

# Structure analysis between the SWAP-70 RHO-GEF and the newly described PLD2-GEF

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Small GTPases like Rac2 are crucial regulators of many cell functions central to life itself. Our laboratory has recently found that phospholipase D2 (PLD2) can act as a guanine nucleotide exchange factor (GEF) for Rac2. PLD2 has a Pleckstrin Homology (PH) domain but does not bear a Dbl homology (DH) or DOCK homology region (DHR) domain. It has, however, a Phox (PX) domain upstream of its PH domain. To better understand the novel finding of PLD2 as an enhancer of GDP/GTP exchange, we modeled the N-terminal portion of PLD2 (as the crystal structure of this protein has not as of yet been resolved), and studied the correlation with two known GEFs, SWAP-70 and the Leukemic Associated RhoGEF (LARG). Structural similarities between PLD2's PH and SWAP-70s or LARG's PH domain are very extensive, while similarities between PLD2's PX and SWAP-70s or LARG's DH domains are less evident. This indicates that PLD functions as a GEF utilizing its PH domain and part of its PX domain and possibly other regions. All this makes PLD unique, and an entirely new class of GEF. By bearing two enzymatic activities (break down of PC and GDP/GTP exchange), it is realistic to assume that PLD is an important signaling node for several intracellular pathways. Future experiments will ascertain how the newly described PLD2's GEF is regulated in the context of cell activation.

## The Enzyme Phospholipase D2 (PLD2) is a GEF

Aside from its well-defined lipase activity, phospholipase D2 (PLD2) has recently been shown to contain an additional and novel catalytic function, that of a guanine nucleotide exchange factor (GEF) for small GTPases, via turnover and release of the inactive GDP-bound GTPase to that of the active GTP-bound GTPase. Our laboratory has demonstrated that the small GTPase targeted by PLD2's activity is Rac2<sup>1,2</sup> and that these two proteins form a complex in living cells,<sup>3,4</sup> whereas Jeon et al. have shown the target to be RhoA.<sup>5</sup> A GEF can be defined as a multidomain-containing protein that speeds up the exchange reaction of GDP by GTP by modifying the nucleotide-binding site, such that the nucleotide affinity is decreased and results in the release of GDP and replacement with GTP. Experimental evidence points at PLD2 being able to complete both functions.

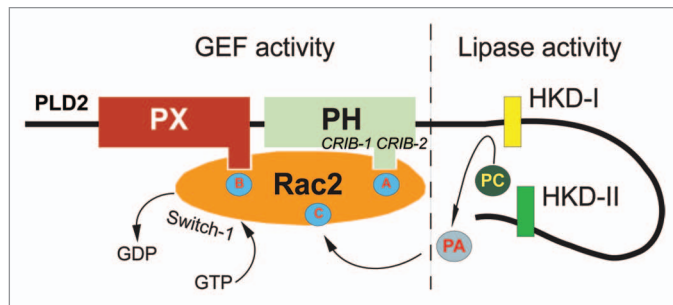
## Sites of Interaction with the Small GTPase RAC2

The first indications that PLD2 has a dual activity (lipase and GEF) were derived from the observation of a PLD2-Rac2 complex formation, both in vitro and in vivo (the latter demonstrated by FRET microscopy).<sup>3,4</sup> PLD2 also contributed to Rac2-mediated phagocytosis, which adds physiological significance to this finding. Our laboratory demonstrated that PLD2 mediates guanine nucleotide exchange by facilitating both GDP dissociation and GTP binding of Rac2 in vitro with recombinant, purified proteins. The presence of PLD2 resulted in a significant increase in GTP bound Rac2, as analyzed by PBD pull-down. PBD pull-down of Rac2 alone with increasing concentrations of GTP $\gamma$ S was significantly increased in the presence of PLD2, suggesting that mere protein-protein interactions between PLD2 and Rac2 induced the GTP binding of Rac2.<sup>1</sup> The interaction involves two newly

described Cdc42- and Rac-Interactive Binding (CRIB) domains in PLD2. We named the first domain "CRIB-1" (<sup>255</sup>ISF VQL FDP GFE VQG V<sup>270</sup>) and the second domain "CRIB-2" (<sup>306</sup>ITE LAQ GPG RDF LQL HRH DSY<sup>326</sup>).<sup>3,4</sup>

The site(s) of interaction of PLD2 with the small GTPases Rac2 or RhoA and its subsequent GEF activity are dependent upon the phox (PX) domain,<sup>1,5</sup> which is located in the N-terminus in PLD2. PLD2 directly binds to nucleotide-depleted RhoA through its PX domain and PLD2-PX overexpression increases cellular GTP-RhoA levels.<sup>5</sup> Our laboratory has expanded the need of other protein motifs, as to include the pleckstrin homology (PH) and residues 263–266 of the CRIB domains also at the N-terminus of PLD2, that mediate PLD2-Rac2 binding.<sup>1,4,5</sup> The new GEF activity is a PLD2 isoform-led effect on the small GTPase, as silencing of the other mammalian isoform, phospholipase D1 (PLD1), had no effect on Rac2.<sup>1</sup> The GEF activity of PLD2 is independent of its lipase activity,

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**Figure 1.** PLD2 has both a lipase and a GEF activity. Both PX and PH protein domains bind to the small GTPase Rac2 (A and B); the interaction PLD2-PH and Rac2 is mediated by two “CRIB” domains in PLD2. The two HKD domains HKD-I and HKD-II are conserved motifs responsible for the lipase activity and conversion of PC into PA. PA is also known to bind to Rac2 (C).

as lipase-dead PLD2 (PLD2-K758R) can still function as a viable GEF for Rac2 similar to that of wild-type while also in the absence of exogenous lipids [phosphatidylcholine (PC) or phosphatidic acid (PA)]. We believe that the region(s) responsible for GEF activity are separated from the two catalytic HKD domains of PLD2<sup>1</sup> and could be localized in the regulatory N-terminal end of the molecule where the PX and PH domains reside (Fig. 1).

### RHO-GEF Modular Domains

As indicated, PLD2 targets the small GTPases Rac2<sup>1</sup> or RhoA<sup>7</sup> both of which belong to the Rho GTPase family, that is comprised of 25 member GTPases.<sup>6</sup> The main GEFs for Rho GTPases are the Dbl (diffuse B-cell lymphoma)-family and the DOCK family [CDM, CED5, DOCK180 (dedicator of cytokinesis) and Myoblast City/DOCK180].<sup>6-12</sup> More than 70 members of the Dbl family have been identified to date,<sup>7</sup> which are typically classified by a ~200 amino acid DH domain adjacent to a ~100 amino acid PH domain in a “classical DH-PH” tandem arrangement. However, the DOCK family of GEFs lacks the DH-PH tandem but has DHR (Dock Homology Regions) that bind specifically to Rac and activate it.<sup>13,14</sup>

Specific activation of Rac2 by the GEF DOCK2 relies on two N-terminal elements found in this small GTPase, divergent amino acid residues in the switch-1 region and in the  $\beta 3$  strand that are necessary for mediating specific recognition by DH domain containing GEFs.<sup>15</sup>

C-terminal tyrosine phosphorylation of a GEF has been shown to be crucial to activation of its GEF function, as tyrosine to phenylalanine point mutations abrogate Vav-1 GEF function and interaction with other protein partners.<sup>16</sup>

### The Particular Architecture of PLD2 as a GEF

In considering PLD2 as a GEF, we have realized that it has some similarity and differences with respect to Dbl and DOCK family. Like Dbl, it has a PH domain and like DOCK it doesn't have a DH domain. However, the DH-PH tandem is far from being a definitive presence for GEF activity. First, and as mentioned above, PLC $\gamma$ 1 mediates the GTP/GDP exchange through its SH3 domain.<sup>17,18</sup> Second, other known GEFs do not possess PH domains. The p164-RhoGEF has a protein of 1510 amino acids (p164), contains a DH domain but lacks a PH domain and still activates RhoA but not Rac1 or Cdc42.<sup>19</sup> Similarly, a novel GEF namely Grinch-GEF has a DH domain, WD-40 domains and two transmembrane domains but lacks a PH domain, is specific to RhoA in vitro and for RhoA, B and C activation in vivo.<sup>20</sup> A GEF named GEF10 also lacks a PH domain.<sup>21</sup> Recent structural studies of GEF-GTPases complexes have also revealed mechanistic features that are shared by all GEFs. This means that they are different in their structures but work in the same way on different types of effectors. Therefore, PLD2 should have a common mechanism of action rather than common structural identity with known GEFs.

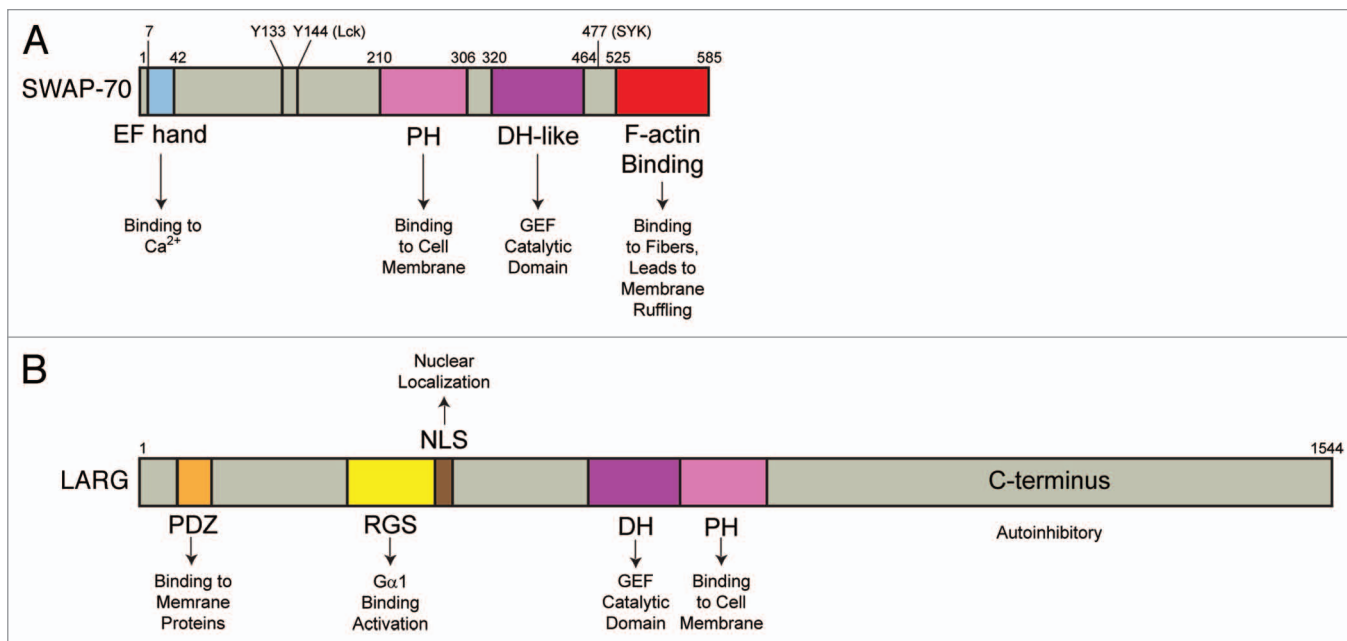
### Looking for Structural Homologies Between GEFs: SWAP-70 and LARG

It is known that SWAP-70 is a GEF that acting on Rac mediates actin cytoskeletal rearrangements found in cell during motility events.<sup>22,23</sup> Based on similar protein function (GEF), physiological function (cell motility), substrate specificity (Rac) and the presence of PH domains, we hypothesized that SWAP-70 was a good candidate for modeling PLD2 and predict structure-function correlations that could be key for cell chemotaxis. It is pertinent to review the biology of SWAP-1 before considering a structural analysis.

SWAP-70 (Switch-associated protein 70; alternate name, KIAA0640) is a phosphatidylinositol-3,4,5-trisphosphate (PI3P)-dependent guanine nucleotide exchange factor (GEF), that mediates integrin-mediated adhesion, cell attachment and membrane ruffling formation.<sup>24-26</sup> It interacts with and regulates proteins of the Rho family, most notably Rac.<sup>26,27</sup> Like many other GEFs, SWAP-70 has a pleckstrin-homology (PH) domain that binds to PI3P and anchors the protein to the cell membrane. A second key domain is the coil-coil sequence with some similarities to a Dbl homology domain (DH). This coil-coil DH-like domain is found to the C-terminus of its pleckstrin homology (PH) domain in SWAP-70 (Fig. 2A) a placement that is reversed from the usual arrangement of other classical Rho-GEFs.<sup>27</sup> SWAP-70 promotes the GDP/GTP exchange in Rac activating it, through an as-yet-unidentified mechanism.

Figure 2B shows the Leukemic Associated RhoGEF (LARG) architecture for comparison with SWAP-70. LARG, a GEF from the Dbl protein family, is a 1544 amino acid protein encoded by the *ARHGGEF12* gene causes cell transformation in hematopoietic cells and cytoskeletal rearrangements.<sup>28,29</sup> It is also found in the leading edge of chemotaxing neutrophils.<sup>30</sup>

In addition to the GEF activity, and closely related to its physiological function, SWAP binds to F-actin. The specific binding of SWAP-70 to F-actin vs. G-actin depends on cell stimulation and



**Figure 2.** Scheme of SWAP-70 and Leukemic-Associated RhoGEF (LARG) architectures with relevant domains. Functional domains of SWAP-70 (A) and LARG (B) RhoGEFs. Numbers correspond to amino acids and abbreviations are: PH, pleckstrin homology; DH, Dbl homology; EF hand, helix-loop helix structural domain; PDZ, post synaptic density, disc large, ZO-1 (zonal occludins); RGS, regulator of a protein signaling and NLS, nuclear localization signal.

is required for actin cytoskeletal architectural remodeling during cell movement<sup>26</sup> and makes SWAP-70 a key link from activated Rac to the actin cytoskeleton and cell motility.<sup>31</sup> In fact, SWAP-70 localizes to loose actin filament arrays located behind actively extending lamellipodia.<sup>32</sup> The C-terminal 60 residues of SWAP-70 (525–585) are responsible for F-actin association and subsequent cell motility.<sup>33</sup>

Regulatory molecules of SWAP-70 are tyrosine kinase receptors and cytosolic kinases such as Syk, Lck and c-kit in hematopoietic cells. C-kit phosphorylates SWAP-70 leading to cell-cell adhesion and migration in mast cells.<sup>31</sup> In B cells, SWAP-70 is tyrosine phosphorylated by Syk at position 517, during cell activation and actin remodeling. Phospho-site mutants of SWAP-70 disrupt B-cell activation and impair migration *in vivo*.<sup>34</sup>

SWAP-70's only close homolog is a protein called Def-6, SLAT or IBP, which was reported to function in T-cell signaling.<sup>35-37</sup> The SWAP-70-like adaptor of T cells (SLAT) is also a guanine nucleotide exchange factor for Rho GTPases that regulates the development of T-helper-cell inflammatory responses.<sup>37,38</sup> Structurally,

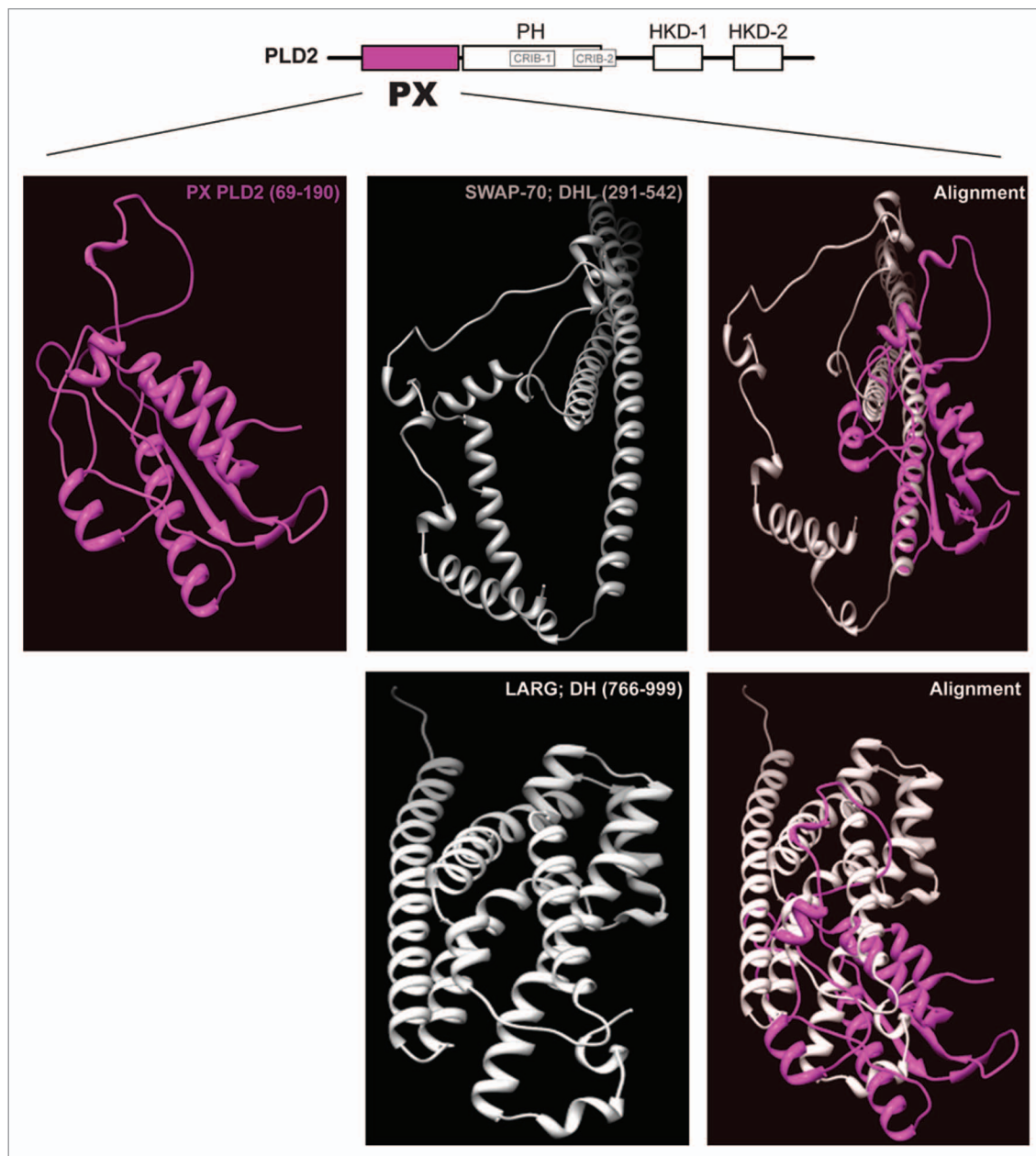
SLAT harbors, from the N terminus: a potential  $\text{Ca}^{2+}$ -binding EF-hand domain, an immunoreceptor tyrosine-based activation motif, and the PH and DH domains necessary for GEF activity.<sup>39,40</sup> There is a strict correlation between the structural features required for SWAP-70/SLAT membrane recruitment and downstream signaling, and require the Lck-dependent phosphorylation of Tyr-133 and Tyr-144.<sup>41</sup> Membrane targeting of the isolated DH domain is sufficient to enhance signaling and physiological responses.

### Modeling the N-terminal Portion of PLD2 and Comparing It to SWAP-70 and to LARG

Going back to PLD and putative similarities with SWAP-70, we realized that SWAP-70, like PLD, was found to have a GEF activity<sup>26</sup> only after its original description as a heavy-chain immunoglobulin class switch in B cells.<sup>24,42</sup> Likewise, PLD was found to have a GEF activity after only its lipase activity had been known. To better understand the novel finding of PXP-PLD2 as a GEF, we considered (Fig. 3 and top) the N-terminal portion of PLD2 that includes the PX

domain and the adjacent PH domain with two CRIB domains (CRIB1 and CRIB2), all of which are the sites where Rac2 binds to PLD2 as our laboratory has shown recently.<sup>3,4</sup> We modeled this portion of PLD2 as the crystal structure of this protein, at the time of writing has not as of yet been resolved. We also looked for putative structural homologies between PLD2 and LARG.

Computer simulations used the Threading ASSEMBLY Refinement algorithm through the server I-TASSER (Center for Computational Medicine and Bioinformatics University of Michigan <http://zhanglab.ccmb.med.umich.edu/I-TASSER>). This program employs a hierarchical protein structure modeling approach based on secondary-structure enhanced profile-profile threading alignment, combined with *ab initio* and Monte Carlo simulation, to arrive at low resolution structural predictions of a query amino acid sequence. We obtained predicted PX-PLD2 and PH-PLD2 structures and approximated them to the crystal structures of known proteins that elicit GTP binding in target GTPases. The images were originally a .pdb (protein data bank) structure file that was opened



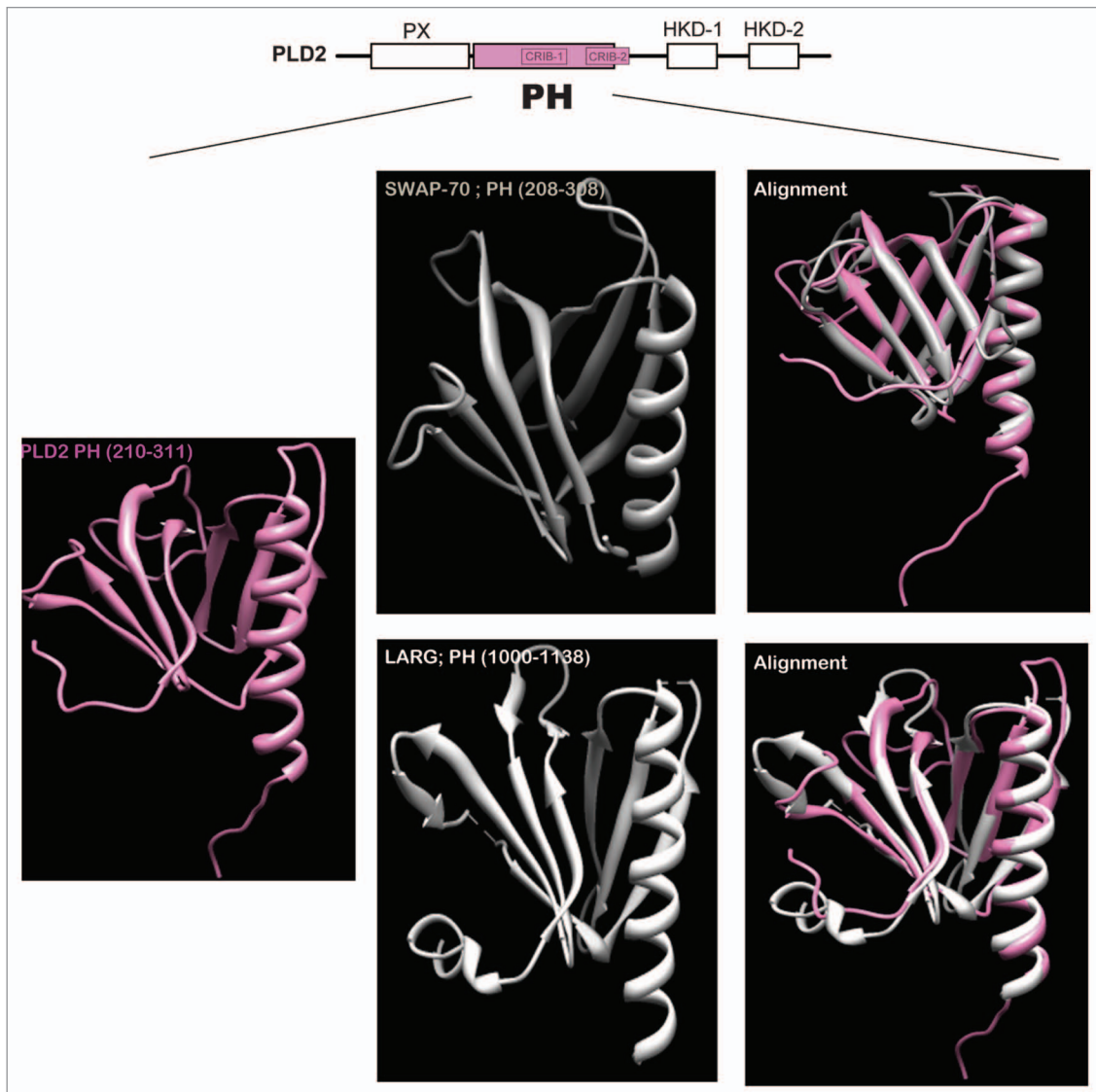
**Figure 3.** Computer generated prediction of match-up between LARG and SWAP-70 with PLD2-PX domain. Top: Schematic model of PLD2 with representation of the principal domains: PX, PH and HKDs (catalytic sites). Right panels: Representation of an image of the NMR solution of DHL domain of SWAP-70 (gray) or the DH domain of LARG (white) superimposed to the PLD2 PX domain (magenta). Both predictions were generated using I-TASSER (Center for Computational Medicine and Bioinformatics University of Michigan <http://zhanglab.ccmb.med.umich.edu/I-TASSER>). Alignments of 3D structures were visualized in “Chimera” <http://www.cgl.ucsf.edu/chimera/download.html>.

using the structure viewer “Chimera” (<http://www.cgl.ucsf.edu/chimera/download.html>).

In **Figure 3**’s left panel, a representative predicted structure of the PX domain (magenta) (amino acids 34–168) of PLD2 is shown, and we aligned it to the F chain of the RHOGEFs SWAP-70 or LARG that interact directly with GTP and Mg. The PX domain is rich in  $\alpha$ -helices like

the DH domain of these two RHOGEFs. We found that PLD2 PX domain has a single  $\alpha$ -helix that modestly aligns with that of the DH domains of SWAP-70 and LARG. The degree of 3D alignment is much lower than that seen in the case of PH. However, experimental evidence demonstrates a GDP/GTP exchange activity in GST-fusion proteins bearing PLD2’s PX domain.<sup>4,5</sup>

In the left panel of **Figure 4**, we modeled the PH domain of PLD2 (amino acids 210–324) (magenta) and superimposed it with the PH domain of SWAP-70 (gray) or LARG (white). These are GEFs that, independently of Ras, transduce signals from tyrosine kinase receptors to Rac. Since each mediates signaling of membrane ruffling and actin cytoskeletal architectural remodeling, these



**Figure 4.** Computer generated prediction of match-up between LARG and SWAP-70 with PLD2-PH domains. Top: schematic model of PLD2 with representation of the principal domains: PX, PH and HKDs (catalytic sites). Orange represents CRIB-1 in PLD2; purple, CRIB-2. Right panels: representation of an image of the NMR solution of the PH domain of SWAP-70 (gray) or LARG (white) superimposed to the PLD2 PH prediction (pink). Predictions and alignments are as indicated in the previous figure legend.

GEFs were good candidates for comparing to PLD2 as the Rac2-PLD2 association is also related to cell chemotaxis. As the figure indicates, the degree of superimposition of the PH domains of PLD2 and SWAP-70 or PLD2 and LARG is very extensive and includes similarities between one  $\alpha$ -helix and one  $\beta$ -barrel. CRIB-1 and CRIB-2 also appear to maintain significant overlap with regions in the tertiary structure of SWAP-70. Extensive similarities exist between the PH domain of PLD2 and other GEFs that possess PH domains, while the similarity between the PX domain of PLD2 and the DH domains

of other GEFs is more modest (only one  $\alpha$ -helix loop).

Both **Figure 3** and **Figure 4** indicate that PLD functions as a GEF utilizing its PH domain and part of its PX domain and possibly other regions. All this makes PLD unique, perhaps in an entirely new class of GEF. By bearing two enzymatic activities (break down of PC and GDP/GTP exchange), it is evident that PLD is an important signaling node for several intracellular pathways. New experiments will ascertain how the newly described PLD2's GEF is regulated in the context of cell activation.

### Specifics of RAC2 Activation

A striking feature of RhoGEFs is that they outnumber their target GTPases by a factor of 3, which means that multiple GEFs are capable of activating the same GTPase. In addition, many GEFs can activate more than one GTPase.<sup>7,43</sup> A recent workshop on exchange factors illustrated the individuality of the regulatory mode and function of each GEF member, as well as the common themes shared by closely related siblings or even evolutionarily diverse distantly related cousins. According to this study, many issues, including how

various intracellular signals converge on the GEFs for their tight regulation, what signals would diverge from their cognate small G-protein substrates and where each GEF resides in the wider context of signaling networks needs to be further tackled in this field. Thus it is not surprising that PLD2 is a GEF for Rac2, which is known to be acted upon by several other GEFs, like Vav1,<sup>44</sup> Tiam1,<sup>45</sup> P-Rex1,<sup>46</sup> SWAP-70<sup>31</sup> and Dock2.<sup>10</sup>

Here, it should be stated that none of the above GEFs are exclusive for Rac2. Indeed, a majority of GEFs activate more than one substrate GTPase. However, there is selectivity based upon the upstream factor that activates the GEF and directs it to the activation of a specific GTPase depending on the cellular function to be activated. This explains why the specificities of GEFs toward GTPases differ *in vitro* in comparison to *in vivo*.

As far as specific structural details of Rac2 activation by GEFs, Haeusler et al. compared the three Rac isoforms 1, 2 and 3 in terms of the nucleotide association and dissociation, specificity to Tiam-1. Rac2 has a decreased nucleotide association constant when compared with Rac1 and Rac3 and Tiam-1 activates preferentially Rac2. When Rac sequences are compared, there are six amino acid deviations in Rac2. However, all the deviations are not in the contact regions of Tiam-1 and Rac2, but, rather at the switch I region, the insert region and the very C-terminal end. The higher flexibility of the switch I regions of Rac1 and 3 when compared with Rac2 explains functional differences of these isoforms.<sup>45</sup> The rigidity in the Rac2 switch I region can also benefit from the PX/PH modular architecture of PLD2. This could position Rac2 as a verified target for more than one GEF and lends support to the recent discovery of PLD2's GEF function.

### PLD2's GEF Activity in Cancer

PLD2's GEF function is physiologically demonstrated in living cells, as silencing PLD2 results in reduced Rac2 activity, while PLD2-mediated Rac2 activation enhanced cell adhesion, chemotaxis and phagocytosis.<sup>1,3,4</sup> Results reported from our lab support the importance of functional

interactions between PLD2 and Rac2 in the biological response of cell migration.<sup>2,47</sup> There is another area in which PLD2 plays an important role, and that is in cancer. Reduced Rac2 expression and/or activation is implicated in diseases like immunodeficiency, whereas upregulated Rac2 activation is reported to be involved in oncogenesis in the case of acute myeloid leukemia, chronic myeloid leukemia and in certain cases of squamous cell carcinomas.<sup>47</sup> Further, cells from colorectal or breast tumors express high levels of PLD2, implicating it in tumor cell invasion/metastasis.<sup>48,49</sup> Since Rho-GTPases are mainly responsible for cell movement through the regulation of actin cytoskeleton, PLD2 is posited to be one of the major GEFs, upstream of these GTPases that induce their activation. Deregulation of RhoGEFs is very common in tumor invasion and metastasis.<sup>50</sup>

### Future Perspectives

All these particulars serve to illustrate the significance of understanding PLD2's newly described GEF activity and its regulation. Dynamics of PLD/PA/GTPases have been proposed in ON/OFF switches that put PLD at the center of integrating and coordinating upstream signals that are channeled to downstream applying pathways.<sup>51</sup> The new discovery of PLD2 as being a GEF broadens even more this landscape. On the basis of homology modeling, PLD2 aligns with 3D structures of the known RhoGEF SWAP-70 and LARG to a great extent through its PH domain and to a lesser extent through its PX domain making PLD2 a unique class of GEF, that uses its PH domain, part of the PX and perhaps other currently unidentified parts of the molecule for the GDP/GTP exchange of Rac2, which warrants further experimental confirmation.

It is becoming evident that this PLD can act as an important signaling node for a several intracellular pathways. The complex regulation of these systems is now beginning to be understood, even though no crystal structure of mammalian PLD2 is available as of yet. Intermolecular interactions and phosphorylation are two of the various mechanisms by which GEFs become active and it will be interesting to

ascertain how PLD2's GEF is regulated in the context of cell activation.

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