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INCREASED CELL GROWTH DUE TO A NEW LIPASE-GEF (PHOSPHOLIPASE D2) FASTLY ACTING ON RAS

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Abstract

We report the novel finding that Phospholipase D2 (PLD2), through its PX and PH domains, binds specifically to Ras and catalyzes the GDP/GTP exchange (i.e., is a GEF), with potency comparable to Ras-GEF-1, a known Ras-GEF. Cells overexpressing PLD2-GEF inactive mutants (F129Y and R172C/L173A) fail to stimulate cell proliferation compared to the wild type-expressing cells. The GEF effect on Ras follows a faster kinetics than other GTPase substrates (such as Rac2 or Rac1) and is a better substrate, too. The GEF action is due to PLD2 (protein) itself, independently of the lipase product PA. PA can still have a fine-tuning regulatory effect on Ras-GTP depending upon its cellular concentration. Rapidly growing cancer cells MDA-MB 231 (but not the slow growing MCF7 counterpart) have high levels of endogenous PLD2-GEF which correlates with high Ras activation. The PLD2-“GEF” activity is even higher than the classical “lipase” activity and is abrogated with GEF single point mutants, particularly F129Y, and concomitantly with a slow rate of cell growth. This can be crucial to cancer biology in that not only Ras mutations explain abnormal growth, but the existence of a new GEF for Ras: a GEF molecule that happens to be a phospholipase.

Keywords

breast cancer; cell proliferation; GEF; phospholipase; Ras

INTRODUCTION

Signal transduction via the Ras-Raf-MEK-MAPK pathway is involved in cell cycle regulation and cell proliferation in response to mitogens [1–4]. This pathway is also key to cell differentiation, cell adhesion, apoptosis and cell migration, as well as tumorigenesis [5–8]. Mutations in specific *ras* genes, most frequently *H-ras*, *K-ras* and *N-ras*, can generate permanently activated Ras proteins [8]. Overactive Ras can put the growth signaling pathway in an “always on” state, even in the absence of incoming signals [9]. These signals could result in uncontrolled cell growth and division, and as such, overactive Ras signaling can ultimately lead to cancer [10, 11].

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As a G-protein, Ras cycles between the active GTP-bound and inactive GDP-bound forms [12]. The process of exchanging the bound nucleotide is facilitated by guanine nucleotide exchange factors (GEFs) that lead to an efficient yield of Ras-GTP from Ras-GDP. Inactivation of Ras-GTP is accomplished by the presence of GTPase activating proteins (GAPs) that leads to hydrolysis of bound GTP and substantially aids Ras intrinsic GTPase activity.

Sos1 and RasGRF1 are the most widely expressed Ras GEFs in mammalian cells [13–15]. They have a modular architecture comprised (from N-terminus) of Dbl homology (DH), pleckstrin homology (PH) and Cdc25 domains [16]. The DH/PH tandem is shared with other GEFs that act upon GTPases of Rho family members such as Vav1 and Tiam1. Recently, our laboratory has reported that the phospholipid lipase PLD2 can act as a GEF for Rac2 [17, 18] effectively binding to it and catalyzing the GDP/GTP exchange both *in vitro* and *in vivo*. PLD2 is also a GEF for RhoA, a protein closely related to Rac2 [19].

PLD2 lipase activity (PA production) is increased during cell proliferation, [20, 21] but whether the newly described activity (PLD2-GEF) is needed in highly proliferative cancer cells has not been addressed. We show the novel finding that PLD2 binds to Ras and is a GEF for this GTPase. The GEF activity of PLD2 is related to cell growth and more so than its lipase (PA production) activity. Further, cancer cell lines that show high levels of proliferation have high levels of PLD2-GEF and hence abnormal Ras activation. Thus in addition to the known Ras mutations that keep the molecule in the GTP-bound state, we are now reporting that PLD2-GEF plays a crucial role in abnormally increased cell growth.

RESULTS

PLD2 binds to Ras via a protein: protein interaction and increases Ras-GTP binding

Cells overexpressing PLD2 show a higher level of cell proliferation than their mock-transfected counterpart (Fig 1A). This enhancing effect of PLD2 is prevented if Ras is silenced with siRNA, which indicates a dependency of PLD2 on Ras for this cell function. In addition (the product of PLD2 lipase catalysis of PC), since cells overexpressing a lipase-inactive mutant (PLD2-K758R) still can proliferate (Fig 1A) suggesting that PLD2-mediated proliferation is only partially due to the presence of PA. Further, exogenous DOPA (a membrane-soluble form of PA) added to the culture media can induce faster growth at low concentrations (1–10 nM) but acts as an inhibitor at higher concentrations. Likewise, PA has a biphasic effect on Ras GTP loading *in vitro* (Fig 1B). Understandably PA exerts its *in vitro* actions at concentrations lower than those needed for *in vivo* (Fig 1A). Nevertheless, PA at >3 nM, a concentration that would be expected in cells overexpressing PLD2 constructs, has a negative effect on Ras-GTP. Clearly, the lipase action of PLD2 cannot adequately explain the enhanced cell proliferation of Fig. 1 (control bars). We reasoned that PLD2's other activity, the recently described GEF activity, had to be at play during cell proliferation.

Overexpression of cells with PLD2 leads to a potent increase in Ras-GTP that peaks at 24 hours after transfection (Fig 1C). Fig 1D indicates that co-transfection of PLD2 plus Ras yields a robust increase in Ras activation (top panel, or “RBD pulldown” that measures the presence of Ras-GTP species); the other three panels in Fig 1D are controls indicating appropriate expression of relevant proteins and equal loading of gels as seen in Western blots using an anti-actin antibody.

PLD2 is a GEF for Ras

The previous results are highly indicative of PLD2 being a GEF for Ras. To show this definitively, we assayed the GTP/GDP exchange reaction in the presence or absence of PLD2 at different time points. Fig 2A indicates that PLD2 exerts a GDP dissociation of Ras

with potency comparable to that of the known GEF for Ras, RasGRF1. Importantly, PLD2GEF uses Ras with faster kinetics than in the case of other small GTPases (Rac2 or Rac1). This indicates that the Ras pathway can be activated immediately upon cell activation of PLD2 within the cell membrane, which is a great biological advantage. Similarly, PLD2 exerts GTP binding on Ras in a time dependent manner, also similar in potency to RasGRF1 (Fig 2B). Results in Fig 2, thus, indicate that PLD2 catalyzes the GTP/GDP exchange on Ras, that is, it is a GEF for the small GTPase Ras. This is independent of its lipase activity product, PA, as the GEF effect is shown in Fig 2 A, B was observed with recombinant PLD2 in *in vitro* reactions. This was accomplished in the absence of PC (the PLD2-lipase substrate, thus ensuring that *de novo* PA was not generated).

The PLD2-PX and -PH domains mediate binding to Ras

That PLD2 is a GEF for Ras presupposes that PLD2 interacts with the GTPase, as it becomes its substrate. To prove this definitively, we conducted immunoprecipitation experiments. A PLD2-Ras protein-protein interaction could be demonstrated (Fig 3) utilizing an array of biological reagents with different tags as summarized in Fig 3A and prepared specifically for this study. PLD2-Ras binding is shown in the second panel of Fig 3B, as Ras was detected with anti-myc antibodies in anti-HA immunoprecipitates. The first and second panels of Fig 3B serve as positive controls for the expression of the relevant proteins, whereas the last panel with actin antibodies is a further control that indicates equal loading of protein in the gels. These immunoprecipitation experiments were conducted in parallel with cells overexpressing Rac2 that served as controls. PLD2-Rac2 binding in Fig 3B (third panel) that our laboratory has previously demonstrated is used as positive control [22, 23].

We next demonstrate that the domains on PLD2 that are implicated in the binding to Ras are PH and PX. Binding between PX/PH and Ras was demonstrated by GST-pulldown experiments, which were performed using GST-PH or GST-PXPH fusion proteins, overexpressed in *E. coli* and affinity purified. As shown in Fig 3C and 3D Ras binds to GST-PH or GST-PXPH at a higher level to that of Rac2, which is a novel finding. Of note, Ras binds strongly to PLD2-GEF than Rac2 does, judging for the intensity level of protein bands in Fig. 3B,C and D, in all cases.

PLD2-GEF catalyzes the GTP/GDP exchange and activation of Ras

Going back to the main biological subject of this study, cell proliferation, we sought to conclusively demonstrate that GEF-PLD2 could account for the Ras-mediated increase in this cellular function. For this, we made use of GEF-PLD2 mutants that involve point mutations in amino acids F107, F129, L166, R172 and L173 on the PX domain (Fig 4A), where we believe the catalytic site resides, as these amino acids are located in highly hydrophobic alpha-helices. Fig. 4B indicates that the representative GEF-PLD2 mutants are GEF inactive and Fig 4C indicates that cells overexpressing these mutations are unable to proliferate as much as that of PLD2 wild-type.

Single point mutations on GEF activity, uncovered in this study for the first time, that lead to slower rate of cell growth

Cell proliferation as assayed by PCNA presence shows that PCNA staining is significantly lower in the case of the GEF-deficient mutants when compared to wild-type controls (Fig 5A), further providing evidence of a key role for PLD2-GEF in cell proliferation. In agreement with this, we have also uncovered that there is a correlation between cell growth (measured as cell number doubling time) (Fig 5B) and the levels of endogenous PLD2 GEF activity (Fig 5C, the lower the bar, the higher the GEF activity) and Ras activation (Fig 5D) in the MDA-MB-231 and MCF-7 cancer cell lines. In a breast cancer cell line known to

have aggressive growth and high metastatic potential, MDA-MB-231, cell growth and Ras activation are both elevated when compared to MCF-7 cells that display low growth/low metastatic potential.

Of most importance, when slow growing MCF-7 cells were transiently transfected with PLD2-GEF (wild type) cell growth was increased by a factor of ~2, which was prevented with cells expressing the GEF mutant F129Y (Figure 5E). This gain-of-function for the GEF wild type and loss-of-function for the GEF mutant was also observed in MDA-MB-231 (Figure 5E), albeit at a lesser extent, due probably that these cells have already reached a regular rate of rapid cell growth that can not be easily increased (but in can be decreased with the mutant Y129F. Thus, PLD2-GEF catalyzes the GTP/GDP exchange and activation of Ras, which is high in highly proliferative and metastatic cell lines. This is summarized in the model of Figure 6.

DISCUSSION

As far as we know, this is the first description of Ras activity being enhanced by a lipase acting as a GEF. This new dual lipase GEF is different from the other classical GEFs that Ras is known to associate with, such as members of the Sos and RasGRF1 families, in that none of those are a phospholipase or have dual activities. The fact that PLD2 has a PH domain that can anchor it to the cell membrane could facilitate the recruitment of Ras to the same environment where the signal transduction between an activated receptor and effectors, mediated by PLD2-GEF, could proceed quickly and efficiently.

Also in this report, by found the unexpected result that Ras-GTP production by PLD2-GEF is being “fine-tuned” by PA as a biphasic effector depending entirely upon its cellular concentration. This role for PA acts directly on Ras itself, not necessarily on its GEF, as Fig 1 shows an effect directly on the amount of GTP loaded onto Ras. The mechanism for this could be based on the earlier observations that PA binds to a related small GTPase, Rac2 [17, 18]. The increment of GTP loaded onto Ras we have found following low PA concentrations is possibly due as a consequence of this binding event mediated by PA, which possibly serves as an allosteric modulator that increases the affinity of the GTPase for GTP. Conversely, at high concentrations or late times of cellular stimulation, PA negatively affects Ras GTP loading possibly by interfering with the binding of GEF-PLD2 to its substrate, Ras.

The importance of the findings reported here is the explanation of increased cell growth (as it occurs in cancer cells) based on the elevated presence and activity of a new GEF for Ras, PLD2-GEF. PLD2 has been traditionally thought of as an enzyme converting PC into PA in cellular membranes, but recent reports have indicated a new GEF activity of Rho GTPases [17, 19]. PLD2 and its lipase activity have been shown before to be elevated in cancer cells [18, 24, 25] and now we report that its GEF activity is greatly elevated in rapidly growing/highly metastatic cancer cells, such as COS-7.

That Ras can be turned on by this new GEF serves to demonstrate that, in addition to mutated Ras that accounts for many forms of abnormally high cell proliferation, the presence of GEF-PLD2 could also play a major role in increased cell growth. In fact, we have uncovered the particular site (F129) that when mutated, abrogates GEF activity and slows down cell growth. We believe that all this have direct implications in trying to manage uncontrolled cell growth (such as cancer) where both GEF-PLD2 and Ras activities are upregulated.

METHODS

Chemicals

Anti-HA and anti-myc IgG antibodies were from Cell Signaling (Danvers, MA). Rhotekin-binding domain (RBD)-agarose was from Millipore (Billerica, MA). [^3H]-guanosine 5'-diphosphate and [^{35}S]-guanosine [γ -thio]-5'-triphosphate were from Perkin-Elmer (Waltham, MA). The RasGRF1 plasmid was from Origene (Rockville, MD). The PLD2 mutant plasmids (pcDNA3.1-mycPLD2- F129Y and -R172C/L173A) were from Mutagenex (Piscataway, NJ).

Cell proliferation assay

COS-7 cells were plated into 24-well plates 24 hr prior to use. Duplicate wells were either mock-silenced or silenced with 300 nM si-RNA specific for Ras and were also simultaneously untransfected or transfected with 2 μg PLD2-WT or PLD-K758R and cell counts were assayed at several time intervals. After incubation, cells were washed 2 $\times$ with PBS and trypsinized and final volume brought up to 1 ml/well using complete DMEM containing 10% FCS. Fifty μl of trypan blue was added to each cell sample and viable cells counted/ml cells $\pm$ SEM.

Ras RBD Pulldown Assays

Ras PBD Pulldown assays were also conducted with purified, recombinant (Ras and PLD2 fusion) proteins to measure the activation of Ras. Five μl of Rhotekin-RBD-agarose was added to each sample and incubated at 4 $^{\circ}\text{C}$ for 30 min in the presence of Magnesium Lysis Buffer (25 mM HEPES, 150 mM NaCl, 1% Igepal CA-630, 10 mM MgCl_2 , 1 mM EDTA and 2% glycerol). Samples were loaded onto gels, transferred to blotting membranes and probed with α -myc antibodies to detect recombinant, GTP-bound Ras. HRP-conjugated secondary antibodies were incubated with PVDFs and products visualized using ECL reagents.

Immunoprecipitation, SDS-PAGE and Western blot analyses

To confirm the presence of overexpressed protein, we performed SDS-PAGE and western blot analyses of myc- or HA-tagged proteins that were overexpressed in COS-7 cells. Two days post-transfection proteins were immunoprecipitated using anti-myc or anti-HA antibodies. Samples were analyzed by SDS-PAGE and Western blot analysis to confirm the presence of PLD2 or Ras proteins in the cell lysates. Immunoprecipitated proteins were also used to measure relative GDP dissociation from Ras.

GEF Activity: GDP dissociation from Ras

To examine the effect of PLD2 or RasGRF1 on [^3H]-GDP dissociation from Ras, 19 pmol of Ras was preloaded with 2 μM [^3H]-GDP. Simultaneously, PLD2 or RasGRF1 was incubated for 10 min in 20 mM Tris-HCl pH 7.5, 0.1 mM DTT, 80 mM NaCl, 0.5 mM MgCl_2 , 0.8 mM AMP-PNP and 1 mM GTP (80 μl volume). PLD2-WT (19 pmol) or Ras alone were used as positive and controls, respectively. Preloaded [^3H]-GDP bound Rac2 samples were mixed with buffer containing PLD2 or RasGRF1 alone from the previous step (100 μl volume). Aliquots were taken at different times to measure the amount of radiolabeled [^3H]-GDP bound to PLD2 or RasGRF1, spotted on Millipore BA85, air-dried, washed 3 $\times$ for 5 min with ice-cold 20 mM Tris-HCl pH 7.5, 100 mM NaCl and 10 mM MgCl_2 . The amount of [^3H]-GDP-bound to Ras was measured by scintillation spectrometry. The assay was performed similarly with immunoprecipitated PLD2 or RasGRF1.

GEF Activity: GTP binding to Ras

To examine the effect of PLD2 or RasGRF1 on GTP binding, [35 S]-GTP γ S bound to Ras in the presence of PLD2 or RasGRF1 was measured. PLD2-WT (19 pmol) or RasGRF1 alone were used as the positive, respectively. Nineteen pmol of Ras was incubated with 8 μ M GDP, 6 mM MgCl₂ (20 μ l volume) for 10 min at room temp. GDP-bound Ras was added to 25 μ l 100 μ M AMP-PNP, 1 mM MgCl₂ and 1 μ M [35 S]-GTP γ S in the absence or presence of PLD2 or RasGRF1 (75 μ l volume). The relative amount of [35 S]-GTP γ S-bound to Ras was measured by scintillation spectrometry. GTP binding assays were performed similarly with the immunoprecipitated PLD2 or RasGRF1.

Generation of GST-tagged fusion proteins using a bacterial expression system

The PX, PH or PXP domain of PLD2 was subcloned into the pGEX-4T-1 vector. When single point mutations were required, mutations were generated by site-directed mutagenesis using pGEX-PXP and were then verified by sequencing the constructs. Individual constructs were transformed into BL21 cells. BL21 culture was induced with 800 μ M (GST-PX) or 100 μ M IPTG (GST-PH and GST-PXP) overnight at 16°C (GST-PX or PH) or 25°C (GST-PXP). Cells were harvested and cell lysates were prepared by resuspending the pellets in lysis buffer (5 mM HEPES, 100 μ M sodium orthovanadate and 0.4% Triton-X100 supplemented with protease inhibitor cocktail just before use). Samples were sonicated to obtain crude lysates, which were then centrifuged at 5000 $\times$ g for 5 min to obtain the clarified lysates. GST-tagged proteins in the clarified lysates were pulled down using glutathione-sepharose beads overnight at 4°C, after which samples were centrifuged to remove the unbound protein and the beads were washed 3 times with 1 $\times$ PBS. GST-proteins bound to glutathione-sepharose beads were analyzed via western-blot analysis using anti-GST antibody.

Cell proliferation markers

De novo DNA synthesis was quantified as protein expression of the proliferation marker PCNA (proliferating cell nuclear antigen) [26], using a specific purified mouse anti-PCNA IgG antibody (PC10, BioLegend, San Diego, CA). Samples were further treated with a goat anti-mouse FITC-conjugated IgG secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and observed by fluorescence microscopy. Positive nuclei were counted under the microscope at 16 $\times$ objective in five different fields and averages were then calculated $\pm$ SEM.

Statistical Analysis

Data are presented as mean $\pm$ SEM. The difference between means was assessed by the Single Factor Analysis of Variance (ANOVA) test. Probability of $p < 0.05$ indicated a significant difference.

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REFERENCES

1. Xu J, Kim S, Chen M, Rockow S, Yi SE, Wagner AJ, Hay N, Weichselbaum RR, Li W. Blockage of the early events of mitogenic signaling by interferon-gamma in macrophages in response to colony-stimulating factor-1. *Blood*. 1995; 86:2774–2788. [PubMed: 7545471]
2. Walker F, Kato A, Gonez LJ, Hibbs ML, Pouliot N, Levitzki A, Burgess AW. Activation of the Ras/mitogen-activated protein kinase pathway by kinase-defective epidermal growth factor receptors

- results in cell survival but not proliferation. *Mol Cell Biol.* 1998; 18:7192–7204. [PubMed: 9819406]
3. Liebmann C. Regulation of MAP kinase activity by peptide receptor signalling pathway: paradigms of multiplicity. *Cell Signal.* 2001; 13:777–785. [PubMed: 11583913]
 4. McCubrey JA, Steelman LS, Chappell WH, Abrams SL, Wong EW, Chang F, Lehmann B, Terrian DM, Milella M, Tafuri A, Stivala F, Libra M, Basecke J, Evangelisti C, Martelli AM, Franklin RA. Roles of the Raf/MEK/ERK pathway in cell growth, malignant transformation and drug resistance. *Biochim Biophys Acta.* 2007; 1773:1263–1284. [PubMed: 17126425]
 5. Ehrenreiter K, Piazzolla D, Velamoor V, Sobczak I, Small JV, Takeda J, Leung T, Baccarini M. Raf-1 regulates Rho signaling and cell migration. *J Cell Biol.* 2005; 168:955–964. [PubMed: 15753127]
 6. Srinivasan R, Zabuawala T, Huang H, Zhang J, Gulati P, Fernandez S, Karlo JC, Landreth GE, Leone G, Ostrowski MC. Erk1 and Erk2 regulate endothelial cell proliferation and migration during mouse embryonic angiogenesis. *PLoS One.* 2009; 4:e8283. [PubMed: 20011539]
 7. Zumkeller W, Schwab M. Insulin-like growth factor system in neuroblastoma tumorigenesis and apoptosis: potential diagnostic and therapeutic perspectives. *Horm Metab Res.* 1999; 31:138–141. [PubMed: 10226794]
 8. Haigis KM, Kendall KR, Wang Y, Cheung A, Haigis MC, Glickman JN, Niwa-Kawakita M, Sweet-Cordero A, Sebolt-Leopold J, Shannon KM, Settleman J, Giovannini M, Jacks T. Differential effects of oncogenic K-Ras and N-Ras on proliferation, differentiation and tumor progression in the colon. *Nat Genet.* 2008; 40:600–608. [PubMed: 18372904]
 9. Adjei AA. Ras signaling pathway proteins as therapeutic targets. *Curr Pharm Des.* 2001; 7:1581–1594. [PubMed: 11562300]
 10. Martinez-Lacaci I, Kannan S, De Santis M, Bianco C, Kim N, Wallace-Jones B, Ebert AD, Wechselberger C, Salomon DS. RAS transformation causes sustained activation of epidermal growth factor receptor and elevation of mitogen-activated protein kinase in human mammary epithelial cells. *Int J Cancer.* 2000; 88:44–52. [PubMed: 10962438]
 11. Steelman LS, Abrams SL, Whelan J, Bertrand FE, Ludwig DE, Basecke J, Libra M, Stivala F, Milella M, Tafuri A, Lunghi P, Bonati A, Martelli AM, McCubrey JA. Contributions of the Raf/MEK/ERK, PI3K/PTEN/Akt/mTOR and Jak/STAT pathways to leukemia. *Leukemia.* 2008; 22:686–707. [PubMed: 18337767]
 12. Lim L, Manser E, Leung T, Hall C. Regulation of phosphorylation pathways by p21 GTPases. The p21 Ras-related Rho subfamily and its role in phosphorylation signalling pathways. *Eur J Biochem.* 1996; 242:171–185. [PubMed: 8973630]
 13. Kumei Y, Shimokawa H, Ohya K, Katano H, Akiyama H, Hirano M, Morita S. Small GTPase Ras and Rho expression in rat osteoblasts during spaceflight. *Ann N Y Acad Sci.* 2007; 1095:292–299. [PubMed: 17404041]
 14. Freedman TS, Sondermann H, Friedland GD, Kortemme T, Bar-Sagi D, Marqusee S, Kuriyan J. A Ras-induced conformational switch in the Ras activator Son of sevenless. *Proc Natl Acad Sci U S A.* 2006; 103:16692–16697. [PubMed: 17075039]
 15. Freedman TS, Sondermann H, Kuchment O, Friedland GD, Kortemme T, Kuriyan J. Differences in flexibility underlie functional differences in the Ras activators son of sevenless and Ras guanine nucleotide releasing factor 1. *Structure.* 2009; 17:41–53. [PubMed: 19141281]
 16. Quilliam LA, Rebhun JF, Castro AF. A growing family of guanine nucleotide exchange factors is responsible for activation of Ras-family GTPases. *Prog Nucleic Acid Res Mol Biol.* 2002; 71:391–444. [PubMed: 12102558]
 17. Mahankali M, Peng HJ, Henkels KM, Dinauer MC, Gomez-Cambronero J. Phospholipase D2 (PLD2) is a guanine nucleotide exchange factor (GEF) for the GTPase Rac2. *Proc Natl Acad Sci U S A.* 2011; 108:19617–19622. [PubMed: 22106281]
 18. Gomez-Cambronero J. The exquisite regulation of PLD2 by a wealth of interacting proteins: S6K, Grb2, Sos, WASp and Rac2 (and a surprise discovery: PLD2 is a GEF). *Cell Signal.* 2011; 23:1885–1895. [PubMed: 21740967]

19. Jeon H, Kwak D, Noh J, Lee MN, Lee CS, Suh PG, Ryu SH. Phospholipase D2 induces stress fiber formation through mediating nucleotide exchange for RhoA. *Cell Signal*. 2011; 23:1320–1326. [PubMed: 21440060]
20. Uchida N, Okamura S, Nagamachi Y, Yamashita S. Increased phospholipase D activity in human breast cancer. *J Cancer Res Clin Oncol*. 1997; 123:280–285. [PubMed: 9201251]
21. Noh DY, Ahn SJ, Lee RA, Park IA, Kim JH, Suh PG, Ryu SH, Lee KH, Han JS. Overexpression of phospholipase D1 in human breast cancer tissues. *Cancer Lett*. 2000; 161:207–214. [PubMed: 11090971]
22. Peng HJ, Henkels KM, Mahankali M, Marchal C, Bubulya P, Dinauer MC, Gomez-Cambronero J. The Dual Effect of Rac2 on Phospholipase D2 Regulation That Explains both the Onset and Termination of Chemotaxis. *Mol Cell Biol*. 2011; 31:2227–2240. [PubMed: 21444720]
23. Peng HJ, Henkels KM, Mahankali M, Dinauer MC, Gomez-Cambronero J. Evidence for two CRIB domains in phospholipase D2 (PLD2) that the enzyme uses to specifically bind to the small GTPase Rac2. *J Biol Chem*. 2011; 286:16308–16320. [PubMed: 21378159]
24. Joseph T, Wooden R, Bryant A, Zhong M, Lu Z, Foster DA. Transformation of cells overexpressing a tyrosine kinase by phospholipase D1 and D2. *Biochem Biophys Res Commun*. 2001; 289:1019–1024. [PubMed: 11741292]
25. Shi M, Zheng Y, Garcia A, Xu L, Foster DA. Phospholipase D provides a survival signal in human cancer cells with activated H-Ras or K-Ras. *Cancer Lett*. 2007; 258:268–275. [PubMed: 17949898]
26. Henkels KM, Short S, Peng HJ, Di Fulvio M, Gomez-Cambronero J. PLD2 has both enzymatic and cell proliferation-inducing capabilities, that are differentially regulated by phosphorylation and dephosphorylation. *Biochem Biophys Res Commun*. 2009; 389:224–228. [PubMed: 19715678]

Highlights

- First report of a new GEF for Ras that happens to be a phospholipase.
- PLD2-GEF acting on Ras follows faster kinetics and more specific than other substrates (Rac2) during cell growth.
- Highly metastatic cancer cells have both high levels of Ras and PLD2-“GEF” activity.
- Single point mutations on GEF activity uncovered in this study for the first time.
- New GEF mutants slow down the rate of growth of breast cancer cells.

Fig 1.

PLD2 binds to Ras via a protein:protein interaction and increases Ras-GTP binding. **(A)** Cells overexpressing PLD2 enhance cell proliferation, which is prevented if Ras is first silenced with siRNA. Right group of bars represent an associated experiment with exogenous DOPA (membrane-soluble PA) added directly to the whole cells. **(B)** PA has a biphasic effect on Ras GTP loading *in vitro* using RBD pull-down assay. **(C)** PLD2 overexpression leads to a potent increase in Ras-GTP as measured by RBD pull-down assay. **(D)** PLD2 and Ras co-overexpression increases Ras activation using RBD pull-down assay

and western-blot loading controls that indicate equal loading of protein Western blots using an anti- PLD2, anti-Ras or anti-actin antibodies. Statistical significance $p < 0.05$.

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Fig 2.

PLD2 is a GEF for Ras. The two reactions that account for a GEF activity (GDP dissociation and GTP binding) were measured. **(A)** PLD2 exerts a GDP dissociation of Ras similar to the classical Ras GEF, RasGRF1 using [^3H]-GDP dissociation assay. **(B)** PLD2 exerts GTP binding on Ras similar to RasGRF1 using [^{35}S]-GTP binding assay. In both cases the experiment were also conducted using GTPases other than Ras, namely Rac2 and Rac1.

Fig 3.

The PLD2-PX and -PH domains mediate binding to Ras. (A) Schematic drawing of expression vectors used for co-immunoprecipitation assays. (B) PLD2-Ras protein-protein interaction demonstrated by immunoprecipitation after the indicated overexpressing proteins. Immunoprecipitation and Western blots were performed with the opposite antibodies. (C,D) GST-PH or GST-PXPH were overexpressed in transformed bacteria and recombinant fusion peptides were isolated and purified. The panel shows GST-pulldown using GST-PH (C) or GST-PXPH (E) fusion proteins and Ras.

Fig 4.

PLD2-GEF catalyzes the GTP/GDP exchange and activation of Ras. **(A)** Schematic drawing of mutated PLD2 protein substrates used for biological assays. **(B)** GEF-PLD2 mutants are GEF inactive as measured by GEF activity assay. **(C)** GEF-PLD2 mutants mediates reduced cell proliferation. Statistical significance $p < 0.05$.

Fig 5.

Single point mutations on PLD2-GEF activity lead to slower rate of cell growth. (**A**) Immunofluorescence of PCNA staining as a measure of cell proliferation assay is lower in the GEF-deficient mutants using Rabbit anti-PCNA IgG antibody. (**B,C,D**) Correlation of cell growth (**B**) in MDA-MB-231 and MCF-7 breast cancer cells with PLD2-GEF activity (Relative [3 H]-GDP dissociation) (**C**) and Ras activation (**D**). Untransfected cells were used to measure the contribution of endogenous proteins. Percent Ras activation of MDA-MB-231 and MCF-7 breast cancer cells, as well as relative endogenous GTP-bound Ras, PLD2, total Ras and actin protein levels as measured by immunoblotting with appropriate

antibodies. In C, the positive control for PLD2-GEF was a recombinant PLD2-GEF protein purified from baculovirus/fp6 insect cell expression system. Lower bars indicate high GEF activity (as GDP dissociation). (E) The cancer cell lines considered were either mock-transfected or they were transiently transfected with PLD2-GEF (wild type) or with single-point mutants PLD2-Y129F that abrogate the GEF function.

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Fig 6.

Model of the correlation between cell growth and the levels of endogenous PLD2-GEF activity and Ras expression. In MDA-MD-231 breast cancer cells that have high growth and metastatic potential, all three parameters are elevated, whereas in MCF-7 cells that display low growth/low metastatic potential, GEF and Ras levels are relatively low, very similar to those of COS-7. Thus, PLD2-GEF catalyzes the GTP/GDP exchange and activation of Ras, with the specific amino acids involved and a notation that this is high in highly proliferative and metastatic cell lines.