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Phospholipase D2 induces stress fiber formation through mediating nucleotide exchange for RhoA

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ABSTRACT

Phospholipase D (PLD) is involved in diverse cellular processes including cell movement, adhesion, and vesicle trafficking through cytoskeletal rearrangements. However, the mechanism by which PLD induces cytoskeletal reorganization is still not fully understood. Here, we describe a new link to cytoskeletal changes that is mediated by PLD2 through direct nucleotide exchange on RhoA. We found that PLD2 induces RhoA activation independent of its lipase activity. PLD2 directly interacted with RhoA, and the PX domain of PLD2 specifically recognized nucleotide-free RhoA. Finally, we found that the PX domain of PLD2 has guanine nucleotide-exchange factor (GEF) activity for RhoA *in vitro*. In addition, we verified that overexpression of the PLD2-PX domain induces RhoA activation, thereby provoking stress fiber formation. Together, our findings suggest that PLD2 functions as an upstream regulator of RhoA, which enables us to understand how PLD2 regulates cytoskeletal reorganization in a lipase activity-independent manner.

1. Introduction

The Rho family GTPases, including Rho, Rac, and Cdc42, are important for multiple cellular processes including the regulation of actin cytoskeletal architecture, cell shape and motility, intracellular trafficking, cell cycle progression, and malignant transformation [1–3]. Especially, RhoA is a key regulator of cellular contractility, producing important actin structures critical for cell migration and adhesion, namely stress fibers (actin–myosin filaments) and focal adhesions [4,5]. Like other GTPases, RhoA acts as a molecular switch by cycling between the inactive GDP-bound state and the active GTP-bound state. Activation of Rho GTPases, a critical step in transducing signals for cellular events, is mediated by guanine nucleotide-exchange factors (GEFs) that catalyze the exchange of GDP for GTP [6]. Classical GEFs for Rho GTPases are the Dbl family of GEFs [7], whereas the Dock (dedicator of cytokinesis) family of GEFs has been characterized as a non-conventional Rho-GEF family [8]. Until now, 69 Dbl family Rho GEFs and 11 Dock family members have been identified in humans.

Phospholipase D (PLD) is an enzyme that hydrolyzes phosphatidylcholine to generate choline and phosphatidic acid (PA) [9]. PLD has been implicated in the regulation of cytoskeleton reorganization that is obligatory for cell adhesion, spreading, morphological changes, and migration [10,11]. For example, PLD has been reported to stimulate actin polymerization and stress fiber formation in fibroblasts and endothelial cells [12–14], and microinjection of PLD2 results in morphological reorganization [15]. In addition, PLD exhibits physical and functional interactions with diverse cytoskeletal proteins including actin [16,17], tubulin [18], α-actinin [19], cofilin [20], gelsoin [21] and fodrin [22]. Several studies have proposed that addition of PLD or PA activates phosphatidylinositol-4-phosphate-5-kinase (PI(4)P 5-kinase), generating cellular $PI(4,5)P_2$, which controls the activities and targeting of actin regulatory proteins [23–25]. Although PLD-mediated $PI(4,5)P_2$ generation has been suggested as a mechanism of PLD-induced actin reorganization, exactly how PLD is involved in cytoskeletal organization is not fully defined.

In this study, we show that PLD2 directly interacts with RhoA, and the interaction results in RhoA activation. We also suggest that the PX domain of PLD2 mediates the interaction and has a GEF-like activity for RhoA, which contributes to stress fiber formation.

2. Materials and methods

2.1. Materials

Anti-RhoA antibody and anti-actin antibody were purchased from Santa Cruz. GDPβS, GTPγS, anti-GFP antibody and anti-tubulin

Abbreviations: PLD, phospholipase D; PA, phosphatidic acid; GEF, guanine nucleotide-exchange factor; PX, phox homology; RBD, Rhotekin Rho-binding domain; GTPγS, Guanosine 5′-(γ-hio) triphosphate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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monoclonal antibody were from Sigma (St. Louis, MO, USA). Polyclonal pan-PLD antibody against the C-terminal region was generated as previously described [26]. Anti-hemagglutinin (HA) 12CA5 antibodies were harvested from the supernatants of hybridoma cell lines. Horseradish peroxidase-conjugated goat anti-mouse IgA + IgM + IgG and peroxidase-conjugated goat anti-rabbit IgG were purchased from Kirkegaad and Perry Laboratories (Gaithersburg, MD). The Enhanced Chemiluminescence kit was purchased from Amersham Biosciences (Buckinghamshire, United Kingdom).

2.2. Cell culture and transfection

HEK293 cells and HeLa cells were cultured at 37 °C in 5% CO_2 in DMEM supplemented with 10% fetal bovine serum. For transfection, cells were transfected using Lipofectamine or Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. To silence PLDs, cells were transfected with synthetic siRNAs specific for human PLDs as described previously [27]. To restore the expressions of PLDs, add-back mutant plasmids of PLD2 that resist siRNA silencing were used. Luciferase GL2 duplex was used as a negative control.

2.3. Plasmids

Glutathione S-transferase (GST)-fused proteins containing phox homology domains (PLDs, $p40^{phox}$, SNX2, SNX7) were constructed as previously described [28]. The full-length cDNA of human PLD2 was digested into fragments containing F1 (a.a. 1–314), F2 (a.a. 315–475), F3 (a.a. 476–612), F4 (a.a. 613–723), F5 (a.a. 724–825) and F6 (a.a. 826–934). Each PLD2 fragment was ligated into the pGEX-4T-3 vector, as previously reported [16,29]. To generate N-terminal green fluorescent protein (GFP)-tagged PLDs, full-length PLD2 or PLD2 fragments including F1 (a.a. 1–314) and PX (a.a. 60–195) were subcloned into the EGFP-C1 (BD Bioscience) vector. HA-RhoA, HA-Rac1 and HA-Cdc42 constructs were purchased from the Guthrie/UMR cDNA Resource Center (Rolla, MO, USA). Human RhoA inserts were ligated into the BamHI and EcoRI sites of the pRSET A vector to produce His_6-tagged RhoA. To generate GST-fused GTPases, human RhoA, Rac1 and Cdc42 obtained by PCR were subcloned into the BamHI and EcoRI sites of the pGEX-4T-1 vector.

2.4. GST-RBD pull-down assay

GTP-bound RhoA was measured by pull-down with glutathione S-transferase fused to the RhoA-binding domain of Rhotekin, GST-RBD [30]. HEK293 cells were serum starved for 24 h and stimulated with or without 10 μM LPA (Avanti). Cells were washed with phosphate-buffered saline (PBS) and lysed in ice-cold cell lysis buffer (50 mM Tris–HCl, pH 7.4, 500 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% Triton X-100, 10 mM $MgCl_2$, 0.2 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride [PMSF], 1 μg/ml aprotinin, and 1 μg/ml leupeptin). Lysates were centrifuged at 14,000 *g* at 4 °C for 10 min. Equal amount of supernatants were incubated at 4 °C for 45 min with glutathione-Sepharose 4B beads coupled with GST-Rhotekin RBD. After incubation, beads were washed four times with washing buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, and 10 mM $MgCl_2$). Bound RhoA was detected by western blotting, and densitometry analysis was performed using ImageJ software (from the NIH). The amount of RBD-bound RhoA was normalized to the total amount of RhoA in cell lysates for comparing activities.

2.5. Immunoprecipitation

Cells were harvested and lysed in cold lysis buffer containing 20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% cholate, 1% TX-100, 1 mM PMSF, 1 μg/ml aprotinin, and 1 μg/ml leupeptin. After brief sonication, cell lysates were centrifuged at 100,000 *g* for 30 min. Supernatants were immunoprecipitated with the indicated antibodies for 4 h at 4 °C under gentle agitation. Immunocomplexes were collected with protein A- (RepliGen) or G-Sepharose beads (Pierce) for 2 h at 4 °C and washed four times with lysis buffer. Whole-cell lysates or immunoprecipitates were subjected to SDS-PAGE and immunoblotted.

2.6. In vitro binding analysis

Hexahistidine (His6)-tagged human PLD2 was expressed in baculovirus-infected Sf9 cells and purified by chelating Sepharose affinity column chromatography, as described previously [31]. GST-tagged or His_6-tagged fusion proteins were expressed in the Escherichia coli strain, BL21. GST-fusion proteins were purified on glutathione-Sepharose 4B (GE Healthcare) and eluted with free glutathione to obtain soluble proteins. To prepare His-tagged RhoA proteins, cell lysates were incubated with Ni-NTA agarose beads (Qiagen), washed with buffer containing 5 mM imidazole, and eluted with 150 mM imidazole buffer.

Preparation of the nucleotide-depleted Rho GTPases was carried out as described for Ras GTPases [32]. Immobilized GST-fusion proteins of RhoA, Rac1, and Cdc42 were incubated in nucleotide binding buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM PMSF, 1 mM DTT, and 0.1% Triton X-100) containing 20 mM EDTA for 45 min. Then, the beads were washed with nucleotide binding buffer without EDTA three times. For nucleotide loading, GST-GTPases (~2 μg per assay) were incubated in 200 μl of nucleotide binding buffer containing 100 μM GTPγS and 10 mM $MgCl_2$ for 1 h at 4 °C with gentle rotation. For the nucleotide-depleted condition, EDTA was added to a final concentration of 10 mM. Purified PLD2 was incubated with either GTP-loaded or nucleotide-depleted immobilized GST-GTPase or GST for 2 h at 4 °C. After three washes, PLD2 retained on GSH-Sepharose was analyzed by anti-PLD immunoblot.

2.7. Guanine nucleotide-exchange assay

The *in vitro* GEF assay was performed as previously described [33]. The purified proteins of RhoA GTPase and hDbs (DH/PH) were purchased from Cytoskeleton (Denver, CO). Exchange activities were followed by fluorescence resonance energy transfer (FRET) between the GTPase tryptophanes ($\lambda ex = 292$ nm) and the methylanthranyloil group of mant-GTP ($\lambda em = 440$ nm). 2 μM GDP-preloaded RhoA was incubated with 1 μM mant-GTP (molecular probe) in reaction buffer (20 mM Tris, pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, and 50 μg/ml bovine serum albumin). All fluorescence measurements were performed with a CARY Eclipse fluorimeter (Varian). The exchange reactions were started by the addition of 0.8 μM hDbs or ~0.3 μM PLD fragments to assay their GEF activity. The data were fit to one phase exponential association curves using GraphPad Prism™.

2.8. Immunocytochemistry

HeLa cells were rinsed with ice-cold PBS and fixed with 4% paraformaldehyde at room temperature. After being rinsed with PBS, cells were permeabilized with PBS containing 0.2% Triton X-100. To visualize actin, cells were incubated with TRITC-labeled phalloidin (Sigma) for 1 h. After being washed with PBS, cells were examined using a confocal microscope (LSM-510 Meta; Carl Zeizz, Jena, Germany).

3. Results

3.1. PLD2 modulates RhoA activity

During the study of the relationship between PLD and RhoA in cells, we observed that PLD2 affected RhoA signaling (data not shown). To investigate the contribution of PLD2 to RhoA signaling in detail, we first examined cellular RhoA activity using Rhotekin RBD pull-down after PLD silencing using small interference RNAs (siRNAs). Lysophosphatidic acid (LPA) is an effective Rho activator, and it has been reported to activate