Activation of Phospholipase D1 by Cdc42 Requires the Rho Insert Region*

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Members of the Rho subfamily of GTP-binding proteins are implicated in the regulation of phospholipase D (PLD). In the present study, we demonstrate a physical association between a Rho family member, Cdc42, and PLD1. Binding of Cdc42 to PLD1 and subsequent activation are GTP-dependent. Although binding of Cdc42 to PLD1 does not require geranylgeranylation, activation of PLD1 is dependent on this lipid modification of Cdc42. Specific point mutations in the switch I region of Cdc42 abolish binding to and, therefore, activation of PLD1 by Cdc42. Deletion of the Rho insert region, which consists of residues 120–139, from Cdc42 does not interfere with binding to PLD1 but inhibits Cdc42-stimulated PLD1 activity. Interestingly, deletion of the insert region from Cdc42 also inhibits activation of PLD1 by Arf and protein kinase C. With the lack of specific inhibitors of PLD activity, the insert deletion mutant of Cdc42 (designated (ΔL8)Cdc42) is a novel reagent for in vitro studies of PLD1 regulation, as well as for in vivo studies of Cdc42-mediated signaling pathways leading to PLD1 activation. Because the insert region is required for the transforming activity of Cdc42, regulation of PLD1 by this region on Cdc42 is of major interest.

Phospholipase D (PLD) catalyzes the hydrolysis of phosphatidylcholine to yield phosphatidic acid and choline. Phosphatidic acid is a second messenger involved in vesicle trafficking and regulated secretion. Phosphatidic acid and one of its metabolic products, lysophosphatidic acid, are potent mitogens and can activate target protein kinases, as well as other agonists involved in cellular responses, including reorganization of the actin cytoskeleton, regulation of gene transcription, and cell cycle progression. Activation of each member of the Rho subfamily, Rho, Rac, and Cdc42, not only leads to distinct outcomes with respect to cytoskeletal rearrangements but also makes a specific and unique contribution to DbI transformation. Recent evidence suggests that Rho and Rac activate PLD in vivo through growth factor receptors and heterotrimeric G proteins. To address the role of Cdc42 activated PLD1 in signaling processes, we have undertaken a domain analysis study of Cdc42 with respect to binding and activation of PLD1.

Several regions on Cdc42 are of potential interest to PLD1 activation because they play important roles in binding and activation of other target effectors, as well as being implicated in activation of PLD1 by RhoA (18). For example, the switch I region, or effector loop, is highly conserved between Cdc42 and RhoA, and mutations in this region alter the regulation of effectors, such as PAK and IQGAP. Of particular interest is a region of 13 amino acids (residues 122–134 in Cdc42) designated the Rho insert region. This region is unique to the Rho subfamily and is not present in Ras and other small G proteins. Although this region does not function as a “switch domain” upon GTP-GDP exchange (19, 20), it may provide a surface for binding interactions with target effectors. A deletion mutant of Cdc42 in which the insert region is replaced with loop 8 of Ras (designated (ΔL8)Cdc42) is capable of both intrinsic and stimulated GTP binding and GTP hydrolytic activities. Furthermore, (ΔL8)Cdc42 can functionally interact with regulators of the GTP-binding and hydrolytic cycle, as well as with all known target effectors of Cdc42. Removal of the insert region does not perturb Cdc42-mediated JNK activation or filopodia formation (21, 22). Interestingly, further characterization of the (ΔL8)Cdc42 mutant implicated the insert region in the transforming ability of Cdc42, specifically interfering with anchorage-dependent growth.

Here, we present evidence that binding of Cdc42 to PLD1 and subsequent PLD1 activation are distinct events. PLD1 utilizes the switch I domain of Cdc42 as part of its binding

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interface but requires the Rho insert region for complete activation by Cdc42, as well as for the synergistic activation of PLD1 caused by Cdc42 working together with either Arf or PKC. The binding of Cdc42 to PLD1 is GTP-dependent but does not require either the geranylgeranyl moiety or the insert region on Cdc42. However, activation of PLD1 by GTP-bound Cdc42 requires both the insert region and geranylgeranylation of the GTPase. To date, PLD1 is the only effector of Cdc42 that requires the Rho insert region for stimulated activity, making PLD1 a candidate effector for Cdc42-mediated signaling events leading to cellular transformation.

EXPERIMENTAL PROCEDURES

Purification of Recombinant PLD1—Monolayers of Spodoptera frugiperda 21 (Sf21) cells were infected with baculovirus encoding human PLD1. Cells were resuspended in Buffer A (8.1 mM NaH₂PO₄, 1.5 mM KH₂PO₄, 137 mM NaCl, 2.7 mM KCl, 2.5 mM EDTA, 1 mM diethiothreitol, protease inhibitor mixture) and lysed by nitrogen cavitation at 4 °C for 30 min. The lysate was centrifuged at 174,000 × g for 1 h to prepare a cytosolic fraction, which was loaded onto a SP-Sepharose column equilibrated with Buffer B (20 mM Hepes, pH 7.5, 1 mM EDTA, 1 mM diethiothreitol). After washing with Buffer B containing 20 mM KCl, the PLD activity was eluted with a linear gradient of 0–100 mM KCl in Buffer B. The baculovirus encoding human PLD1 was a kind gift from Drs. Michael Frohman and Andrew Morris at SUNY Stony Brook.

Purification of Recombinant Cdc42Hs—Glutathione S-transferase (GST)-tagged Cdc42Hs constructs were expressed in Escherichia coli and purified by glutathione-agarose affinity chromatography as described (23). Cloning of wild type and (∆L8)Cdc42Hs baculoviruses were described previously (21), and cloning of (Q61L), (T17N), and (F37A/Y40C)Cdc42Hs into the insect cell baculovirus vector pVL1392 was described previously (21). Cloning of wild type and (∆L8)Cdc42Hs baculoviruses were described previously (21), and cloning of (Q61L), (T17N), and (F37A/Y40C)Cdc42Hs into the insect cell baculovirus vector pVL1392 was achieved using the same protocol. For the production of geranylgeranylated Cdc42Hs, monolayers of Sf21 cells were infected with baculovirus encoding hexahistidine-tagged Cdc42Hs constructs. Cells were lysed by Dounce homogenization in binding buffer (20 mM Tris-Cl, pH 8.0, 500 mM NaCl, 5 mM imidazole, 0.4% CHAPS, 100 μM GDP, protease inhibitor mixture). The lysate was centrifuged at 27,000 × g for 45 min at 4 °C, and the supernatant was loaded onto a HiTrap chelating column (Amersham Pharmacia Biotech) equilibrated with Buffer C. The column was washed in Buffer C, and the protein was eluted with a linear gradient of 0–500 mM KCl in Buffer C. The PKC αII baculovirus was a kind gift from Dr. Alexandra Newton at the University of California, San Diego.

In Vitro Binding Experiments—Binding experiments with GST-tagged Cdc42Hs were carried out essentially as described previously (26). PLD1 binding to Cdc42 was analyzed by Western blot analysis using polyclonal antibodies raised against the carboxyl terminus of human PLD1.

Coimmunoprecipitation of Cdc42 and PLD1—COS7 cells or NIHpcpcsrc527/foe/ep/bI cells (NIH3T3–527 cells) (27) were cotransfected with pKHe3-Cdc42Hs constructs and pCMV3-PLD1 using LipofectAMINE according to the manufacturer’s standard protocols (Life Technologies, Inc.). Cells were washed in cold phosphate-buffered saline and lysed in 1% Nonidet P-40 lysis buffer (50 mM Tris-Cl, pH 8.0, 50 mM KCl, 1 mM EDTA, 1% Nonidet P-40, protease inhibitor mixture). The lysates were preclarified by centrifugation for 30 min at 4 °C, followed by incubation with 10% (w/v) protein A-Sepharose beads in immunoprecipitation buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% Tween-20) for 1 h at 4 °C. The beads were pelleted, and the supernatant was incubated with antibody to the hemagglutinin (HA) tag on Cdc42Hs for 3 h at 4 °C. Following primary antibody incubation, protein A beads were added, and the mixture was incubated for 1 h at 4 °C. The beads were washed three times with immunoprecipitation buffer and three times with phosphate-buffered saline and resuspended in sample buffer. The immunoprecipitated proteins were separated by SDS-polyacrylamide gel electrophoresis and analyzed by Western blotting with anti-HA (Berkeley Antibody Co.) or anti-PLD1 antibodies.

Exogenous Substrate Assay of PLD Activity—PLD activity was measured essentially as described previously (25), using the SP-Sepharose peak described above. Reactions were incubated for 30 min at 37 °C with 10 μM GTPγS.

RESULTS AND DISCUSSION

We initially set out to examine which regions on Cdc42, a Rho family GTPase, were involved in binding to PLD1 by examining the ability of different forms of GST-Cdc42 fusion proteins to precipitate ectopically expressed PLD1 from COS7 cell lysates. As shown in Fig. 1, the constitutively active, GTP-bound (Q61L)Cdc42 mutant more effectively bound PLD1 compared with wild type Cdc42. Deletion of the Rho insert region does not interfere with the binding of Cdc42 to PLD1. However, two effector loop mutations (Y40C and F37A) abolish the GTP-dependent binding of Cdc42 to PLD1. RhoA and Cdc42 exhibit higher sequence similarity in the effector loop, or switch I region, than other members of the Rho subfamily, and identical
mutations (Y42C and F39A) in RhoA were previously shown to interfere with the activation of PLD1 by RhoA (18). The GTP-dependent binding of Cdc42 to PLD1 does not require the geranylgeranylation modification of the GTPase, because the binding results shown in Fig. 1 were carried out using bacterially expressed Cdc42 constructs.

The specificity of Cdc42 binding to PLD1 was verified by co-expressing these proteins in cells (Fig. 2A). HA-tagged GTPase defective (Q61L)Cdc42 was more effective in co-immunoprecipitating PLD1 than was wild type HA-Cdc42 (compare lanes 1 and 3). Here again, this binding does not require the insert region of the GTPase (compare lanes 3 and 5). The effector loop region is required for in vivo binding, because identical mutations to those used in the in vitro binding experiments abolished the interaction of Cdc42 and PLD1 in vivo (data not shown). Finally, endogenous PLD1 from NIH3T3–527 cells also associated with GST-Cdc42 in a GTP-dependent manner independent of the insert region (Fig. 2B). Previous reports have demonstrated associations between RhoA and PLD1 by two-hybrid analysis (28). Yamazaki et al. (29) showed that RhoA interacts with a carboxyl-terminal fragment of human PLD1 and that this interaction was decreased by deletion of the CAAX box from RhoA. Our findings demonstrate a physical association between full-length PLD1 and a Rho family activator, Cdc42.

We next examined the ability of specific Cdc42 mutants to activate PLD1 in an in vitro system. Unlike binding of Cdc42 to PLD1, activation of PLD1 by this Rho family GTPase requires geranylgeranylation. Isoprenylation of RhoA is also required for activation of PLD1, suggesting that protein-lipid interactions, as well as protein-protein interactions, may be required for activation of PLD1 by Rho family members (18). Consistent with the binding data, activation of PLD1 by Cdc42 is GTP-dependent. Stimulation of PLD1 activity in vitro by Cdc42 requires either an activated form of the GTPase (either wild type preloaded with a GTP analog or the Q61L mutation) or the addition of GTPyS to the assay (25). Furthermore, the switch I mutations that abolish binding of Cdc42 to PLD1 are incapable of activating PLD1 (data not shown). Although wild type Cdc42 activates PLD1 in the presence of exogenous GTPyS, (ΔL8)Cdc42 inhibits PLD1 under the same conditions (Fig. 3, top panel). Activation of PLD1 by Cdc42 is synergistic with Arf and PKC as shown previously by Singer et al. (10). In contrast, (ΔL8)Cdc42 inhibits both Arf1 and PKC activation in a dose-dependent manner (Fig. 3, middle and bottom panels, respectively). The ability of the (ΔL8)Cdc42 mutant to inhibit both Arf- and PKC-mediated activation of PLD1 activity suggests that the insert region on Cdc42 is not only essential for activation of PLD1 by Cdc42 but also plays an essential role in the formation of an activated Cdc42/Arf/PLD or Cdc42/PKC/PLD complex. Nonproductive binding of (ΔL8)Cdc42 to PLD1 in the presence of GTPyS can be reversed by the addition of an activated form of Cdc42 (Q61L Cdc42) that stimulates PLD activity (Fig. 4). In the absence (top panel) or presence (bottom panel) of Arf1, (Q61L)Cdc42 (closed circles) activates PLD1 in a

FIG. 3. Deletion of the Rho insert region from Cdc42 abolishes its ability to activate PLD1 in vitro. Purified PLD1 (10 nM) was assayed for catalytic activity in the presence of modified Cdc42 (○) or (ΔL8)Cdc42 (□) under basal (top panel) or activated conditions (250 nM Arf1, middle panel; 70 nM PKCβII, bottom panel). The PLD1 activity data were plotted using a simple equilibrium model assuming a bimolecular interaction between Cdc42 and PLD1 (PLD1 + Cdc42 ↔ PLD1/Cdc42). Curves were obtained using the iterative, nonlinear least squares fitting regime in the IGOR Pro software package (Wave Metrics).

FIG. 4. Inhibition of PLD1 by (ΔL8)Cdc42 is reversed in the presence of an activated mutant (Q61L) of Cdc42. Purified PLD1 (20 nM) was assayed for catalytic activity in the absence (○) or presence (□) of a fixed concentration of (ΔL8)Cdc42 (40 nM). Increasing amounts of modified (Q61L)Cdc42 were added in the absence (top panel) or presence (bottom panel) of 250 nM Arf1. The PLD1 activity data were plotted using a simple equilibrium model (PLD1/(Q61L)Cdc42 + (ΔL8)Cdc42 ↔ PLD + (Q61L)Cdc42 + (ΔL8)Cdc42 + (Q61L)Cdc42). Curves were obtained using the iterative, nonlinear least squares fitting regime in the IGOR Pro software package (Wave Metrics).
dose-dependent manner. In the presence of a fixed concentration (40 nM) of (L8)Cdc42 (open circles), (Q61L)/Cdc42 reverses the (L8)Cdc42 inhibition of PLD1. This reversal is competitive, demonstrating the specificity of the (L8)Cdc42 inhibition of PLD1 activity.

Taken together, these data indicate that binding of PLD1 to Cdc42 and subsequent activation of PLD1 by this GTPase are distinct events. First, unprenylated Cdc42 can bind PLD1 in a GTP-dependent manner but is unable to stimulate substrate hydrolysis. Unmodified Cdc42 interacts with all target effectors, including PLD1 as demonstrated here. The requirement of the geranylgeranyl moiety on Cdc42 specifically for activation of PLD1 (but not binding to PLD1) suggests that this lipid modification may serve a dual role in the activation of this lipase. The modified Cdc42 may help to position PLD1 in a favorable orientation with respect to its lipid substrate in the membrane, as well as inducing a catalytically active conformation in the lipase itself. Second, the switch I region on Cdc42 mediates GTP-dependent binding to PLD1, but the insert region is required for complete activation of PLD1. That is, primary interactions between PLD1 and Cdc42 are mediated through the classical effector loop, but secondary interactions between PLD1 and the insert region of the fully modified Cdc42 are necessary for PLD1 to adopt a catalytically active conformation. Furthermore, these secondary interactions are important in regulating the synergistic activation of PLD1 by Cdc42, Arf, and PKC. The ability of (L8)Cdc42 to inhibit both Arf and PKC-stimulated PLD activity suggests that the insert region of Cdc42 is involved in the synergistic stimulation of PLD by these activators. Specifically, the insert region may indirectly influence the binding sites on PLD for Arf and PKC or may itself contribute to the interaction surface, leading to the observed cooperativity between Cdc42 and these activators with respect to PLD stimulation. With the lack of specific inhibitors of PLD1 activity, the (L8)Cdc42 mutant provides a novel reagent for studying the mechanisms of PLD1 activation by Cdc42 alone and in combination with Arf and PKC.

Activating mutations in Rho, Rac, and Cdc42 that mimic their in vivo activation by exchange factors, such as the Dbl oncoprotein, result in specific and unique contributions of each Rho family GTPase to Dbl-induced cellular transformation (15). This observation raised the issue of whether specific downstream targets mediate the particular signal contributed by each GTPase. In the presence of a specific inhibitor of the RhoA effector, p160-ROCK, Dbl-induced focus formation was blocked, suggesting that focus formation induced by RhoA is mediated by this target kinase (30). Cdc42 was implicated in anchorage-independent growth induced by the Dbl oncogene, result in specific and unique contributions of each Rho family GTPase to Dbl-induced cellular transformation (32). Furthermore, Rac-induced elevation of superoxide levels plays a critical role in Ras-mediated transformation (33). Similar to the demonstration that a specific effector of Rac, the NAPDH oxidase, mediates Rac-induced transformation events, we demonstrate here that PLD1 is a candidate effector for the potentiation of Cdc42 induced anchorage-independent growth. Future studies will be directed at establishing the in vivo relationship between Cdc42-PLD1 interactions and Cdc42-mediated anchorage-independent growth.

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