Phospholipase D2 (PLD2) is a guanine nucleotide exchange factor (GEF) for the GTPase Rac2

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We have discovered that the enzyme phospholipase D2 (PLD2) binds directly to the small GTPase Rac2, resulting in PLD2 functioning as a guanine nucleotide exchange factor (GEF), because it switches Rac2 from the GDP-bound to the GTP-bound states. This effect is large enough to be meaningful (~72% decrease for GDP dissociation and 300% increase for GTP association, both with PLD2), it has a half-time of ~7 min, is enhanced with increasing PLD2 concentrations, and compares favorably with other known GEFs, such as Vav-1. The PLD2-Rac2 protein–protein interaction is sufficient for the GEF function, because it can be demonstrated in vitro with just recombinant proteins without lipid substrates, and a catalytically inactive lipase (PLD2-K758R) has GEF activity. Apart from this function, exogenous phosphatidic acid by itself (300 pM) increases GTP binding and enhances PLD2-K758R–mediated GTP binding (by ~34%) but not GDP dissociation. Regarding the PLD2-Rac2 protein–protein association, it involves, for PLD2, residues 263–266 within a Cdc42/Rac interactive binding region in the PH domain, as well as the PX domain, and it involves, for Rac2, residue N17 within its Switch-1 region. PLD2's GEF function is demonstrated in living cells, because silencing PLD2 results in reduced Rac2 activity, whereas PLD2-initiated Rac2 activation enhances cell adhesion, chemotaxis, and phagocytosis. There are several known GEFs, but we report that this GEF is harbored in a phospholipase. The benefit to the cell is that PLD2 brings spatially separated molecules together in a membrane environment, ready for fast intracellular signaling and cell function.

The small GTPase Rac is a crucial regulator of actin cytoskeletal rearrangement and plays an important role in cell spreading, migration, mitogenesis, phagocytosis, superoxide generation, and axonal growth (1). Activation of Rac occurs because of GTP exchange factors [guanine nucleotide exchange factors (GEFs)], and inactivation is mediated by the intrinsic GTPase enzymatic activity of Rac, greatly aided by GTPase activating proteins (GAPs). Further regulation of the cycle is mediated by guanosine nucleotide dissociation inhibitors (GDIs) that antagonize both GEFs and GAPs. There are three families of GEFs for monomeric GTPases that include Cdc25, Dbl homology (DH), and Sec7 domains (2–4).

Clear advances have been made in the molecular mechanisms that control the targeting of GEFs to their effectors, such as Rac, to the plasma membrane, but only a handful of cases remain clearly mapped out. For Sos, the recruitment of phosphatidylinositol 3,4,5 phosphate (PIP_3) concurs to unmask its Rac-GEF activity in vitro (5). Actin-binding ezrin promotes recruitment of GEF-Dbl to the plasma membrane and to lipid raft microdomains where it can act upon Cdc42 and the downstream effector PAK-1 (6). Also, activation of ARFs catalyzed by the characteristic Sec7 domain of ARF-GEFs occurs before recruitment of their effectors and membrane trafficking and vesicle formation (7).

Several studies have implicated phospholipase D (PLD) in cell signaling, as it relates to Rac. The C-terminal polybasic motif of Rac1 interacts with phosphatidic acid (PA), and PLD serves to recruit Rac to the cell membrane (7), but how this affects the GEFs for Rac is not known. However, PLD-derived PA acts upon GEFs. PA activates the unconventional DOCK2-GEF, which then targets GTPase(s) (8). Our laboratory demonstrated that PLD binds to Grb2, which recruits the GEF Sos (8), and it is also known that PA binds directly to Sos (9). All of the mechanisms that imply translocation to the membrane or membrane trafficking that place GEF close to its effectors are complex and rely on the formation of multimeric signaling proteins.

We show here that PLD2 functions as a GEF, because it switches Rac2 from the GDP-bound to the GTP-bound state. There are a number of known GEFs, but none of the three families of GEFs for monomeric GTPases is a phospholipase, making this study unique. A further biological significance is that signals from the cell receptor are routed to Rac with an economy of molecular machinery without any intermediary factor(s). Last, our results are demonstrated first in vitro and then extended to the physiology of leukocytes, implicating PLD2 as a GEF for Rac in key cellular functions: cell adhesion, chemotaxis, and phagocytosis.

Results

Rac2 and PLD2 Interact in Vivo and in Vitro. Initially, we hypothesized that both PLD2 and Rac2 could form an interaction within the cell and must, therefore, be in close spatial proximity. Fig. 1*A* demonstrates that Rac2 and PLD2 are associated in the cell by immunofluorescence microscopy. FITC-labeled Rac2 and tetramethyl rhodamine isothiocyanate (TRITC)-labeled PLD2 colocalize to the perinuclear region in the cell, though some Rac2 also has a punctate nature localized to endosomes. To further study the association of Rac2 and PLD2, and to ascertain the stoichiometry of the binding, we used recombinant proteins from a baculovirus/insect cell expression system, as our laboratory recently documented (10). This process involved expression and purification of C-terminal 6xHN-tagged proteins by a TALON cobalt metal affinity resin. PLD2 was >87% pure (Fig. 1*B*), whereas Rac2 was >95% pure (Fig. 1*C*). To ascertain the stoichiometry of PLD2 and Rac2 binding, we used these recombinant proteins in an ELISA binding assay. We added increasing concentrations of PLD2 to the plate and determined the bound fraction. Then, we added a constant and excess amount of Rac2 and measured the molar concentration of Rac2 bound to the PLD2 by direct UV absorbance (Fig. 1*D*). With this technique, we calculated that PLD2 binds to Rac2 at a molar ratio of PLD2:Rac2 between 1:1.3 and 1:1.7. Results in Fig. 1 thus indicate that Rac2 and PLD2 form a heterodimer.

PLD2 Is a Bona Fide GEF for Rac2. To investigate the possible functional consequences of a Rac2-PLD2 interaction on Rac2 functionality, we designed a loss-of-function cell model in Rac2 GTPase activity. For this, we used RNA gene expression silencing and analyzed Rac2 activation [protein binding domain (PBD) pull-down]. As shown in the Western blots depicted in Fig. 2*A*,

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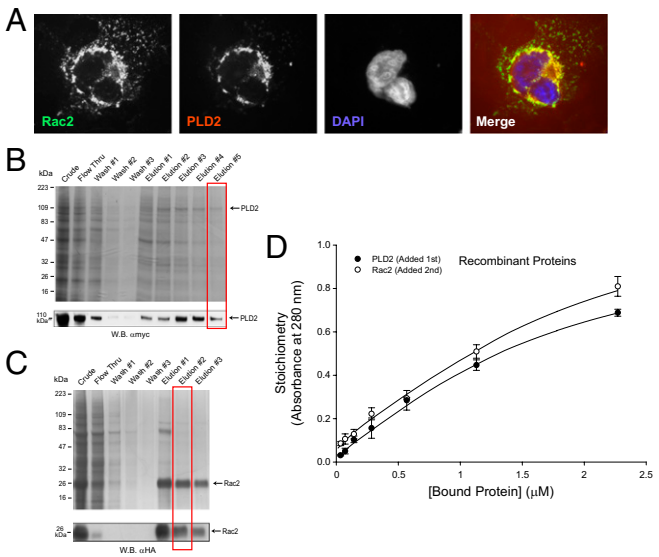

Fig. 1. Demonstration of a molecular association between Rac2 and PLD2. (*A*) Immunofluorescence microscopy of COS-7 cells cotransfected with both myc-Rac2 and HA-PLD2 alone. FITC, Rac2 fluorescence; TRITC, PLD2 fluorescence; DAPI, nuclei staining; merged, overlay of three fluorescent images. (*B* and *C*) Purification of recombinant baculovirus PLD2 (*B*) and Rac2 (*C*) shown by Coomassie blue-stained SDS/PAGE and Western blots developed with antibodies to the corresponding tags. The red rectangles indicate the elution samples that were used for subsequent experiments. (*D*) Stoichiometry of PLD2 and Rac2 binding. Purified, recombinant PLD2 was first bound to the ELISA plate (●), followed by addition of purified, recombinant Rac2 that bound directly to the prebound PLD2 (○). Triplicate results are mean ± SEM and are expressed in terms of binding absorbance at 280 nm.

PLD2 was effectively and significantly silenced (>80%) using increasing concentrations of siRNA. Fig. 2*C* shows that PLD2 had a positive effect on Rac2 activation, because its lack of expression led to a diminished Rac2 activation. Conversely, silencing with siPLD1 RNA (Fig. 2*B* for immunoblot controls) or siScrambled RNA had no effect on Rac2 activity (Fig. 2*C*). This in vivo study suggests a PLD2-led GEF effect on Rac2 (but not a PLD1-led effect) with functional consequences. Thus, we concentrated on the PLD2 isoform for all subsequent experiments.

The first evidence that PLD2 acted as a GEF for Rac2 is shown in Fig. 2 *D–G* and represents a series of GTP loading experiments in the presence of increasing PLD2 concentrations. In an in vivo approach shown in Fig. 2*D*, Sf21 cells were coinfected with fixed amounts of baculoviral Rac2 and varying amounts of baculoviral PLD2. Increasing expression of PLD2 led to an increase in GTP/GDP exchange loading activity of Rac2. PLD2 had an overall positive effect on Rac2 activity at a multiplicity of infection (MOI) ≥ 10:1. This effect was also observed in COS-7 cells transfected with PLD2 and/or Rac2 (Fig. 2*E*).

The use of recombinant, purified proteins (Rac2 and PLD2) indicated that GTPγS alone had a weak effect on GTP loading of Rac2 at low nanomolar concentrations (Fig. 2*F*), which increased concomitantly with increasing concentrations of recombinant PLD2 (Fig. 2*G*). It is noteworthy that the results had no exogenous lipids (neither phosphatidylcholine, the substrate of PLD2 action, nor the product, PA), indicating that the effect of PLD2 on Rac2 can occur even in the absence of the lipase enzymatic activity. In conclusion, Fig. 2 indicates that PLD2 directly affects an increase in vivo and in vitro of GTP loading of Rac2.

Purified, Recombinant PLD2 Leads to a GDP/GTP Exchange in Rac2. Fig. 3 is a series of GTP/GDP exchange experiments, the hallmark of GEF function (11–15). Fig. 3*A* indicates that PLD2 elicited dissociation of the relative amount of bound [^{3}H]GDP from Rac2 with a half-time of 7 min and a total decrease on GDP dissociation of ~72%. This in vitro assay with recombinant proteins had no exogenous lipids, indicating that the effect of PLD2 on Rac2 can be accomplished without intervening enzymatic (lipase) activity. We nevertheless asked whether the

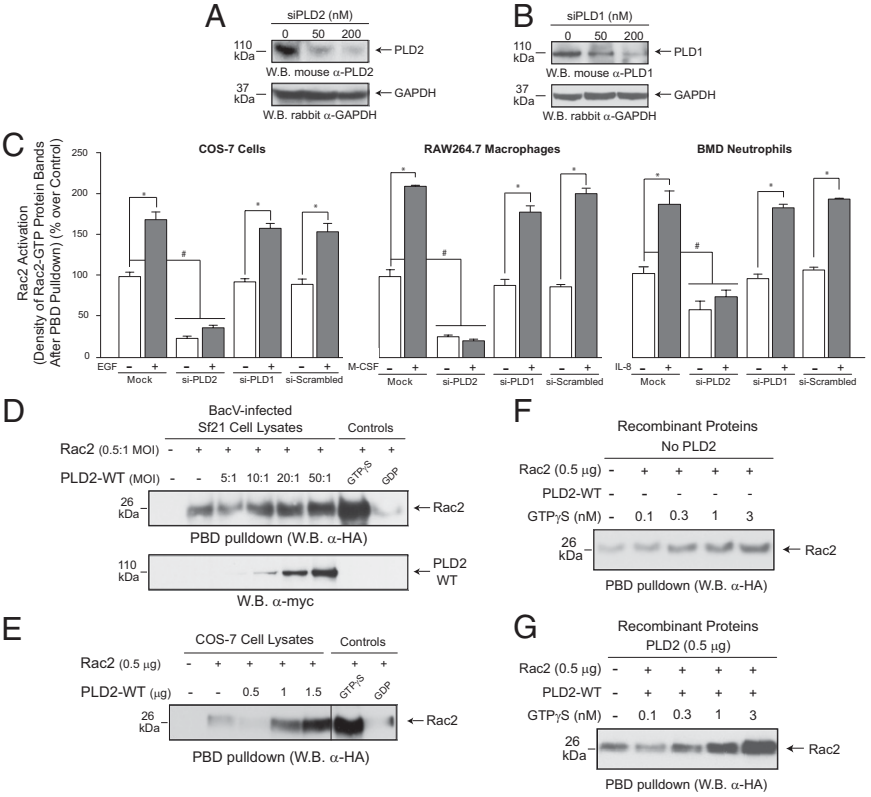

Fig. 2. Functional effect of PLD2 on GTP loading of Rac2. Effect of silencing PLD2 on protein expression (*A* and *B*) and on Rac2 PBD pull-down assays (*C*). (*A* and *B*) Cells were silenced with 0, 50, or 200 nM siRNA specific for exon 15 on PLD2 (*A*) or exon 10 on PLD1 (*B*). (*C*) COS-7 or phagocytes were mock transfected or transfected with siPLD2, siPLD1, or siScrambled at a final concentration of 300 nM siRNA for 3.5 d. After silencing, cells were stimulated with 3 nM EGF, 3 nM M-CSF, or 10 nM IL-8, respectively, harvested, and then lysates prepared and used for Rac2 PBD pull-down assays. Triplicate results are mean ± SEM and are expressed in terms of Rac2 activation (PBD pull-down). (*D–G*) PLD2 increases GTP loading on Rac2 both in lysates and purified proteins. (*D*) Sf21 cells were coinfected with fixed amounts of baculoviral Rac2 and varying amounts of baculoviral PLD2 and used for a PBD pull-down assay. (*F* and *G*) Experiments with purified, recombinant proteins. (*F*) Control with no PLD2. (*G*) Test with PLD2. Purified, recombinant baculoviral proteins were used for a PBD pull-down assay to ascertain the effect of PLD2 on GTP binding to Rac2.

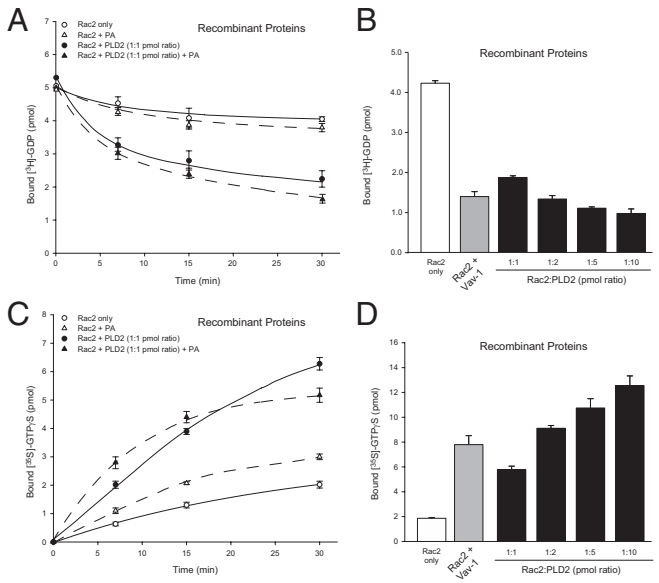

Fig. 3. GTP/GDP exchange activity of Rac2 is increased in the presence of PLD2. Recombinant, purified Rac2 and PLD2 proteins were used for the hallmark measurement of GEF activity (GTP/GDP exchange). (*A*) [^{3}H]GDP dissociation of Rac2, alone or with PLD2, without any further treatment (solid lines), or in the presence of 300 pM PA (dashed lines), was measured at various time points as indicated. (*B*) Increasing GEF effect (GDP dissociation) of PLD2 on Rac2 with concomitant increases in PLD2 concentration at maximal time (30 min). (*C*) [^{35}S] GTPγS binding of Rac2, alone or with PLD2, without any further treatment (solid lines), or in the presence of 300 pM PA (dashed lines), was measured at various time points as indicated. (*D*) Increasing GEF effect of PLD2 for Rac2 GTP binding as PLD2 concentrations increase at maximal time (30 min).

addition of lipids (particularly phosphatidic acid, PA, the product of PLD reaction) would have an additional role in GDP

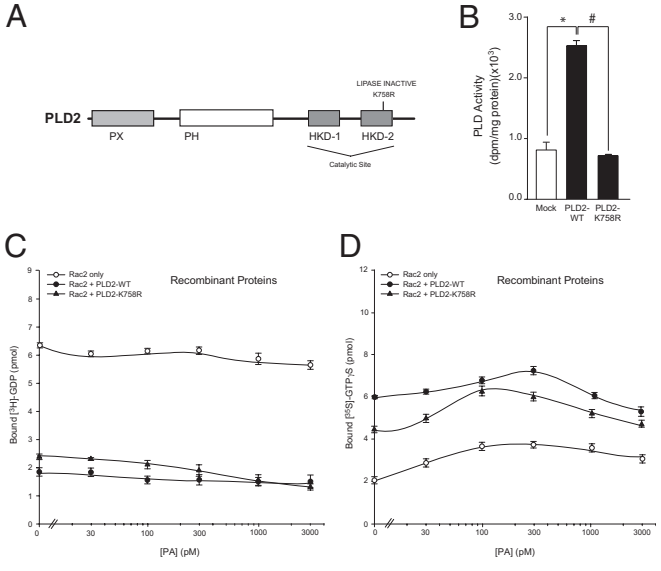

Fig. 4. PA aids in GEF activity by PLD2, particularly in GTP binding. Recombinant, purified Rac2, PLD-WT, and PLD2-K758R proteins were used throughout this figure. (*A*) Schematic representation of PLD2 and its domains, with special marking of the K-to-R mutation at amino acid 758 on the HKD-2 catalytic domain that renders the enzyme inactive. (*B*) Purified, recombinant PLD2–K758R lipase-inactive mutant has no lipase activity compared with wild-type protein. Triplicate results are mean ± SEM and are expressed in terms of PLD2 activity (dpm/mg protein). (*C*) Dose-dependent effect of PA on Rac2 GDP dissociation in the presence of PLD2-WT and -K758R. (*D*) Dose-dependent effect of PA on Rac2 GTP binding in the presence of PLD2-WT and -K758R.

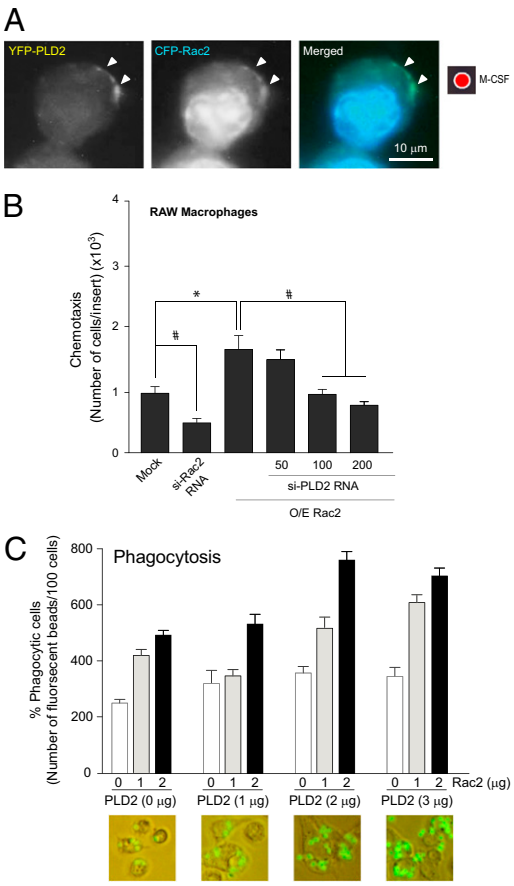

Fig. 5. PLD2 GEF effect of Rac2 affects physiological processes. (*A*) Functional effect of Rac2 and PLD2 on directionality during chemotaxis. Cells transfected with YFP-PLD2 and CFP-Rac2 were locally stimulated with M-CSF by a red dot (*Right*). Colocalization occurred along the plasma membrane (white triangles). (*B*) Effect of silencing Rac2 and/or PLD2 on chemotaxis of RAW264.7 mouse macrophages. (*C*) Functional effect of Rac2 and PLD2: phagocytosis. RAW264.7 macrophages were transfected simultaneously with increasing PLD2 and constant Rac2 at three different concentrations, and at 48 h posttransfection, cells were incubated with opsonized zymosan A (*S. cerevisiae*) fluorescein conjugate. Cells were manually counted, and the phagocytic index was calculated.

dissociation. PA alone has a small (~13%) effect on GDP dissociation (Fig. 3*A*) over the already robust decline caused by PLD2 alone.

To further prove the GEF effect, Fig. 3*B* shows an increasing GDP dissociation with concomitant increases in PLD2 concentration. Additionally, PLD2 measures up well with a positive control, Vav-1, which is a well known and previously characterized Rho-GEF (16).

We next concentrated on the second part of a GEF reaction (binding of GTP). As shown in Fig. 3*C*, there was a large increase (~300%) in the binding of [^{35}S]GTPγS to Rac2 in the presence of PLD2. This effect was observed in recombinant proteins in the absence of lipids, meaning that a protein–protein interaction between PLD2 and Rac2 is enough for this activity. Nevertheless, another set of experiments was conducted with PA. PA alone (no PLD2 present) did cause a significant increase (60%) in GTP binding (Fig. 3*C*, open triangles), whereas PA and PLD2 show an initial fast GTP binding of Rac2 followed by a diminished binding (dashed lines). The reason for this complex, biphasic effect, is not understood at present. Apart from this, and as observed earlier for GDP dissociation (Fig. 3*B*), the PLD2 effect for GTP binding increased as PLD2 concentrations increased (Fig. 3*D*), and also compares well with the positive

control of the known GEF, Vav-1, further proving the GEF function of PLD2.

In conclusion, PLD2 drives the dissociation of GDP and augments Rac2 GTP binding, both hallmarks of GEF activity. Both effects (particularly the first one) can be largely accomplished without PA. The GEF effect is large enough to be meaningful (~72% decrease for GDP dissociation and 300% increase for GTP association, in the presence of PLD2), has a reasonable kinetic behavior with a half-time of 7 min, which reached saturation at 15–30 min and was enhanced concomitantly with increasing PLD2 concentrations.

Lipase-Inactive Mutant of PLD2 Is also a GEF for Rac2. To further explore the effect of PA on the GEF function of PLD2, we used a lipase-inactive PLD2 mutant (PLD2-K758R) with a key substitution in the HKD-2 domain that renders it incapable of producing PA (Fig. 4*A*). As a necessary control, we verified that purified, recombinant PLD2-K758R has no lipase activity compared with wild type (Fig. 4*B*). Fig. 4 *C* and *D* confirms that a protein–protein interaction between PLD2 and Rac2 is sufficient to initiate the GTP/GDP exchange and can be accomplished with either a fully active or a lipase-inactive enzyme, albeit with a slightly lower capacity in the latter. The GEF effect of PLD2 on K758R was aided by PA, as shown in Fig. 4 *C* and *D* with increasing concentrations of PA at different extents, with an estimated 34% improvement for GTP binding (second GEF reaction step) but with no significant effect for GDP dissociation (first GEF reaction step). Therefore, the product of lipase activity of PLD2, PA, is mostly involved (as a "booster") in the second step of the GEF reaction.

Physiological Implications of PLD2 as a Rac2 GEF: Cell Polarization, Chemotaxis, and Phagocytosis. Because Rac2 has been extensively implicated in cell migration (17–19) and phagocytosis (20, 21) in leukocytes, we chose to study these functionalities using several approaches after demonstrating that PLD2 acts as a GEF for Rac2 in vitro. During macrophage chemotaxis, polarization of the cell occurred in response to a chemoattractant and resulted in colocalization of the Rac2 and PLD2 proteins as evidenced by immunofluorescence microscopy (Fig. 5*A*). In macrophage colony-stimulating factor (M-CSF)-stimulated cells, ~15–20% of either protein signal migrated to the plasma membrane (white triangles). Thus, subcellular localization of PLD2 and Rac2 is dependent on the presence of a chemoattractant and is directly related to its concentration. The effect of silencing either Rac2 or PLD2 was documented during chemotaxis of RAW 264.7 mouse macrophages (Fig. 5*B*). When Rac2 was silenced, chemotaxis decreased by ~50% compared with the negative mock control.

Last, in Fig. 5*C*, another physiological function of macrophages, phagocytosis, is shown. The percent of phagocytic cells increased proportionally with increasing concentrations of PLD2 (Fig. 5*C*, *Inset*). This data demonstrates that PLD2 and Rac2 colocalize in the cell and lead to augmentation of normal physiological functions of the cells: adhesion, chemotaxis, and phagocytosis.

Site of Binding of PLD2 to Rac2. A final question we asked in this study was regarding the site of binding of PLD2 to Rac2, particularly because we have demonstrated the preeminence of a protein–protein interaction. As shown in Fig. 6*A*, a Rac2 mutant was used that was modified in the Switch-1 domain near the N terminus (preventing binding to GTP), whereas a PLD2

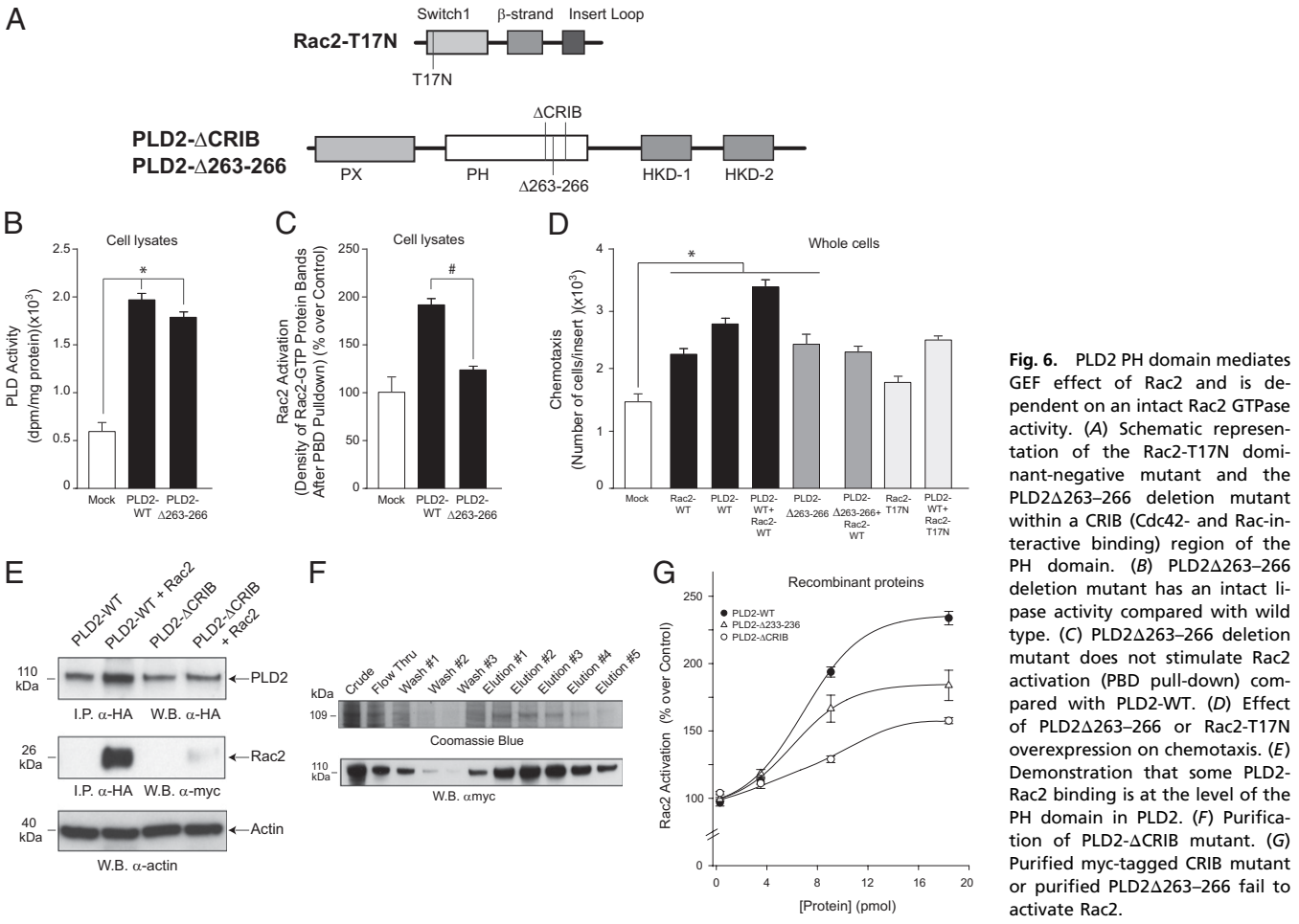

Fig. 6. PLD2 PH domain mediates GEF effect of Rac2 and is dependent on an intact Rac2 GTPase activity. (*A*) Schematic representation of the Rac2-T17N dominant-negative mutant and the PLD2Δ263–266 deletion mutant within a CRIB (Cdc42- and Rac-interactive binding) region of the PH domain. (*B*) PLD2Δ263–266 deletion mutant has an intact lipase activity compared with wild type. (*C*) PLD2Δ263–266 deletion mutant does not stimulate Rac2 activation (PBD pull-down) compared with PLD2-WT. (*D*) Effect of PLD2Δ263–266 or Rac2-T17N overexpression on chemotaxis. (*E*) Demonstration that some PLD2-Rac2 binding is at the level of the PH domain in PLD2. (*F*) Purification of PLD2-ΔCRIB mutant. (*G*) Purified myc-tagged CRIB mutant or purified PLD2Δ263–266 fail to activate Rac2.

mutant was generated in the PH domain [within a Cdc42- and Rac-interactive binding (CRIB) region] (10). As shown in Fig. 6*B*, the PLD2Δ263–266 deletion mutant had a conserved and intact lipase activity compared with wild type. When the PLD2Δ263–266 deletion mutant was used in the Rac2 activation assay via PBD pull-down, the PLD2 GEF activity of Rac2 was lost (Fig. 6*C*).

Overexpression of Rac2-WT, PLD2-WT, or both proteins simultaneously resulted in significantly increased chemotaxis (Fig. 6*D*). When PLD2Δ263–266 and Rac2-WT were simultaneously overexpressed, chemotaxis did not additionally increase as the PLD2-WT and Rac2-WT combination did. The PLD2Δ263–266 deletion mutant cannot interact with Rac2, which highlights the crucial importance of the PH domain in the GEF function of PLD2. Using GST-PX, we have indicated earlier that Rac2 also binds to PLD2 at the PX site (22) and, as such, both the PH and the PX domains are implicated in the Rac2-PLD2 protein–protein interaction.

Because it can be argued that the PH domain is simply necessary to bring PLD2 to the membranes that contain Rac2, we conducted additional experiments to further demonstrate the Rac2-PLD2 interaction first with coimmunoprecipitation and then in vitro with recombinant proteins. As shown in Fig. 6*E*, PLD2-Rac2 binding is at the level of the PH domain in PLD2, because association occurred in intact proteins but not in the CRIB deletion mutant. Purified deletion proteins (such as presented in Fig. 6*F*) were used for the Rac2 activation assay; the results of Fig. 6*G* indicate that they failed to fully activate Rac2 compared with the PLD2-WT protein control. In summary, we provide functional evidence of the sites of interaction between PLD2 and Rac2, which occurs via the PH domain of PLD2 (complementing the participation of the PX domain) and via the N-terminal region of Rac2 in the Switch-1 domain.

Discussion

We report here a unique function of a phospholipase: that of it being a GEF. Based on in vitro and in vivo experiments, PLD2 acts as a bona fide GEF for Rac2 by increasing GTP loading and decreasing GDP dissociation. This report of a phospholipase acting as a GEF opens unexpected consequences in cell regulation, some of which are studied here (cell polarization, chemotaxis and phagocytosis). Further, it has been observed that leukocytes from RacGEF KO mice show persistent Rac2 activity, implicating that an unknown GEF is still present. We posit that this GEF is PLD2.

The data presented here indicates that PLD2 effectively affects the two steps of a GEF reaction: GDP dissociation and GTP binding. The GEF role is largely accomplished by PLD2-Rac2 protein–protein interactions, partially aided by the enzymatic reaction of PLD2 (PA production). We propose the model as shown in Fig. 7*A* in which PLD2 executes the two GEF sequential steps and the aiding effect of PA, which bears its effects in the second reaction step of GEF, that of GTP binding.

We are also reporting the site of binding between the two proteins. In the case of PLD2, the amino acids 263–266 are within a CRIB region of the PH domain (Fig. 7*B*), which is in addition to the PX domain, as indicated earlier in our laboratory, because both GST-PH and GST-PX bind to Rac2 (22). The existence of a PH domain is shared by all known GEFs, and Fig. 7*B* presents the PH structure of Vav-1, the GEF used as positive control in this study, and a predicted model of PLD2's PH domain. The similarities between the α-helix and the β-sheet are obvious. However, Rho-GEFs have a DH domain upstream of the PH domain. In the case of PLD2, there is no DH domain. Nevertheless, atypical GEFs, such as DOCK, have been discovered in the recent past (23), suggesting that Rho-GEFs can be of various types based on their structure and specificity toward their GTPases. In some cases, the PH domain, apart from its established docking protein-lipid domain, can be at the center of the catalytic activity of GEFs. In the case of Dbs (24), the PH

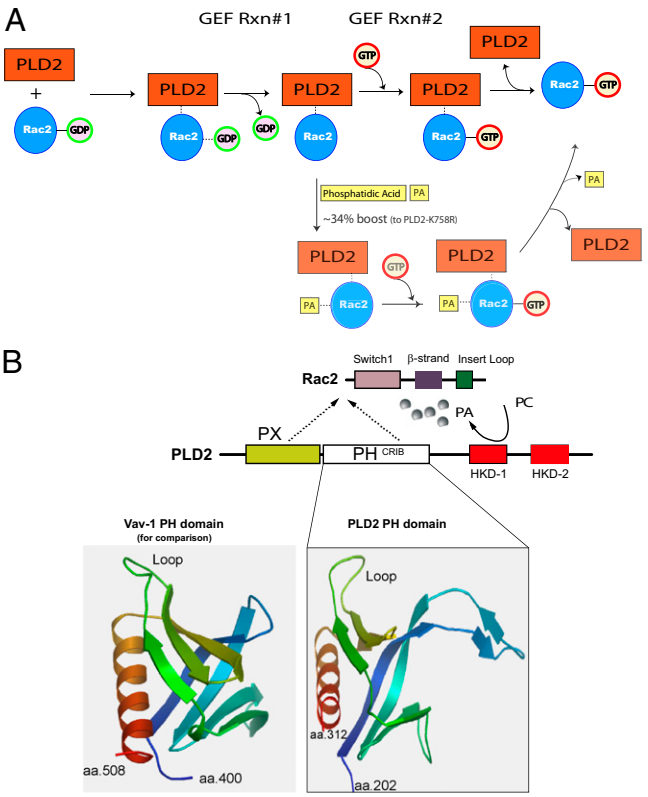

Fig. 7. Proposed mechanism of the PLD2 GEF reaction on Rac2 and comparison of computer-generated predictions of PH domains of Vav-1 and PLD2. (*A*) A theoretical model of the mechanism of the Rac2 GTP/GDP exchange reaction as performed by PLD2, which functions as a GEF that acts upon GDP dissociation (GEF Rxn#1) and GTP binding (GEF Rxn#2). (*Lower*) The "aiding" role of PA is described that is mainly involved in GEF Rxn#2 and is derived from data with the phospholipase-inactive mutant PLD2-K578R. (*B*) Representation of an image of the NMR solution Vav-1 PH domain (*Left*) with the PLD2 PH prediction (*Right*; amino acids 210–324). The model was created with SWISS-MODEL (32–34) and http://swissmodel.expasy.org/workspace. The participation of PLD2's PX and PH domains (and at some level, the enzymatic product, PA) to the GDP/GTP exchange of Rac2 is also depicted.

domain also interacts with the substrate GTPase Cdc42 along with its DH domain, and is also responsible for catalytic activity. We propose that PLD2 acts as a GEF toward Rac2 with the help of the PH domain and the PX domain (a role for both domains is depicted as dotted lines in Fig. 7*B*) and perhaps the participation of PA (gray spheres in Fig. 7*B*), particularly for GTP binding to Rac2. The exact binding of these three components to the Rac2 molecule remains to be elucidated.

We can speculate a molecular mechanism for the effect of PA on GTP binding: it can facilitate docking via binding of positively charged domains of target proteins to the inner leaflet of the cell membrane and directs the membrane localization of various GEFs (25). Our data are in agreement with a positive role of PA, because it can increase the GTP loading of Rac2. The importance of Rac2-PLD2 in a cell membrane environment is underscored by the fact that in stimulated cells PIP_3 can bind to PLD2 (26). The small GTPase Cdc42 can act as a competitive inhibitor of Rac1 and Rac2 binding and subsequent activation (27). Similar to what is seen in PLD activity, GTP-bound Rac is necessary for sustained Nox enzyme activity (28). Phosphorylation allows the protein to bind and activate its target (29).

The DOCK family is a part of the Rho-GEF family. Following PIP_3 phospholipid formation, DOCK2 (as a GEF) is recruited to the plasma membrane. Subsequently, PLD is activated, which then mediates the hydrolysis of PC to form PA. This phospholipid is

thought to bridge DOCK2 toward activation of Rac to stimulate leading-edge actin polymerization to drive neutrophil chemotaxis (30). One can attempt to explain that the results presented herein are due to PA affecting Rac2 through DOCK2, which is needed as an intermediary signaling link. The use of recombinant purified proteins in the present study rule out this possibility, arguing in favor of a direct effect of PLD2 on Rac2 as a bona fide GEF. In summary, PLD acts as a GEF for small GTPases, such as Rac2, both in vitro and in vivo in leukocytes. The functional consequences of a GEF-PLD–mediated activation of Rac2 are an increased adhesion, phagocytosis, and chemotaxis of leukocytes that are at the center of the innate immune system's functions.

The GEF function of PLD2 could also be implicated in cancer metastasis. Cells from colorectal or breast cancer exhibit high PLD2 levels (31). PLD2 in these cells could serve to constitutively activate Rac2, a GTPase known to have a role in cell movement and cancer metastasis. Hence, characterizing the GEF activity of PLD2 further aids in understanding and unraveling the still-unknown mechanisms of both leukocyte migration and cancer metastasis to devise novel therapeutic approaches.

Methods

A full description of methods is provided in *SI Methods*.

Murine BM cells were harvested from mice, and neutrophils were differentiated in vitro with IL-3 and granulocyte CSF. Cells were nucleofected with siRNA for the last 3 d of differentiation to allow silencing of PLD2. For cell migration assays, RAW264.7 macrophage cells were placed in 8-mm pore, polycarbonate membrane Transwell inserts and migrated against M-CSF into the lower wells of 24-well plates for 1 h. For adhesion experiments, cells were plated onto collagen-coated coverslips at a concentration of 5×10^4 cells/mL and incubated at 37 °C for specific times. Adherent cells were fixed, hematoxylin stained, and counted. For phagocytosis, zymosan A (*Saccharomyces cerevisiae*) fluorescein conjugates were opsonized by incubating with zymosan A bio particles/opsonizing agent and mixed with cells.

Purified, recombinant PLD2 and Rac2 proteins were prepared from a baculovirus/insect cell expression system: pBac-C1-HA-Rac2 and pBac-C1-myc-PLD2-WT recombinant viruses to infect Sf21 cells. 6xHN-tagged proteins in the lysates were bound by TALON resin and purified. Binding/stoichiometry was performed in an ELISA plate setting. Increasing concentrations of baculoviral, purified PLD2 were incubated with PVC-grade, 96-well plates. Unbound PLD2 was determined by measuring absorbance at OD_{280}. For Rac2 PBD pull-down assays, 2×10^6 Sf21 insect cells were infected with Rac2 baculovirus at a MOI of 0.5:1. Proteins were mixed with PAK-1 PBD agarose. Samples were loaded onto gels, transferred to blotting membranes, and probed with either α-HA antibodies to detect GTP-bound Rac2 or α-myc antibodies to detect PLD2 that interacted with Rac2 in the pull-down assay.

GTP/GDP exchange was measured with purified, recombinant proteins (PLD2 and Rac2) used in an in vitro assay that had no phosphatydilcholine, the substrate of PLD2 action, so that no enzymatic production of PA was possible. These reactions contained no PA, unless expressly added exogenously and, as such, indicated in *Results* and figure legends. For GDP dissociation, 0.5 μg of Rac2 was preloaded with 2 μM [^{3}H]GDP and then incubated with 2 μg PLD2. Aliquots were taken at different times to measure the amount of radiolabeled [^{3}H]GDP bound to Rac2. To examine if PLD2 could exchange GDP, 0.5 μg of Rac2 was incubated with 8 μM GDP and [^{35}S]GTPγS ± 2 μg PLD2. In either case, the amount of GTP- or GDP-bound Rac2 was measured by scintillation spectrometry.

For PLD lipase activity assay, purified baculoviral PLD2 (-WT, -Δ263–266, or -KR) was processed in PC8 liposomes and [^{3}H]l-butanol (8). Reactions were stopped and lipids were then isolated and resolved by TLC. The amount of [^{3}H]PBut that comigrated with PBut standards was measured by scintillation spectrometry.

Statistical analysis data are presented as mean ± SEM. The difference between means was assessed by single-factor ANOVA. Probability of $P < 0.05$ indicated a significant difference.

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