Specificity of Rho Insert-mediated Activation of Phospholipase D1*

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Members of the Rho subfamily of GTP-binding proteins regulate phospholipase D1 (PLD1) activity and signaling. In previous work, we demonstrated that binding of the Rho family member Cdc42 to PLD1 and the subsequent stimulation of its enzymatic activity are distinct events. Deletion of the insert helix from Cdc42 does not interfere with its switch I-mediated, GTP-dependent binding to PLD1 but inhibits Cdc42-stimulated PLD1 activity. To understand the mechanism of the insert-mediated activation of PLD1 by Cdc42 and to develop reagents to study Cdc42-activated PLD1 in cellular signaling events, we have undertaken a mutational analysis of the Rho insert region of Cdc42 and examined the specificity of the insert helix requirement in the other Rho family members, RhoA and Rac1. Here, we identify a critical residue, serine 124, in the Cdc42 insert helix central to its activation mechanism. Further, we examine this activation mechanism with respect to other members of the Rho family and demonstrate that each Rho protein activates PLD by distinct mechanisms, potentially allowing for unique signaling outcomes in the cell.

Phospholipase D (PLD) catalyzes the hydrolysis of phosphatidylcholine to yield phosphatic acid and choline. Phosphatidic acid is a second messenger involved in membrane-remodeling events that are critical to cell growth, such as vesicle trafficking and regulated secretion. Because of the important cellular functions of PLD and its products, the enzymatic activity of PLD is tightly regulated by a variety of hormones, growth factors, cytokines, and other agonists involved in cellular signaling (1–3). The human PLD1 (hPLD1) isosform is directly activated by several protein activators, including the Rho (4, 5) and Arf (6, 7) families of small GTP-binding proteins and protein kinase C (PKC) (5, 8), and this regulated activity is dependent on a lipid activator, phosphatidylinositol 4,5-bisphosphate (5, 6). Given the importance of the lipid products generated by PLD activation, we aim to understand the signaling events that control the enzymatic activity of PLD through these protein activators. In particular, we are interested in the connection between PLD activity and the cellular events mediated by the Rho family of GTPases.

PLD is unique among effectors for the Rho GTPases in that all three major Rho proteins, Rho, Rac, and Cdc42, are capable of binding to PLD and stimulating its enzymatic activity. In contrast to most other Rho effectors, PLD does not contain a recognizable binding motif, such as a CRIB domain, responsible for its GTP-dependent interaction with the switch I region of these GTPases. To study the specificity and contribution of each Rho family protein to PLD signaling necessitates an understanding of the protein-protein interactions required for the Rho proteins to bind PLD, as well as an understanding of the mechanism by which these interactions stimulate PLD activity. In previous work, we investigated the activation of hPLD1 by Cdc42 and demonstrated that there are three key features to the activation mechanism (9). First, activation of hPLD1 by Cdc42 requires prenylation of the GTPase. Cdc42 is geranylgeranylated at its carboxyl terminus, and this lipid modification, whereas not necessary for Cdc42 binding to hPLD1, is necessary for potent stimulation of PLD activity by Cdc42, suggesting that colocalization of Cdc42 and hPLD1 to phosphatidylinositol 4,5-bisphosphate-containing membranes is essential to the activation mechanism. Second, the binding of Cdc42 to hPLD1 and its subsequent activation are GTP-dependent. This GTP-dependent binding event is mediated by the switch I region of Cdc42, and a specific point mutation (Y40C) in the switch I region of Cdc42 renders Cdc42 unable to bind or activate hPLD1. Finally, in contrast to mutations in the switch I region, deletion of the entire Rho insert region from Cdc42 (designated Cdc42 ΔI8), a 13-amino acid insert helix unique to Rho family proteins, does not interfere with the GTP-dependent binding of Cdc42 to hPLD1 but inhibits Cdc42-stimulated hPLD1 activity. Interestingly, binding of Cdc42 ΔI8 to hPLD1 inhibits both the basal activity of PLD as well as the synergistic activation of hPLD1 by Arf and PKC. This work suggests that the binding of Cdc42 to hPLD1 and subsequent activation are separable and distinct events, with the switch I region mediating the GTP-dependent binding, while the insert helix of Cdc42 acts as an activation domain.

During this previous study, several papers had already demonstrated that the insert helix of Cdc42 is not required for regulation of the GTPase by GEFs or GAPs, nor is it required for Cdc42-mediated signaling events, such as Pak activation or cytoskeletal rearrangements (10, 11). Deletion of the insert helix from Cdc42 did, however, interfere with Cdc42-mediated transformation (11). The fact that deletion of the insert region does not perturb most conventional Cdc42 signaling pathways implies that a specific effector that utilizes the insert helix for activation is essential to Cdc42-mediated transformation. The requirement for the Cdc42 insert helix in activation of PLD activation suggests a role for PLD in Cdc42-mediated transformation. To study the role of PLD activation by Cdc42 in cellular signaling events, it is important to understand the underlying mechanism of this interaction, as well as the specificity of the insert helix in activation of PLD.

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To better understand the mechanism of PLD1 activation by the insert helix, we have conducted a mutational analysis of the Rho insert region of Cdc42. Specifically, we have individually mutated the solvent-exposed residues along the Cdc42 insert helix (Ser-124, Glu-127, Lys-128, Lys-131, Asn-132) to alanine to assess their role in PLD1 activation. Mutations E127A, K128A, K131A, and N132A are right shifted in their ability to activate PLD1 in a dose-dependent manner. These results suggest that a mechanism in which a single residue, Ser-124, makes an essential contact with hPLD1 for activation of its enzymatic activity. Charge-charge interactions between the large charged residues on the surface of the Cdc42 insert helix and hPLD1 are likely responsible for properly positioning this key activation residue.

As noted above, PLD is unique among Rho-family effectors in its ability to interact with all three members of the Rho family. To assess the general importance of the Rho insert region in PLD activation, we performed mutational analysis of the insert helices of RhoA and Rac1. Our data demonstrates that unlike Cdc42, where the insert helix plays an essential role in PLD activation, the insert helix of RhoA is important to but not essential for the ability of RhoA to activate hPLD1. In contrast to Cdc42 and RhoA, Rac1 activation of hPLD1 shows no dependence on its insert helix. These results suggest that the specificity of signaling to hPLD1 through the Rho family proteins may be regulated not only by specific upstream signaling pathways but also through the interaction between a given protein activator and the Rho insert region of Cdc42. Specifically, we have individually mutated the insert helix and PLD1 and the underlying mechanism of this interaction.

### EXPERIMENTAL PROCEDURES

#### Mutagenesis and Molecular Biology—The Cdc42, Rho, and Rac constructs/proteins used in these studies were derived from human sequences. Construction of the Cdc42 ΔL8 mutant was described previously (10). All mutations (including the ΔL8 mutations of RhoA and Rac1 and the 5A mutant of Cdc42) were generated using the QuikChange protocol (Stratagene) with the constructs in pet15b as templates (Table I). Mutations were confirmed by restriction digest analysis, when applicable, and sequence analysis in all cases. The generation of hexahistidine-tagged Cdc42, Rac, and Rho constructs in the pVL1392 vector (PharMingen) and subsequent virus production were completed as previously described (9).

The D4 PLD1 fragment was generated using PCR with full-length hPLD1 in the pcMVM vector as the template. The D4 PLD1 product was inserted into pet15b using NdeI sites engineered during the PCR reaction.

#### Protein Expression—Expression and purification of recombinant hPLD1, Cdc42, PKCβII, and Arf1 were carried out as described previously (9). Recombinant human RhoA and Rac1 were expressed and purified using the same protocol as that for Cdc42 expression and purification. Briefly, for the production of the geranylgeranylated GTPases, monolayers of S21 cells were infected with baculovirus encoding the hexahistidine-tagged constructs. Cells were lysed by Dounce homogenization in a binding buffer (20 mM Tris-Cl, pH 8.0, 500 mM NaCl, 5 mM MgCl₂, 1.5 mM imidazole, 0.2% Triton X-100, 0.4% CHAPS, 100 µg/mL protease inhibitor mixture). The lysate was centrifuged at 27,000 × g for 45 min at 4 °C and the supernatant was loaded onto a 1-ml Hitrap chelating column (Amersham Biosciences) charged with 50 mM nickel sulfate and equilibrated with binding buffer. The column was washed with binding buffer followed by wash buffer (20 mM Tris-Cl, pH 8.0, 500 mM NaCl, 60 mM imidazole, 0.2% N-acytly-l-p-glucopyranoside). The protein was eluted using the same buffer supplemented with 250 mM imidazole.

Hexahistidine-tagged D4 PLD1 was expressed in the Escherichia coli strain BL21 DE3 (Novagen). Protein expression was induced with 0.3 mM isopropyl-1-thio-β-D-galactopyranoside at room temperature for 12–16 h. Cell pellets were lysed with binding buffer supplemented with 10% glycerol followed by centrifugation at 45,000 × g for 1 h at 4 °C. The supernatant was loaded onto a 5-ml HiTrap chelating column charged with 50 mM nickel sulfate and equilibrated with binding buffer. The loaded column was washed with 100 ml of binding buffer followed by 100 ml of wash buffer. The protein was eluted using a gradient of imidazole (0–1 M). Fractions containing D4 PLD1 were pooled, concentrated, and snap frozen.

#### PLD Activity Assay—Activity in the exogenous substrate assay was measured as described previously (9, 12). The hPLD1 source for the exogenous assays was cytosol from S21 cells infected with hPLD1 baculovirus, and all assays contained 3 µg of this cytosol unless otherwise noted on the graph label. The data in Fig. 6 was generated with an SP-Sepharose-purified fraction of S21 cytosol expressing hPLD1; this fraction and its purification were described previously (9). Reactions were incubated for 30 min at 37 °C with 10 µM GTPγS in the presence of lipid vesicles containing radiolabeled substrate ([3H]dipalmitoyl phosphatidylcholine). Activity was measured using scintillation counting of the released, soluble product, [3H]choline. The PLD activity data were plotted using a simple equilibrium model assuming a bimolecular interaction between the protein activator (i.e. Cdc42) and hPLD1. Curves were obtained using the IGOR pro software package (Wave Metrics).

### RESULTS

#### Residues of the Insert Helix Are Essential to Cdc42 Activation of hPLD1—Previous data from our laboratory demonstrated
that a construct of Cdc42 in which the entire Rho insert helix and an additional seven residues were deleted and replaced with loop 8 of Ras (Cdc42 ΔL8) retains GTP-dependent binding to hPLD1 but is unable to stimulate the enzymatic activity of hPLD1 (9). In fact, binding of Cdc42 ΔL8 to PLD inhibits both basal and stimulated PLD activity. This data established a role for the Rho insert helix of Cdc42 not as a binding region for PLD but as an activation domain in the regulation of PLD activity. Here we further demonstrate that this regulation is the result of a specific interaction between Cdc42 ΔL8 and PLD by coupling the ΔL8 mutation with a mutation in the switch I region (Y40C), which abolishes Cdc42 binding to hPLD1. This Cdc42 Y40C/ΔL8 construct neither activates nor inhibits hPLD1 activity (Fig. 1). This result clearly demonstrates that the inhibition of PLD activation by Cdc42 ΔL8 requires switch I-dependent binding to PLD.

The ΔL8 construct removes the insert helix as well as a significant number of surrounding residues by replacing residues 120–139 with loop 8 from Ras. To specifically implicate residues within the insert helix in PLD activation, we generated a Cdc42 mutant in which the five solvent-exposed residues on the insert helix (Ser-124, Glu-127, Lys-128, Lys-131, Asn-132) were mutated in combination to alanine (designated Cdc42 S124A). As shown in Fig. 1, Cdc42 S124A does not activate hPLD1. Thus, the inability of the Cdc42 ΔL8 construct to activate hPLD1 can be attributed to the loss of solvent-exposed residues within the insert helix itself.

The Ser-124 Residue in the Insert Helix Is Essential for Activation of hPLD1 by Cdc42—To further delineate the contribution of individual residues on the insert helix of Cdc42 to hPLD1 activation, we singly mutated each of the solvent-exposed residues on the helix to alanine. Mutation of the large charged residues Glu-127, Lys-128, Lys-131, and Asn-132 results in a right shift of the dose-dependent activation of hPLD1 by Cdc42, while mutation of Ser-124 completely blocks hPLD1 activation (Fig. 2A). This data suggests that the interaction between the insert helix and hPLD1 is bifunctional. A single residue, Ser-124, is critical for the insert-mediated activation of hPLD1 by Cdc42. Charge-charge interactions between surrounding residues of the insert helix and hPLD1 likely play a role in orienting this critical activation residue to stimulate the full catalytic activity of PLD. It is interesting to note that unlike Cdc42 ΔL8, which inhibits Arf and PKC stimulation of PLD, Cdc42 S124A has no effect on Arf or PKC activation (Fig. 2B). This suggests that the residues from loop 8 of Ras, which replace the insert helix in the ΔL8 construct, may perturb the site on hPLD1 that normally binds to Ser-124 in its proper orientation. It also demonstrates that binding of Cdc42 to PLD without activation (S124A) is insufficient for the synergistic effects normally observed with wild type Cdc42 in combination with Arf or PKC.

Deletion of the insert helix from Cdc42 results only in a loss of PLD activation; binding is unaffected. This loss of activation can now be attributed to a single residue, Ser-124. To date, this mutation in Cdc42 appears to only affect activation of PLD. Furthermore, just as the ΔL8 mutation does not interfere with regulation of the nucleotide cycle of Cdc42 (nucleotide exchange or hydrolysis) or most Cdc42-mediated signaling events (10, 11), the S124A mutations do not interfere with exchange or hydrolysis, nor do they interfere with conventional effector interactions (i.e. association with the CRIB domain of PAK) (data not shown).

Distinct Roles for the Insert Helices of the Different Rho Family Proteins in the Activation of hPLD1—Having examined the role of the insert helix of Cdc42 in hPLD1 activation in detail, we examined the requirement of the insert helix for other Rho family members, RhoA and Rac1, in PLD activation. We constructed and expressed the ΔL8 versions of RhoA and Rac1 to assess the roles of their insert helices in hPLD1 activation. Deletion of the insert helix from Cdc42 results in inhibition of hPLD1 activity. However, this is not true for either RhoA or Rac1 (Fig. 3). Deletion of the insert from RhoA results in a partial loss of hPLD1 activation; thus, the insert helix on RhoA seems to be involved in but not necessary for RhoA activation of hPLD1. Deletion of the insert helix from Rac1 has little or no effect on its ability to activate hPLD1, as wild type...
and ΔL8 Rac1 activate hPLD1 to the same extent. These results clearly demonstrate differential requirements for the insert helix in activation of PLD by different Rho family proteins. It suggests that the specificity of activation of PLD, unlike other Rho GTPase effectors, results from the concerted action of the switch I region and the insert helix.

RhoA Activation of hPLD1 Utilizes a Mechanism Distinct from That of Cdc42 Activation

Because deletion of the insert helix from RhoA resulted in a partial loss of hPLD1 activation, we examined the contribution of individual residues along the RhoA insert to this activation event. The insert helices of the Rho proteins are most divergent in sequence at the amino-terminal end of the helix (Fig. 4A); therefore, we singly mutated the solvent-exposed residues Glu-125, His-126, and Thr-127 at the amino-terminal end of the RhoA insert helix to alanine. Mutations H126A and T127A lead to a right shift in the dose-dependent RhoA activation of hPLD1, similar to mutations of the large, charged residues of the Cdc42 insert helix (Fig. 4B). Interestingly, RhoA E125A was no better than RhoA ΔL8 in activating hPLD1 (Fig. 4B). It seems that this residue is a critical residue for the insert helix-mediated activation of hPLD1 by RhoA, similar to Ser-124 of Cdc42. Because deletion of the insert helix of RhoA does not result in the complete loss of PLD activation, as is the case for Cdc42, this residue is not the sole determinant of RhoA activation. Thus, although both Cdc42 and RhoA share a common requirement for a specific residue in insert activation of hPLD1, the mechanisms by which these two proteins activate hPLD1 are distinct.

The Carboxyl Terminus of hPLD1 Differentially Interacts with Individual Rho GTPases—Our results demonstrate that the specificity of activation of hPLD1 by individual Rho proteins results from differing requirements for the insert helix. Activation of hPLD1 by all Rho GTPases requires an initial GTP-dependent binding event mediated by switch I of the Rho protein. Several groups have shown that RhoA binds in a GTP-dependent manner to residues within the carboxyl-terminal region of PLD (residues 712–1074 in hPLD1) (13–15). Mutations in this region of PLD1 that block RhoA binding also block activation of PLD by RhoA, suggesting that this carboxyl-terminal region of PLD contains the switch I binding site for RhoA. The assumption is that this region is also the site for binding of Cdc42 and Rac1 through their switch I domains. Here we test this hypothesis by examining the competitive effect of a carboxyl-terminal domain of hPLD1 (D4 PLD1) on
the activation of full-length hPLD1 by RhoA, Cdc42, and Rac1 (Fig. 5). In agreement with previous data, we show that addition of D4 PLD1 in increasing amounts does not compete for activation of hPLD1 by Arf or PKC. We also confirm that the carboxyl terminus of PLD1 is an interaction site for RhoA, as D4 PLD1 competes in a dose-dependent manner for activation of hPLD1. Deletion of the insert helix from RhoA seems to be involved in but not necessary for RhoA activation of hPLD1. Deletion of the insert helix from Rac1 has little or no effect on its ability to activate hPLD1, as wild type and ΔL8 Rac1 activate hPLD1 to the same extent.

**Fig. 3.** Deletion of the insert helices from RhoA and Rac1 has differential effects on hPLD1 activation. Unlike Cdc42 ΔL8 (top panel), neither RhoA ΔL8 (middle panel) nor Rac1 ΔL8 (bottom panel) inhibit PLD activity. Deletion of the insert helix from RhoA results in a partial loss of hPLD1 activation; thus, the insert helix on RhoA seems to be involved in but not necessary for RhoA activation of hPLD1. Deletion of the insert helix from Rac1 has little or no effect on its ability to activate hPLD1, as wild type and ΔL8 Rac1 activate hPLD1 to the same extent.

**DISCUSSION**

PLD is unique among effectors for the Rho GTPases in that all three major Rho proteins, RhoA, Rac1, and Cdc42, are each capable of binding to PLD and stimulating its enzymatic activity. In contrast to most other Rho-family effectors, PLD does not contain a recognizable binding motif, such as a CRIB do-
main, responsible for its GTP-dependent interaction with the switch I region of these GTPases. Given the unique nature of this effector interaction and the importance of lipid products generated by PLD in cellular events, we are interested in understanding the mechanisms underlying the ability of Rho GTPases to stimulate PLD activity, the specificity of the interaction between the Rho GTPases and PLD, and the contribution of each Rho-family protein to PLD signaling. In previous work we implicated the Rho insert helix of Cdc42 in PLD activation (9). Here, we have further delineated the mechanism by which Cdc42 activates PLD, identifying a critical residue in the insert helix central to the activation mechanism. We also examined this activation mechanism with respect to other members of the Rho family and demonstrated that each Rho protein activates PLD by distinct mechanisms, allowing for unique signaling outcomes in the cell.

Fig. 4. RhoA activation of hPLD1 utilizes a distinct mechanism from that of Cdc42 activation. A, sequence alignment of the insert helices from RhoA, Rac1, and Cdc42. The line below the alignment spans the Rho insert region, while the shaded bar above denotes the section of the insert region that forms the secondary helical structure. The dots above the sequence mark the solvent-exposed residues of the helix. Conserved and similar residues are bolded. B, activation of PLD by RhoA constructs with insert helix mutations. The solvent-exposed residues at the amino terminus of the RhoA insert helix (Glu-125, His-126, and Thr-127) were each mutated to alanine to disrupt potential interactions required for RhoA activation of PLD1. Despite utilizing the insert helix in PLD activation, Cdc42 and RhoA differ in the mechanism by which they use this region.

Fig. 5. The carboxyl terminus of hPLD1 differentially interacts with individual Rho GTPases. A carboxyl terminal domain of hPLD1 (D4 PLD1), previously shown to compete for RhoA binding to and activation of full-length PLD1, interacts differently with Cdc42 and Rac1. SP-Sepharose purified hPLD1 (640 ng) was assayed in the presence of 28 nM PKCβII, 350 nM Arf1, 300 nM RhoA, 300 nM Cdc42, or 300 nM Rac1 with increasing amounts of D4 PLD1. PLD activity was measured as described under “Experimental Procedures” and is represented here as a percentage of the activity stimulated by each activator at the concentrations listed above in the absence of D4 PLD1.
A number of groups, including our own, have demonstrated that this event is mediated through a GTP-dependent conformational change in the switch I region of the GTPase (9, 13), as specific point mutations in switch I abrogate both binding and activation. The region on PLD that binds to the active conformation of the switch I region is still not clear. Several groups have shown that RhoA binds in a GTP-dependent manner to residues within the carboxyl-terminal region of PLD (residues 712–1074 in hPLD1) (13–15). Mutations in this region that block RhoA binding in RhoA activation of PLD1 do not further increase PLD activity. Activation of PLD1 by Cdc42 only occurs when the insert helix interacts with PLD1; this activation utilizes a critical serine residue (Ser-124, represented by a star) in the amino terminus of the insert helix.

In the work described here, we demonstrate that following the GTP-dependent binding event, the mechanism of PLD activation by each Rho-family GTPase is distinct. Specifically, we demonstrate that Cdc42, RhoA, and Rac1 exhibit a differential requirement for the insert helix in stimulating the enzymatic activity of PLD, as summarized in the model in Fig. 6. Deletion of the insert helix from each Rho protein shows that Cdc42 absolutely requires the insert helix for activation of PLD, Rac1 activation of PLD does not involve the insert helix at all, and the insert helix of RhoA contributes to PLD activation but is not essential. We have identified a single residue within the insert helix of Cdc42 (Ser-124) that is critical to the activation mechanism, as mutation of this residue to alanine completely blocks the ability of Cdc42 to activate PLD. In addition, surrounding residues in the insert helix appear to play a role in charge-charge interactions with PLD that serve to orient the critical serine residue.

Deletion of the insert helix from RhoA compromises its ability to stimulate the enzymatic activity of PLD relative to wild type RhoA. Unlike Cdc42 ΔL8, RhoA ΔL8 is able to stimulate PLD activity, achieving a level of activation similar to wild type Rac1 or Cdc42. However, with the insert helix, RhoA stimulates PLD approximately four to five times better. Similar to Ser-124 in Cdc42, a single residue, Glu-125, makes a significant contribution to the insert-mediated stimulation of PLD by RhoA, and its interaction with PLD is facilitated by charge-
charge interactions between surrounding residues of the helix and PLD. Furthermore, there is clearly a contribution of the switch I region, or possibly other regions of RhoA, to the activation of PLD. A secondary contribution may come from an interaction with switch II on RhoA, as a single residue, Asp-76, in RhoA has been shown to contribute to the activation of PLD (16). In contrast to RhoA and Cdc42, the Rac1 insert makes no contribution to the activation of PLD. It seems that the switch I of Rac1, or possibly the switch I combined with a different secondary region, is solely responsible for the activation of PLD.

The loss of PLD1 activation by mutations in the Cdc42 insert helix is the first demonstration of a Cdc42 effector whose activity is completely dependent on residues within the insert helix. This data is also the first demonstration that the underlying mechanisms involved in activation of PLD by individual Rho family GTPases are distinct for each protein. In combination with work from other laboratories on the insert helix and effector interactions, our previous (9) and current work suggests that the Rho insert helix is not an effector-binding region but serves as an activation domain for a variety of effectors. Recent work from Zong et al. (17) studied the role of the insert helix of RhoA in effector binding and activation. Similar to our earlier results on the Cdc42/PLD interaction (9), they showed that the insert helix was not required for binding to the Rho effector, Rho kinase, but was essential for stimulation of its kinase activity, as well as for Rho-mediated foci formation in vivo (17).

Similarly, work from both the Lambeth and Bar-Sagi laboratories independently demonstrated that the insert region in Rac is essential to activation of the NADPH oxidase (18) and is critical for the ability of Rac to promote cell cycle progression, as well as mitogenesis through regulation of superoxide production (19). Furthermore, Rac-induced elevation of superoxide levels plays a critical role in Ras-mediated transformation (20). Recent work from Diebold and Bokoch showed that activation of the oxidase by Rac2 involves an insert helix-dependent interaction between Rac2 and cytochrome b (21). This interaction is subsequent to a GTP-dependent interaction between the switch I region of Rac2 and another component of the oxidase system, p67phox.

Interestingly, like PLD, p67phox does not contain a conventional Rho-protein binding domain. Switch I of Rac binds to a TPR motif on p67phox (22), a unique mode of effector binding to its associated GTPase. This binding event, and the subsequent activation of the oxidase complex through contacts between the Rac insert helix and cytochrome b, is specific to Rac; neither Cdc42 nor Rho can stimulate superoxide production. Two residues in Rac, Ala-27 and Gly-30, are responsible for this specificity. In fact, changing the equivalent residues in Cdc42, Lys-27 and Ser-30 to alanine and glycine, respectively, allows Cdc42 to bind p67phox and activate the oxidase system (22, 23). In this system, the specificity of signaling resides in the switch I region of the GTPase. This is in contrast to PLD, which can be activated by a range of Rho proteins, with the specificity in signaling arising from distinct activation mechanisms employed by each Rho GTPase.

Taken together, our data suggests a unique and specific role of the insert helix of each Rho GTPase in signaling to PLD1. It is interesting to speculate that the distinct mechanisms of PLD activation by each Rho protein may result in distinct outcomes in cell signaling. We are specifically interested in the unique cellular outcomes of Cdc42 signaling to PLD, and in particular, that the role PLD plays in Cdc42-mediated transformation. Because the Rho insert helix of Cdc42 is required not as a binding region but as an activation domain for PLD, the most interesting finding is that Cdc42 shows an absolute dependence on a critical residue in its insert helix for stimulation of PLD1 activity. The insert helix on Cdc42 is absolutely required for its ability to transform cells (11) and for its ability to activate PLD (9). PLD is the only known Cdc42 effector that requires the insert helix for its activation. These observations implicate PLD as a highly specific effector for a Cdc42-mediated signaling pathway that, when up-regulated, may lead to transformation. This potential role for PLD in Cdc42-mediated transformation, which is dependent on the insert helix of Cdc42, is analogous to the activation of both the NADPH oxidase system by Rac and activation of Rho kinase activity by RhoA. In these interactions the insert helix acts as an activation domain, responsible for stimulating the activity of the effector. Interestingly, in each case, the insert helix is involved in effector activation events that can be linked to signaling pathways leading to enhanced cell growth and transformation through the activated effector. The studies described here represent an important step in understanding the cell biology of PLD activation by the Rho GTPases. The realization that each Rho protein utilizes a distinct mechanism in activating PLD suggests that these underlying mechanisms may impart specificity to PLD signaling through the Rho GTPases. The identification of a critical residue in the Cdc42 insert helix that is responsible for the activation of PLD by Cdc42 provides a powerful reagent for the study of the specific cellular events regulated by Cdc42 activation of PLD.

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