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PLD2 has both enzymatic and cell proliferation-inducing capabilities, that are differentially regulated by phosphorylation and dephosphorylation

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Abstract

Phospholipase D2 (PLD2) overexpression in mammalian cells results in cell transformation. We have hypothesized that this is due to an increase of *de novo* DNA synthesis. We show here that overexpression of PLD2-WT leads to an increased DNA synthesis, as measured by the expression levels of the proliferation markers PCNA, p27^{KIP1} and phospho-histone-3. The enhancing effect was even higher with phosphorylation-deficient PLD2-Y179F and PLD2-Y511F mutants. The mechanism for this did not involve the enzymatic activity of the lipase, but, rather, the presence of the protein tyrosine phosphatase CD45, as silencing with siRNA for CD45 abrogated the effect. The two Y→F mutants had in common a YxN consensus site that, in the phosphorylated counterparts, could be recognized by SH2-bearing proteins, such as Grb2. Even though Y179F and Y511F cannot bind Grb2, they could still find other protein partners, one of which, we have reasoned, could be CD45 itself. Affinity purified PLD2 is indeed activated by Grb2 and deactivated by CD45 *in vitro*. We concluded that phosphorylated PLD2, aided by Grb2, mediates lipase activity, whereas dephosphorylated PLD2 mediates an induction of cell proliferation, and the specific residues involved in this newly discovered regulation of PLD2 are Y¹⁷⁹ and Y⁵¹¹.

Keywords

PCNA; cell proliferation; phospholipase; tyrosine; phosphatase

INTRODUCTION

Phospholipase D (PLD) catalyzes the hydrolysis of phosphatidylcholine to generate the lipid second messenger phosphatidic acid (PA), and choline, in response to mitogenic signals such as PDGF, EGF, FGF, insulin, IGF, VEGF and serum [1-9]. The expression levels of the PLD gene are under developmental control and can also vary during the different stages of cell differentiation. Cell differentiation is accompanied with a very large increase in PLD2

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expression. It has been demonstrated that PLD can also contribute to increased cell transformation [10]. Rat fibroblasts are transformed during elevated expression of Src or EGF-R and overexpression of PLD1 or PLD2 [11-13]. PLD activity has been reported to be elevated in cells transformed by v-Src, v-Fps, v-Ras and v-Raf and tyrosyl-phosphorylated PLD2 has been reported [14-19]. In general, the phosphotyrosine motif *p*-YxN serves as docking sites for recruitment of SH2-domain containing proteins like the growth factor receptor bound protein 2 (Grb2) [20]. As indicated previously by our laboratory, PLD2 exists as a complex with Grb2 and PTP1b [21]. Also, Grb2 is found complexed with Sos *in vivo* [22-24] and PLD2 binds Grb2 and recruits Sos [25]. PLD-produced PA can bind directly to PLD [26] and it has been proposed that once activated, the cell could follow different paths alternatively during different stages of development: PLD2/Grb2/Sos and, through PA binding to Sos, resulting in either case in the activation of Ras and the ERK pathway [27]. Nevertheless, the signaling mechanisms that are in play for cell proliferation are not completely understood.

We have hypothesized that an increase in cell transformation in PLD2-overexpressing cells could be due to an increase of *de novo* DNA synthesis induced by PLD2. We uncovered an unexpected dual regulation by phosphorylation and dephosphorylation of lipase activity and proliferation-inducing capabilities of the human isoform PLD2 and show the specific tyrosine residues involved in those functions: Y¹⁷⁹ and Y⁵¹¹.

METHODS

Cells

COS-7 cells (ATCC) were initially seeded at 1×10^5 cells/well in 6-well tissue culture plates, in 2 ml D-MEM containing 10% FBS and non-essential amino acids. Cells were grown at 37 °C in a CO₂ incubator until they were 60-70% confluent (~36 h). Human breast cancer MCF-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM), phenol free, with 10% bovine calf serum.

Affinity purification of PLD2

Six well plates of COS-7 cells (~60% confluency) were transfected with pcDNA3.1-mycPLD2 (0.3 ml of a lipid-DNA complex with 2 µg pcDNA-mycPLD2 plasmid, 3 µl lipofectamine (Invitrogen, Carlsbad, CA) and 5 µl "plus" reagent (Invitrogen, Carlsbad, CA) in Opti-MEM (Invitrogen, Carlsbad, CA), previously mixed in sterile glass test tubes. After a 3-hour incubation, cells were washed and re-fed with DMEM/FBS complete media). After 36 hours, cells were harvested, washed 2x in PBS and lysed in lysis buffer (0.3% Triton X-100, 5 mM HEPES, pH 7.4, plus freshly added 1 µg/ml aprotinin, and 1 µg/ml leupeptin). The lysate (1.0-1.5 mg/ml protein) was sonicated, and the supernatant was applied to a 8 µl batch of α-myc agarose matrix (Santa Cruz, Santa Cruz, CA) and rotated for 2 hr at 4 °C. Immunocomplex beads were then washed with LiCl (100 mM Tris-HCl, pH 7.4, 400 mM LiCl) and NaCl (10 mM Tris-HCl, pH 7.4 100 mM NaCl, 1 mM EDTA) Wash Buffers. PLD2-bound α-myc agarose was resuspended in 100 µl of 5mM HEPES, pH 7.5. Reactions were split into 2 × 50 µl aliquots for various assays. Purity of purified PLD2 was assessed by immunoblotting. Samples were loaded onto a 4-20% gradient SDS gel and electrophoresed, transferred onto PVDF membrane and immunoblotted with 1:1,000 α-myc rabbit antibody and 1:3,000 Sheep anti-rabbit HRP IgG antibody. The immunoreactivity was visualized using ECL reagents (GE Healthcare, Piscataway, NJ).

Phospholipase activity

Measurement of lipase activity began with the addition of the following reagents (final concentrations): 3.5 mM PC8 phospholipid, 45 mM HEPES (pH 7.8), and 1 µCi [³H]n-Butanol in a liposome form, as indicated in [28,29]. Samples were incubated for 20 minutes at 30 °C

with continuous shaking. Addition of 0.3 ml ice-cold chloroform/methanol (1:2) stopped the reactions. Lipids were then isolated and resolved by thin layer chromatography. The amount of [³H]-PBut that co-migrated with PBut standards was measured by scintillation spectrometry. Control reactions lacking PC8 were used to remove background counts.

Cell proliferation markers

De novo DNA synthesis was quantified as protein expression of the proliferation marker PCNA (proliferating cell nuclear antigen) [30], using a specific purified anti-PCNA antibody (PC10, BioLegend, CA). Samples were further treated with FITC-conjugated secondary antibodies and observed by fluorescence microscopy. Positive nuclei were counted under the microscope at 16x objective in 5 different fields and averages were then calculated. P27^{KIP1} and phospho-histone-3 were monitored by their expression levels after PLD2 overexpression by immunoprecipitation and Western blotting using specific antibodies.

Preparation of phosphorylation-deficient PLD2 mutants

The construct pcDNA-mycPLD2 [26] was used as a template to create four Y→F point substitutions (Y179F, Y296F, Y415F and Y511F) following the QuickChange XL Site-Directed mutagenesis protocol (Stratagene, La Jolla, CA), using the following sets of primers: Y179F-sense: TCTTGACCATGTCTTTCTTTTCGAAACTACCATGCC, Y179F-antisense: GGCATGGTAGTTTCGAAAGAAAGACATGGTCAAGA; Y296F-sense: CTCAAGTGCAGCAGCTTTTCGGCAGGCACGGTGG, Y296F-antisense: CCACCGTGCCTGCCGAAAGCTGCTGCACTTGAG; Y415F-sense: GGCATCAACAGTGGCTTTAGCAAGAGGGCG, Y415F-antisense: CGCCCTCTTGCTAAAGCCACTGTTGATGCC; Y511F-sense: GGCTGGGCAAGGACTTCAGCAATCTTATCACC, Y511F-antisense: GGTGATAAGATTGCTGAAGTCCTTGCCCAGCC. All oligonucleotides and their reverse complements were PAGE/HPLC purified (Integrated DNA Technologies, Coralville, IA). Molecular identity of the three pcDNA-mycPLD2 mutants was confirmed by direct sequence analysis (Agencourt Bioscience Corporation, Beverly MA).

RESULTS

As indicated by several authors [11,12,32,33], PLD2 overexpression induces transformation. We began our experiments with the hypothesis that PLD2 expression would increase *de novo* DNA synthesis. We transfected COS-7 with the PLD2-wild type and saw that the cell proliferation marker PCNA (as positive stained nuclei) was elevated in PLD2-WT expressing cells with respect to mock controls (Figure 1A,B). PCNA is a nuclear protein whose expression is elevated when cells are committed to enter the S phase of the cell cycle as is taken here as an index for *de novo* DNA synthesis. As demonstrated in earlier work from our laboratory [25], PLD2 is regulated by phosphorylation, with the Y179 residue playing an important role. We have prepared phosphorylation-deficient mutant PLD2-Y179F as well as three new Y→F point mutants (PLD2-Y296F, PLD2-Y415F and PLD2-Y511F) targeting suspected sites of tyrosine kinase phosphorylation. Figure 1C-F shows that while PLD2-Y296F and PLD2-Y415F failed to elicit a proliferation response, PLD2-Y179F and PLD2-Y511F, show an increase in proliferation-inducing capabilities even larger than that observed for PLD2-WT. A quantification of PCNA positive nuclei with all PLD2 constructs is presented in Figure 1G. Additionally, Figure 3H presents two other cell cycle markers (for Y179F, as an example), p27^{KIP1} and phosphohistone. The figure shows an elevation of p27^{KIP1} expression in WT with respect to mock, and more so in Y179F, and an increase of phospho-histone expression with respect to mock of both PLD2-wt and PLD2-Y179F, all consistent with an induction of cell cycling as seen in cell proliferation.

We next investigated the possible mechanism that could govern the observed PLD2-proliferation inducing effect, first looking at the activity of the enzyme. Transfection of COS-7 cells with PLD2-WT led to a robust increase in lipase activity, as expected. Transfection with any of the four mutants resulted in a near-abrogation of PLD2 activity (Figure 2A), indicating that Y¹⁷⁹, Y²⁹⁶, Y⁴¹⁵ and Y⁵¹¹ may need to be phosphorylated in order for PLD2 to exhibit its high lipase activity. Comparing these results with those of Fig. 1, a preliminary correlation between PLD activity and induction of *de novo* DNA synthesis could not be derived, as Y179F and Y511F had enhancing effects on PCNA but had lost their enzymatic activity. We therefore explored other signaling mechanisms.

A close look at the PLD2 amino acid sequence indicated that the only trait that set PLD2-Y179F/PLD2-Y511F and PLD2-Y296F/PLD2-Y415F apart was that the former set had the two tyrosines within the consensus site YxN. This site is precisely recognized by SH2-bearing proteins, one of those being Grb2 [25]. However, PLD2-Y179F and PLD2-Y511F are phosphorylation-deficient mutants and cannot be phosphorylated, making binding to Grb2 unlikely. Nevertheless, nothing prevents those mutants from binding to other proteins, and we hypothesized that the protein phosphatases could be at play. The protein tyrosine phosphatase-1B has been reported to form a complex with PLD2 *in vivo* [21]. As Figure 2B presents, silencing CD45 expression with ds-RNA led to a significant decrease in PCNA induction of the Y→F mutants, but not in the wild type. This indicates that the proliferation-inducing capabilities of PLD2 are CD45-dependent.

We reasoned then that if de-phospho-PLD2 could associate with CD45, then the phospho-PLD2 form with Y¹⁷⁹ and Y⁵¹¹ residues fully phosphorylated within the pYxN consensus sites, would be targets for docking SH2-bearing proteins, such as Grb2. We tested this hypothesis in two systems: lysates from cells overexpressing PLD2-wt and Y→F mutants, and with a purified enzyme. In the first approach, lysates from PLD2-overexpressing cells were mixed with or without recombinant Grb2 and taken for PLD activity. As Figure 3A shows, Grb2 had a positive effect on the enzymatic activity in the wild type PLD2, but not in the PLD2-Y179F or PLD2-Y511F mutants, indicating that it is only the phosphorylated form that binds to Grb2. The PLD2 activity was expressed in cells as a phosphorylated enzyme [21].

Lastly, we utilized the same myc-pcDNA3-PLD2a-WT overexpression construct [31] in COS-7 cells and generated an immunoaffinity-purified enzyme taking advantage of the myc tag during immunoprecipitation and affinity-purification procedures. Figure 3B shows purity of PLD2. This preparation bears a relatively high level of lipase activity (~1,800 dpm/mg protein) indicating that the cells produced a constitutively active enzyme that retained its activity throughout the process of affinity purification and dialysis. The protein tyrosine phosphatase CD45 at 5 µg/ml caused a loss of activity of ~40% of the immunoaffinity purified PLD2 enzymatic activity (Figure 3C). The effect was abrogated with pretreatment of CD45 with a cocktail of phosphatase inhibitors. Conversely, the addition of recombinant Grb2 further increased the activity of the lipase. The positive effect of Grb2 on purified PLD2 is significantly larger than that seen in Fig 3A in crude cell lysates. This is probably due to that the basal level of the purified enzyme was not masked by co-factors left behind during purification/dialysis. As Figure 3C clearly indicates, the level of enzymatic activity is fully restored and dramatically inhibited with the exogenously added Grb2 or CD45, respectively.

DISCUSSION

The results presented in this study point at a dual phosphorylation-dephosphorylation mechanism that finely regulate PLD2. Grb2 could dock to tyrosine-phosphorylated PLD2 with the *p*-YxN motifs Y¹⁷⁹ and Y⁵¹¹. Conversely, these residues, when dephosphorylated, would mediate other biological activities, particularly and as shown here, *de novo* DNA synthesis.

Our laboratory has shown earlier that phosphorylated PLD2 complexes with Grb2 [21] through two newly defined SH2 recognition sites on PLD2 that in turn bind to Sos [25]. Importantly, PLD2-derived phosphatidic acid (PA) directly serves to modulate Sos and Ras GTP/GDP exchange that brings the PLD system to the Ras/MEK/ERK pathway [26]. As the Foster group has demonstrated [14,31] PLD2 overexpression induces DNA proliferation and cell transformation. We studied DNA *de novo* synthesis and thought initially that we could correlate enhanced lipase activity to a cell function. PLD2 was capable of increasing cell growth (as measured by PCNA-positively stained nuclei) in agreement with previous reports showing that PLD2 overexpression induces transformation of cells [11,12,33] and that two phosphorylation-deficient mutants, with low lipase activity had low proliferation-inducing actions.

However, PLD2-Y179F and PLD2-Y511F, furthered the proliferative inducing increase observed in PLD2-wild type and, as such, the regulation had to be more complex than initially thought. When fully phosphorylated, Y¹⁷⁹ and Y⁵¹¹ could be the site for binding of PLD2 to Grb2 as the two tyrosines are within a YxN SH2-recognition site (Figure 4A). The situation reverses itself when the residues are dephosphorylated as CD45 silencing inhibited PLD2-mediated proliferation. We have concluded that phosphorylated PLD2, aided by Grb2, mediates lipase activity, whereas dephosphorylated PLD2 mediates an induction of cell proliferation. The model presented in Fig. 4B incorporates the specific residues involved in this newly discovered regulation of PLD2: Y¹⁷⁹ and Y⁵¹¹.

This could be important for cell transformation, for example. PLD promotes cell proliferation and suppresses the default apoptotic programs that prevent cancer. PLD activity is further elevated in cancer cell lines in response to the stress of serum withdrawal [10]. Dephosphorylation of Y¹⁷⁹ or Y⁵¹¹ could be the signal for both decreasing PLD activity and concomitant high proliferation level that can help prevent the onset of cell transformation. Understanding signals activated in human cancers offers interesting therapeutic opportunities, since suppression of these signals (such as dephosphorylation) could theoretically lead to deactivation of proliferative capabilities of certain cancer cells.

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Figure 1. Study of DNA synthesis-inducing capabilities of PLD2 and four phosphorylation-deficient mutants

COS-7 cells were either mock transfected or transfected with PLD2-WT or each one of the four phosphorylation-deficient mutants (PLD2-Y179F, PLD2-Y296F, PLD2-Y415F and PLD2-Y511F) that were derived from myc-pcNA3-PLD2-WT by site-directed mutagenesis. *De novo* DNA synthesis was quantified as protein expression of the proliferation marker PCNA. Positive nuclei were counted under a fluorescence microscope in five separate fields. The (A-F) panels present one such representative field for each transfection. (G) Shows the quantitation of PCNA staining as manual counting of positive nuclei. (*) Denote difference between means that were statistically significant ($p < 0.05$) by ANOVA. (H) Western blots of

samples of transfected cells that were lysed, electrophoresed and immunoblotted with α -myc antibodies (as positive control of PLD2 expression), α -p27^{KIP1} or α -phospho-histone-3 antibodies.

Figure 2. Identification of the mechanism of PLD2-induced DNA synthesis

(A) Role of lipase activity. COS-7 cells were transfected with PLD2-wt or each of the four phosphorylation-deficient mutants for 48 hs, after which, cells were harvested and lysates used for PLD2 activity in PC8 liposomes and [3 H]n-butanol. Results represent mean $\pm$ SEM of 4 independent experiments in duplicate. 100% is equivalent to $3,328 \pm 290$ dpm/mg protein. ANOVA tests were performed and when statistically significant differences ($p < 0.05$) with respect to wild type were found, those points were denoted with an asterisk (*). (B) Effect of CD45 gene expression knockdown on PLD2-induced DNA synthesis. 150 nM CD45-siRNAs (a pool of 4 target-specific 20-25 nt siRNA from Santa Cruz Biotechnology, Inc.) or a negative control siRNA (scrambled sequence) were transfected into COS-7 cells for four days. During

the last two days, the cells were also transfected with the indicated PLD2 constructs. De novo DNA synthesis was quantified as protein expression of the proliferation marker PCNA. (*) denote difference between means that were statistically significant ($p < 0.05$) by ANOVA.

Figure 3. Cofactors needed for full PLD2 activity

(A) Study in transfected cells. COS-7 cells were transfected with the indicated constructs for 48 hs, after which, cells were harvested and lysates were mixed butanol in the presence or absence of recombinant Grb2 and used for PLD activity in PC8 liposomes and [3 H]n-butanol.

(B,C) Study with affinity purification of PLD2 and effect of phosphatase *in vitro*. (B) Western blot of α -myc immunopurified PLD2 from COS-7 cell lysates following transfection with pcDNA-mycPLD2-WT. The three lanes are: "load" (= crude homogenate), "flow thru" (= unbound protein) and "elution" (= dialyzed, purified PLD2).

(C) Immunoaffinity purified PLD2 was treated *in vitro* with 5 μ g/ml CD45 in 25 mM Tris-HCl, pH 7.6 (from stocks of 50 mU/ μ g, one unit capable of hydrolyzing 1 μ mol of phosphopeptide substrate/min at pH 7.2); with CD45 and a cocktail of phosphatase inhibitor-II (Sigma, St Louis, MO) at a 1:10 dilution; or with recombinant Grb2 (0.1 μ g/ml), for 30 min at 30 $^{\circ}$ C. Samples were then processed for PLD2 activity. Results represent mean $\pm$ SEM of 3 independent experiments in duplicate. (*) denotes statistically significant differences ($p < 0.05$) (ANOVA) between controls and treated samples.

A

151 GPEGSTRHAASKQKYLENYLNCLLTMSFYRNYHAMTEFLEVSQLSFIPDL
 201 GRKGLEGMIRKRSGGHRVPGLTCCGRDQVCYRWSKRWL VVKDSFLLYMCL
 251 ETGAISFVQLFDPGFQVQVGRSTEARHGVRIDTSHRSLILKCSSYRQAR
 301 WWAQEITELAQQPGRDFLQLHRHDSYAPPRPGTLARWVNGAGYFAAVAD
 351 AILRAQEEIFITDW WLSPEVYLKRPAHSDDWRLDIMLKRKAEEGVRSIL
 401 LFKEVELALGINSGYSKRALMLLHPNIKVMRHPDQVTLWA HHEKLLVVDQ
 451 VVAFLGGLDLAYGRWDDLHYRLTDLGDSSESAASQPPTPRPDSPATPDLS
 501 HNQFFWLGKDYSNLITKDWVQLDRPFEDFIDRETTTPRMPWRDVGVVVHGL

B **Figure 4. Model**

(A) Partial amino acid sequence of PLD2 indicating the four Y targets of this study. Underlined are the two tyrosines within a *YxN* consensus site from which the PLD2-Y179F and PLD2-511F mutants were derived. (B) Proposed model that takes into account the results presented in this study. Phosphorylated PLD2, aided by Grb2, mediates lipase activity, whereas dephosphorylated PLD2 mediates an induction of cell proliferation and the specific residues involved in this mechanism are Y¹⁷⁹ and Y⁵¹¹.