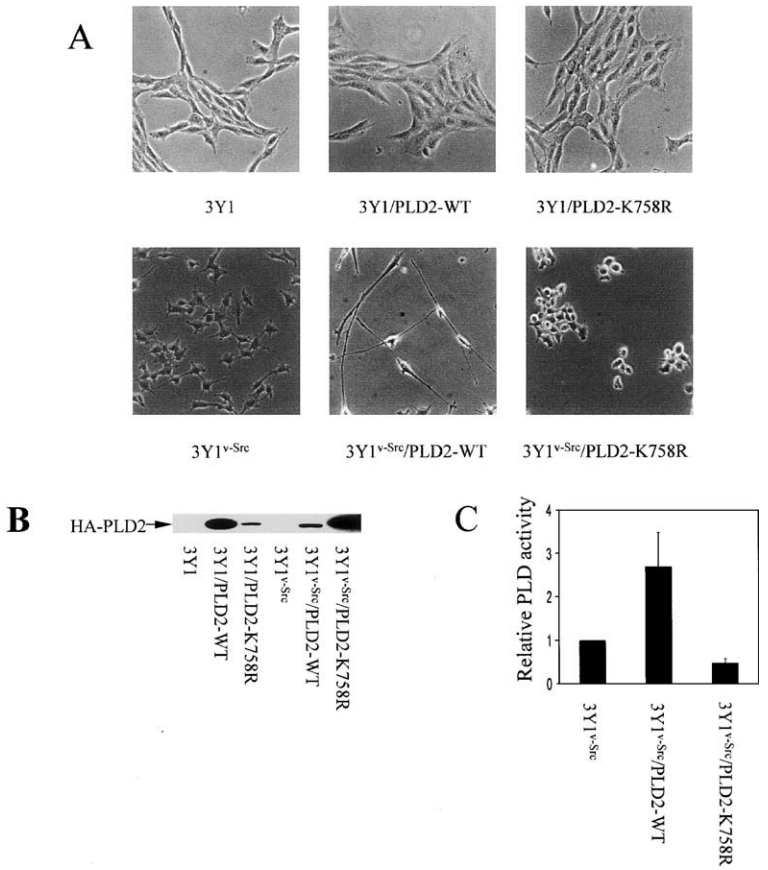

Fig. 1. PLD2 regulates v-Src-induced cell protrusion in 3Y1 cells. (A) Parental and v-Src-transformed 3Y1 cells, stably transfected with a flu-tagged PLD2 and catalytically inactive PLD2 expression vectors were analyzed for PLD2 expression by Western blot analysis using an antibody raised against the flu epitope. (B) The morphology of the cells characterized in (A) was examined microscopically. The data shown are representative of the morphology observed for two other independently derived clones. (C) PLD activity in the cells used in (A) and (B) was determined using the transphosphatidylation reaction and normalized to either the PLD activity in the 3Y1 cells or the $3Y1^{v\text{-}Src}$ cells. Cells were prelabeled with [^{3}H]myristate for 4 h and then incubated with 1-BtOH (1% v/v) for 15 min. Generated PBt was resolved by thin layer chromatography and visualized by autoradiography. The PBt was quantified by densitometry as described in Materials and methods. Error bars represent the standard deviation of three independent experiments performed in duplicate.

effects of PLD2 and the catalytically inactive PLD2 seen in Fig. 1 were due to the PLD activity and further indicate a requirement for PLD activity for the cell protrusions observed in the $3Y1^{v\text{-}Src}$ cells.

v-Src-induced cell protrusions are dependent upon microtubule assembly

To characterize cytoskeletal components responsible for the cell protrusions, we employed compounds that interfere with the polymerization of actin filaments or microtubules. Cytochalasin B, which blocks actin polymerization, rounded up the cell bodies but did not retract the cell protrusions in the $3Y1^{v\text{-}Src}$ cells overexpressing PLD2 (Fig. 3A). In contrast, colchicine, an inhibitor of microtubule polymerization, caused complete retraction of the protrusions without rounding up cell bodies (Fig. 3B). These data indicate that microtubule formation is required for maintaining the protrusions. The data do not rule out a role for actin in the formation of the protrusions, but do suggest that actin polymerization is not required for maintenance of the structure.

Discussion

In this report, we have provided evidence that cell protrusions observed in v-Src-transformed rat fibroblasts are microtubule-based structures and are dependent upon PLD2. Overexpressed PLD2 enhanced these structures and a dominant-negative PLD2 abolished them. These cell protrusions were dependent upon both v-Src and PLD2. PLD2 did not stimulate protrusions in non-transformed cells. Thus, while PLD2 is necessary for the cell protrusions, it is not sufficient and therefore Src is not stimulating these protrusions simply by activating PLD2.

At this point, it is not clear how v-Src and PLD are cooperating to generate cell protrusion, however, Src

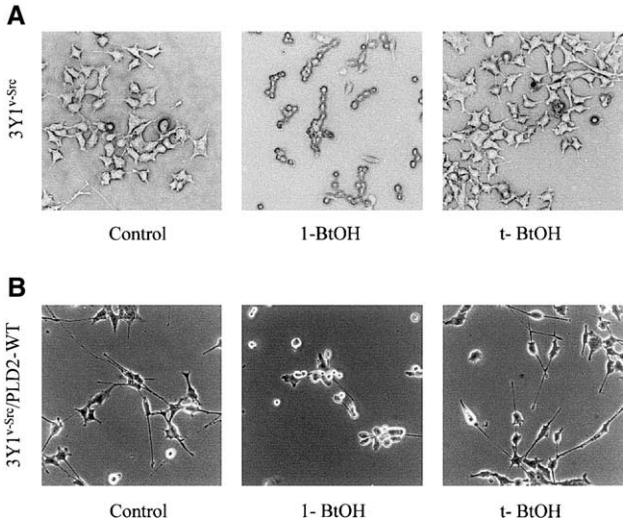

Fig. 2. v-Src-induced cell protrusions are dependent upon PLD activity. (A) $3Y1^{v\text{-}Src}$, $3Y1^{v\text{-}Src}$-P2, and $3Y1^{v\text{-}Src}$-P2-K758R cells were prelabeled with [^{3}H]myristate for 4 h and then incubated with 1-BtOH (1% v/v) for 15 min. Membrane lipids were subjected to thin layer chromatography and PBt levels were determined by densitometric analysis of autoradiographs as described in Materials and methods. PBt values (PLD activity) were normalized to the PLD activity in the $3Y1^{v\text{-}Src}$ cells, which was given a value of 1. Error bars represent the standard deviation for three independent experiments performed in duplicate. (B) $3Y1^{v\text{-}Src}$ and $3Y1^{v\text{-}Src}$-P2 cells were either untreated or treated with either 1-BtOH (primary alcohol) or *t*-BtOH (tertiary alcohol) (1% v/v) for 15 min at which the morphology of the cells was examined. Shown are fields revealing the morphology representing the majority of the cells. The data presented are representative results of an experiment that has been repeated many times.

has been shown to phosphorylate tubulin on tyrosine residues and this posttranslational modification of tubulin is thought to regulate microtubule dynamics [37]. Src deficiency can cause similar motility defects as those caused by microtubule depolymerization [11], indicating a role for Src in the growth of microtubules. Thus, Src could play a direct role in the formation of microtubule through interaction with and phosphorylation of microtubules.

Cell protrusion has been implicated in cell motility and metastasis [9,12,17]. We reported previously that RalA, a Ras family GTPase, was critical for metastasis of both Src- and Ras-transformed cells in a mouse model system [38]. RalA has also been implicated in signaling pathways that regulate invasion [39]. However, RalA interacts with PLD1, rather than PLD2 [40,41]. This raises a question on the specificity of PLD1 and PLD2. We did not see the extended protrusions in cells overexpressing PLD1 that were observed with PLD2. However, overexpression of PLD1 is not tolerated well by cells, and while we demonstrated previously that overexpressed PLD1 could cooperate with the EGF receptor to transform 3Y1 cells [4], these transformed cells frequently became highly vesiculated and stopped dividing [4]. Similar observations were seen in this study with PLD1 expression in the $3Y1^{v\text{-}Src}$ cells. Most of the v-Src-transformed cells that expressed high levels of PLD1 became heavily vesiculated. Thus, the accumulation of vesicles in cells overexpressing PLD1 could have masked any effect on cell protrusion. Therefore these data do not exclude a role for PLD1 in the generation of the cell protrusions reported here.

The data presented here demonstrated that inhibiting microtubule formation, retracted cell protrusions. Thus, continued microtubule assembly is apparently required for maintaining the cell protrusions in Src-transformed cells. Since it widely believed that the role of microtubules in cell migration is secondary to the driving force provided by actin filament formation [10–16], there is

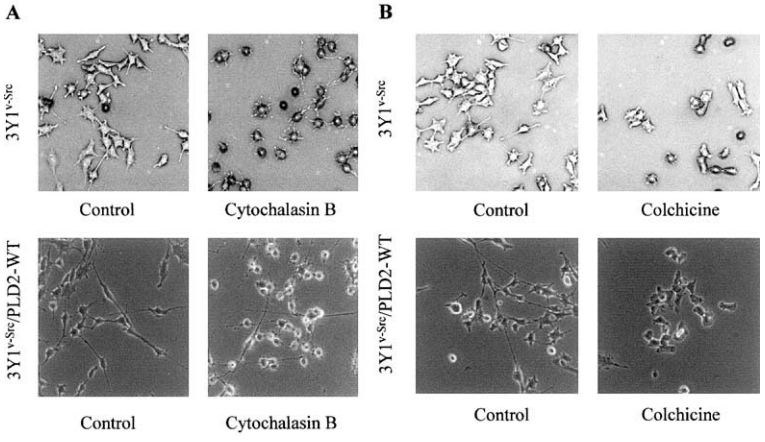

Fig. 3. Inhibition of microtubule formation retracts the v-Src-induced cell protrusions. (A) $3Y1^{v\text{-}Src}$ and $3Y1^{v\text{-}Src}$-P2 cells were treated either with DMSO (Control) or with cytochalasin B (15 μM in DMSO). After 30 min, the cells were visualized microscopically. Fields revealing the morphology of the majority of the cells are shown. (B) $3Y1^{v\text{-}Src}$ and $3Y1^{v\text{-}Src}$-P2 cells were treated either untreated (Control) or treated with colchicine (50 μM) for 30 min. Fields revealing the morphology of the majority of cells are shown. The data presented are representative results of experiments repeated three times.

likely a role for the actin cytoskeleton as well. Consistent with this hypothesis, PLD activity has been implicated in the reorganization of actin cytoskeleton [28–33,42]. Thus, it is possible that in v-Src-transformed cells, PLD2 may regulate both actin and microtubule cytoskeletal networks to manipulate morphological and migratory behaviors.

Tumor invasion is a multiple step process. First, tumor cells must recognize and interact with the surrounding extracellular matrix; second, they must degrade or remodel the extracellular matrix; and finally, tumor cells must migrate through the dissolved extracellular matrix [9]. PLD has been implicated in each of these steps. PLD activity was shown to correlate with the production of CD44, a transmembrane hyaluronan receptor that mediates cell–matrix interaction [43,44]. PLD was also reported to be required for the secretion of matrix metalloproteinases and urokinase plasminogen activator, proteases that dissolve the interstitial matrix, and basement membranes to allow invasion [38,45–47]. Data presented here provide evidence that PLD2 and v-Src, working together, lead to the generation of cell protrusions, which are implicated in cell motility and invasion. We speculate that the metastatic and invasive behaviors of v-Src-transformed cells may involve PLD2 and the generation of cell protrusions.

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