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Inhibition of PLD1 activity causes ER stress via regulation of COPII vesicle formation

Hiroshi Nakagawa [a, *], Kazuki Hazama [a], Katsumasa Ishida [a], Masayuki Komori [b], Kazuhiko Nishimura [a], Saburou Matsuo [a]

[a] Laboratory of Toxicology, Graduate School of Veterinary Sciences, Osaka Prefecture University, Japan
[b] Laboratory of Cellular and Molecular Biology, Graduate School of Veterinary Sciences, Osaka Prefecture University, Japan

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ABSTRACT

Phospholipase D (PLD) plays a crucial role in the regulation of some cellular processes, including autophagy and apoptosis. Accumulation of protein in the endoplasmic reticulum (ER) lumen causes ER stress. Although ER stress is a principal cause of apoptosis and autophagy, the relationship between PLD activity and ER stress remains unclear. Protein transport from the ER to the Golgi apparatus is conducted by coat complex II (COPII) transport vesicles. Here, we demonstrated that inhibition of PLD1 activity or PLD1 knockdown suppressed COPII vesicle transport in normal rat kidney (NRK) cells. COPII vesicle coat proteins are composed of Sar1 as well as Sec23/24 and Sec13/31 complexes. For COPII vesicle formation on the ER membrane, Sar1, Sec23/24, and Sec13/31 are sequentially recruited from the cytosol to the ER membrane. Using a cell-free COPII coat protein recruitment assay, we demonstrated that inhibition of PLD1 activity suppressed Sec13/31 recruitment from the cytosol to the ER membrane in COPII vesicle formation. PLD1 knockdown in NRK cells was associated with increased expression of the ER stress marker GRP78 and apoptosis. Taken together, these results suggest that PLD1 activity regulates COPII vesicle transport from the ER to the Golgi apparatus by regulating Sec13/31 recruitment from the cytosol to the ER membrane during COPII vesicle formation.

1. Introduction

Phospholipase D (PLD) catalyzes the formation of phosphatidic acid (PA) to elevate the acidic lipid composition of the cell membrane [1]. Two PLD isoforms, PLD1 and PLD2, are localized not only at the plasma membrane but also at intracellular membranes, including the endoplasmic reticulum (ER) membrane [2,3]. Phospholipase D plays a crucial role in the regulation of cellular processes, such as autophagy [4] and apoptosis [5]. The ER is responsible for folding newly synthesized proteins [6], and accumulation of newly synthesized protein in the ER lumen causes ER stress [7,8]. Although ER stress is a principal cause of apoptosis and autophagy [9], the relationship between PLD activity and ER stress remains uncharacterized.

In eukaryotic cells, transport between the cellular compartments is mediated by vesicles that perform vectorial transfer of cargo molecules. These vesicles are generated on the donor membrane by recruitment of cytoplasmic coat proteins that deform the membrane to drive vesicle formation [10]. The ER exports newly synthesized proteins to the Golgi apparatus via coat complex II (COPII) transport vesicles. The COPII protein comprises Sar1, the Sec23/24 complex, and the Sec13/31 complex [11]. The COPII protein drives the formation of transport vesicles at a specific location of the ER membrane known as the ER exit site (ERES) [12,13]. The assembly of COPII vesicles is initiated by recruitment of Sar1 from the cytosol to the ER membrane [14,15]. In the second step, the Sec23/24 complex is recruited from the cytosol to the ER membrane and forms the inner COPII coat [16]. In the third step, the Sec13/31 complex is recruited from the cytosol to the ER membrane and forms the outer COPII coat [17]. For COPII vesicle formation at the ERES, appropriate membrane phospholipid characteristics are crucial. For example, COPII components need acidic phospholipids, including PA, to bind the ER membrane [18].

In the present study, we hypothesized that PLD activity regulates COPII vesicle assembly machinery as well as COPII vesicle transport. To test this latter hypothesis, we determined whether

* Corresponding author. Rinku-ourai-kita 1-58, Izumisano, Osaka 598-8531, Japan.
E-mail address: nakagawa@vet.osakafu-u.ac.jp (H. Nakagawa).

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inhibition of PLD activity caused ER stress, which would suggest that newly synthesized proteins were accumulating in the ER lumen rather than being exported by COPII vesicles. We found that PLD1 activity regulated Sec13/31 recruitment in COPII vesicle formation and that inhibition of PLD1 activity suppressed COPII vesicle transport and caused ER stress.

2. Material and methods

2.1. Reagents

The pan-PLD inhibitor 5-fluoro-2-indolyl des-chlorohalopemide (FIPI), the PLD1 inhibitor CAY10593, and the PLD2 inhibitor VU0364739 were purchased from Cayman (Ann Arbor, MI). Brefeldin A (BFA) was purchased from Calbiochem (San Diego, CA). The following primary antibodies were used: PLD1 (1:1000) (Cell Signaling Technology, Danvers, MA), PLD2 (1:1000) (Abnova, Taipei, Taiwan), Sec23 (1:1000) (Abcam, Cambridge, UK), Sec13 (1:500) (Santa Cruz, Santa Cruz, CA), 58K Golgi protein (1:100), GRP78 (1:1000), actin (1:10,000), and calnexin (1:1000) (Sigma, St. Louis, MO). The Sar1 antibody used in the present study was previously developed in our laboratory [19]. The following secondary antibodies were used: horseradish peroxidase (HRP)-conjugated anti-mouse IgG (1:2000), HRP-conjugated anti-rabbit IgG (1:1000) (both purchased from Cappel, Aurora, OH), and Texas Red-conjugated anti-mouse IgG (1:100) (Jackson ImmunoResearch, West Grove, PA).

2.2. Animals

The experiments were conducted with the approval of the Animal Research Control Committee and in accordance with the Guidelines for Animal Experiments of Osaka Prefecture University. All efforts were made to minimize the number of animals used and their suffering. Eight-week-old male Sprague Dawley rats (Slc:SD) were obtained from Japan SLC (Hamamatsu, Japan) and acclimatized for more than 1 week. The animals were kept in an air-conditioned room that was maintained at 22 ± 1 °C and 60% ± 5% relative humidity on a 12 h light/dark cycle and were allowed free access to solid chow (CE-2 rodent chow, Clea Japan, Tokyo, Japan) and tap water.

2.3. Cell culture

Normal rat kidney (NRK) cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum.

2.4. siRNA experiments

Rat PLD1 siRNA (SASI_Rn02_00264,158) was purchased from Sigma. The siRNA negative control (SI03650318) was purchased from Qiagen (Hilden, Germany). Transfection of siRNA was performed using RNAiMAX (Invitrogen, Carlsbad, CA) following the manufacturer's instructions.

2.5. COPII recruitment assay in a cell-free system

The COPII coat protein recruitment assays were performed in microsomes, a cell-free system, as previously described [20] and modified [19]. The system consisted of rat liver cytosol (source of Sar1, Sec23, Sec24, Sec13, and Sec31) and NRK cell microsomes (source of ER membrane) in a reaction mixture. Microsome membranes (40 μg) were incubated with cytosol (200 μg) in a final volume of 60 μl of reaction mix in the presence or absence of 100 μM GTPγS. The PLD inhibitor CAY10593 or VU0364739 was added to the reaction mixture containing GTPγS. After being incubated at 32 °C for 15 min, the reaction samples were layered on a 15% sucrose cushion (180 μl) containing 75 mM KOAc and 2 mM MgOAc in a 1500 μl microtube, and were centrifuged at 16,000 × *g* and 4 °C for 15 min. The membrane-containing pellet was solubilized with Laemmli SDS sample buffer and analyzed by quantitative western blotting. The amounts of the microsome-bound proteins (Sar1, Sec23, and Sec13) were quantified by densitometry of the respective protein-positive bands using Multi Gauge software (FUJIFILM, Tokyo, Japan). To normalize the densitometric values of the positive bands in each experiment, the density values of the positive protein bands were normalized to that of calnexin, an ER membrane marker protein, in the corresponding lane.

2.6. Quantification of phospholipids in ER membranes

Phospholipids were extracted from microsome membranes in a COPII recruitment assay by the Bligh-Dyer method [21]. Extracted phospholipids were separated using thin layer chromatography and visualized by primuline staining.

2.7. Whole cell lysate preparation

Briefly, cells were rinsed twice with PBS and then incubated in lysate buffer (20 mM HEPES-KOH, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM EDTA, 10 mM sodium pyrophosphate, 100 mM NaF, 17.5 mM glycerol-2-phosphate disodium salt hydrate, 1 mM PMSF, 4 μg/ml aprotinin, 2 μg/ml pepstatin). The supernatant collected after centrifugation at 16,000 × *g* and 4 °C for 15 min was used as whole cell lysates. Whole cell lysates were solubilized with Laemmli SDS sample buffer and analyzed by quantitative western blotting using antibodies against GRP78, PLD1, or actin.

2.8. Evaluation of COPII vesicle transport in a cell system

COPII vesicle transport was evaluated by examining the reassembly of BFA-treated Golgi components in NRK cells. The NRK cells were treated for 30 min with BFA (10 μg/ml). After the BFA was washed out, the reassembly of the Golgi apparatus was induced in DMEM with or without PLD inhibitors for 40 min. After methanol fixation and 0.3% Triton X-100 permeabilization, the cells were incubated with a 58K Golgi protein antibody (1:100) in PBS containing 0.3% Triton X-100 and 0.1% BSA overnight at 4 °C and then incubated with a Texas Red-conjugated secondary antibody (1:100) for 2 h at room temperature. The architectural changes in the *cis* Golgi apparatus following BFA treatment and BFA washout were observed using this immunocytochemical assay for the 58K Golgi protein and evaluated according to the following modified classification scheme that was originally proposed by Sonoda et al. [22]: Grade 1, the majority of the Golgi structures were concentrated at the perinuclear region similar to that seen under basal conditions in wild-type cells; Grade 2, partially dispersed Golgi structures were loosely concentrated at the perinuclear region; Grade 3, dispersed Golgi structures were located throughout the perinuclear region; Grade 4, the majority of the Golgi structures were fragmented and appeared in small punctate patterns throughout the cytoplasm. More than 200 cells in each drug treatment group were evaluated and scored using this classification scheme to determine the morphological changes in the *cis* Golgi apparatus.

2.9. Statistical analysis

Data represent means ± S.D. of at least three independent experiments for each experimental condition. Student's *t*-tests or a