Mammalian patatin-like phospholipase domain-containing proteins (PNPLAs) are lipid-metabolizing enzymes with essential roles in energy metabolism, skin barrier development, and brain function. A detailed annotation of enzymatic activities and structure–function relationships remains an important prerequisite to understand PNPLA functions in (patho-)physiology, for example, in disorders such as neutral lipid storage disease, non-alcoholic fatty liver disease, and neurodegenerative syndromes. In this study, we characterized the structural features controlling the subcellular localization and enzymatic activity of PNPLA7, a poorly annotated phospholipase linked to insulin signaling and energy metabolism. We show that PNPLA7 is an endoplasmic reticulum (ER) transmembrane protein that specifically promotes hydrolysis of lysophosphatidylcholine in mammalian cells. We found that transmembrane and regulatory domains in the PNPLA7 N-terminal region cooperate to regulate ER targeting but are dispensable for substrate hydrolysis. Enzymatic activity is instead mediated by the C-terminal domain, which maintains full catalytic competence even in the absence of N-terminal regions. Upon elevated fatty acid flux, the catalytic domain targets cellular lipid droplets and promotes interactions of PNPLA7 with these organelles in response to increased cAMP levels. We conclude that PNPLA7 acts as an ER-anchored lysophosphatidylcholine hydrolase that is composed of specific functional domains mediating catalytic activity, subcellular positioning, and interactions with cellular organelles. Our study provides critical structural insights into an evolutionarily conserved class of phospholipid-metabolizing enzymes.

Mammalian patatin-like phospholipase domain-containing proteins (PNPLAs) constitute a family of lipid-metabolizing enzymes with critical roles in energy metabolism, skin barrier development, and brain function (1–4). The patatin-like phospholipase domain (Pfam01734) is named after a homologous module in human PNPLA6 and PNPLA7 constitute a subgroup within the patatin-like phospholipase domain proteins (PNPLAs) constitute a family of lipid-metabolizing enzymes with critical roles in energy metabolism, skin barrier development, and brain function (1–4). The patatin-like phospholipase domain (Pfam01734) is named after a homologous module in human PNPLA6 and PNPLA7 constitute a subgroup within the patent-like phospholipase domain proteins (PNPLAs) that have been assigned hydrolytic, transacylase, or acyltransferase activities with diverse lipid substrates such as phospholipids, acylglycerols, and retinoids (6–9). These reactions have been proven essential for the turnover of cellular membranes, mobilization of storage lipids, and generation of signaling molecules (10–12). The physiological relevance of PNPLAs is illustrated by a diverse spectrum of inherited disorders that have been associated with mutations in human PNPLA genes including neutral lipid storage disease, non-alcoholic fatty liver disease, ichthyosis, hereditary spastic paraplegia, and other neurodegenerative syndromes (2, 3, 13, 14). Of note, defects in enzymatic function and subcellular localization have been identified as common molecular mechanisms in the onset and progression of PNPLA-related disorders (4, 15, 16). PNPLA6 and PNPLA7 constitute a subgroup within the PNPLA family that has been remarkably conserved during evolution with orthologous proteins in yeast, nematodes, and flies (17). PNPLA6 acts as a (lyso)phospholipase and is involved in the degradation of membrane lipids such as phosphatidylcholine (PC) and lysophosphatidylcholine (LPC) (6, 11). Human PNPLA6 is a primary substrate of organophosphates (OPs) that
cause a neurotoxic syndrome termed OP-induced delayed neuropathy. Inhibition of neuronal phospholipid homeostasis initiate OP-induced delayed neuropathy. Accordingly, PNPLA6 has been traditionally referred to as neuropathy target esterase (18, 19).

In contrast to PNPLA6, little is known about the molecular and physiological function(s) of the closely related PNPL7 (also termed neuropathy target esterase-related esterase). Both proteins share a highly conserved domain architecture that is assembled of the enzymatic patatin-like phospholipase domain and extensive “non-enzymatic” segments of poorly defined function including three putative cyclic nucleotide monophosphate (cNMP)-binding sites (1, 25). Initial in vitro studies identified PNPL7 as a lysophospholipase whose transcript expression is highly responsive to feeding/fasting transitions and insulin concentrations (25, 26). In mammalian cells, PNPL7 localizes to the ER and lipid droplets (LDs), which are cellular lipid storage organelles with pivotal functions in energy metabolism and lipid trafficking (25, 27). Although these observations closely link PNPL7 to lipid and energy metabolism, it is presently unknown how fluctuations in PNPL7 expression or subcellular distribution affect lipid homeostasis of cells or tissues (25).

In this study, we further characterized the enzymatic function of PNPL7 in cellular lipid metabolism and established detailed structure–function relationships among domain architecture, subcellular positioning, and enzymatic activity of the protein. We confirm that PNPL7 acts as a potent intracellular lysophospholipase and identify LPC as a major substrate of PNPL7 in living cells. Moreover, we demonstrate that PNPL7 is composed of specific functional parts mediating ER targeting, interactions with LDs, and substrate hydrolysis. Our study provides novel structural insights into an evolutionarily conserved class of phospholipid-metabolizing enzymes.

Results

PNPL7 expression affects lysophospholipid metabolism in mammalian cells

To assess possible function(s) of PNPL7 in cellular lipid metabolism, we first created cell lines stably expressing PNPL7-EGFP or EGFP, respectively, and subjected them to enzyme activity assays and lipid analysis. Consistent with previous studies, cell homogenates expressing PNPL7-EGFP exhibited increased hydrolytic activity toward several lysophospholipid species as compared with EGFP-expressing control homogenates including C18:1 LPC (4.6-fold), C18:1 lysophosphatidylethanolamine (LPE; 4.0-fold), and C18:1 lysophosphatidylserine (LPS; 2.7-fold) (25). In addition, PNPL7-EGFP-expressing homogenates showed a low but significant increase in the hydrolytic activity toward C18:1/C18:1 PC (1.5-fold) and C18:1/C18:1 phosphatidylethanolamine (PE; 1.2-fold), whereas the activity toward C18:1/C18:1 phosphatidylserine (PS) was not different from EGFP-expressing controls (Fig. 1A). The lipid hydrolase activity of PNPL7-EGFP was 2.6-fold higher toward C18:1 LPC as compared with C18:0 LPC, suggesting a preference of the enzyme for unsaturated compared with saturated species (Fig. 1B). Lipid analysis revealed that stable expression of PNPL7-EGFP significantly reduced cellular LPC levels (−34%) as compared with EGFP-expressing control cells but did not affect total cellular levels of LPE, LPS, PC, PE, or PS (Fig. 1C). The decrease in LPC levels observed in PNPL7-EGFP-expressing cells was mainly due to a reduction in unsaturated LPC species containing C18:1 (−35%), C18:1 (−43%), and C18:2 (−52%) fatty acids (FAs) (Fig. 1D). Although a trend toward decreased cellular levels was also observed for other LPC species, the difference was not significant when compared with controls (Fig. 1D). To further investigate the function of PNPL7 in cellular LPC metabolism, we depleted endogenous PNPL7 in murine AML12 cells by RNAi and assessed cellular LPC hydrolase activities and LPC levels. Stable expression of two independent shRNA constructs targeting PNPL7 (shRNA1 and shRNA2) decreased cellular Pnpla7...
PNPLA7 is an integral membrane protein with a luminal N and a cytosolic C terminus

To link the lipid-metabolizing function of PNPLA7 to a specific cellular site, we next assessed its subcellular localization and the topological orientation of its domains. The arrangement of possible functional domains within the PNPLA7 polypeptide is shown in Fig. 3A. The N-terminal half of the protein includes a single predicted transmembrane (TM) domain close to the N terminus and a putative “regulatory” (R) domain, which harbors three predicted cNMP-binding domains (CBDs). The C-terminal half (C domain) includes the patatin-like phospholipase domain predicted to mediate enzymatic activity (Fig. 3A). Subcellular fractionation of Neuro-2a postnuclear supernatants (PNSs) and subsequent immunoblotting showed that endogenous PNPLA7 was enriched in the membrane fraction but undetectable in the cytosol (Fig. 3B). Likewise, a PNPLA7-EGFP fusion protein transiently expressed in COS-7 cells was exclusively recovered in the membrane fraction (Fig. 3B), indicating a similar subcellular distribution of endogenous PNPLA7 and ectopically expressed PNPLA7-EGFP. Membrane-associated PNPLA7-EGFP was partially solubilized with Triton X-100 but not with NaCl or Na2CO3, similar to the integral membrane protein Calnexin but unlike the peripheral membrane protein DDHD2 (28, 29) (Fig. 3C). Next, we used proteinase K protection assays to assess the topological orientation of PNPLA7 within cellular membranes. Incubation of isolated microsomes with increasing concentrations of proteinase K provoked a dose-dependent depletion of the signal corresponding to full-length PNPLA7-EGFP in our immunoblots (Fig. 3D). This was accompanied by the generation of a ~25-kDa fragment likely representing EGFP, which is known to be resistant to proteinase K digestion, indicating proteolytic degradation and therefore cytosolic orientation of the C terminus of PNPLA7 (Fig. 3D). Using antibodies directed toward the C domain or the R domain of PNPLA7, we failed to detect additional protein bands eluding proteolytic digestion of PNPLA7-EGFP, suggesting that these domains are also largely exposed to the cytosol and thus accessible to proteolytic degradation (Fig. 3D). Integrity of the isolated microsomes was confirmed by the apparent protection of the 66-kDa luminal domain of Calnexin from proteolytic degradation (28) (Fig. 3D). To address whether the N terminus of PNPLA7 is oriented toward the cytosol or the lumen, we repeated the proteinase protection assay with microsomes isolated from COS-7 cells expressing PNPLA7 harboring an N-terminal HA tag (HA-PNPLA7). As shown in Fig. 3E, proteinase K treatment depleted the signal corresponding to full-length HA-PNPLA7 and led to the accumulation of an immunoreactive peptide of less than 10 kDa consistent with a luminal orientation of the N terminus. Addition of Triton X-100 to solubilize microsomal membranes rendered this peptide susceptible to proteolytic digestion, suggesting that microsomal integrity is indeed required to protect the N-terminal part of PNPLA7 from proteolysis. In an independent approach, we permeabilized COS-7 cells expressing HA-PNPLA7 either with Triton X-100 or digitonin and costained the cells with antibodies directed toward the HA tag and the C domain (Fig. 3F). Permeabilization with Triton X-100 rendered both epitopes accessible to antibody staining, resulting in a reticular staining pattern reminiscent of the ER and an apparent overlap of both signals. In contrast, digitonin treatment, which is used to selectively permeabilize the plasma membrane, rendered only the C region but not the N-terminal HA tag accessible to antibody staining (Fig. 3F). Taken together these results suggest that PNPLA7 is a bilayer-spanning TM protein that orients its N terminus toward the lumen but exposes its R and C domains largely toward the cytosol.
The N-terminal region targets PNPLA7 to the ER

We next addressed the functional contribution of individual protein domains to the subcellular positioning of PNPLA7. To this end, we constructed EGFP-tagged PNPLA7 variants lacking individual domains (Fig. 4A) and assessed their subcellular localization in living COS-7 cells by confocal fluorescence microscopy. Consistent with the results described above, ectopically expressed full-length PNPLA7-EGFP exhibited a reticulate pattern that colocalized with an ER marker protein in COS-7 cells (Fig. 4B). A PNPLA7 mutant lacking the C domain (PNPLA7-N-EGFP) exhibited a similar subcellular distribution as full-length PNPLA7-EGFP and colocalized with the ER, indicating that the C domain is dispensable for ER association of PNPLA7. Furthermore, sole expression of the N-terminal TM domain (PNPLA7-TM-EGFP) was sufficient to target EGFP to the ER, whereas deletion of the TM domain (PNPLA7-ΔTM-EGFP) compromised ER localization of PNPLA7, suggesting an essential requirement of the TM domain for ER targeting of PNPLA7. Notably, expression of a construct harboring an internal deletion of the R but not the TM domain resulted in aggregation of the recombinant protein that failed to properly distribute to the ER network. This suggests that both the TM
domain and the R domain contribute to the positioning of full-length PNPLA7 at the ER. Consistent with these findings, a mutant lacking both the TM and R domains (PNPLA7-C-EGFP) distributed diffusely in the cytoplasm and showed only minimal overlap with the ER marker (Fig. 4B). As a complementary approach, we used subcellular fractionation and immunoblotting to assess membrane association of PNPLA7 mutant proteins. As shown in Fig. 4C, PNPLA7-EGFP, PNPLA7-N-EGFP, PNPLA7-TM-EGFP, and PNPLA7-ΔR-EGFP were recovered exclusively in the membrane fraction, whereas PNPLA7-ΔTM-EGFP and PNPLA7-C-EGFP distributed between the soluble and particulate fractions, suggesting impaired membrane association of these mutants. Thus, our data suggest that the N-terminal TM domain mediates efficient membrane tethering of PNPLA7 and that both the TM and R domains but not the C domain control the subcellular positioning of PNPLA7 at the ER.

The C domain of PNPLA7 associates with LDs

In addition to the ER, PNPLA7 has been shown to localize to a subset of cellular LDs upon incubation of cells with FAs (25). To address the structural requirements for LD association of PNPLA7, we induced LD formation in COS-7 cells transfected with EGFP-tagged PNPLA7 or PNPLA7 truncation mutants and reassessed subcellular localization of the recombinant proteins by confocal fluorescence microscopy. PNPLA7-EGFP exhibited a reticular distribution and only marginal overlap with LDs, suggesting that PNPLA7 remains predominantly ER-associated also upon increased LD formation (Fig. 5A). Likewise, neither PNPLA7-N-EGFP nor PNPLA7-ΔTM-EGFP exhibited apparent colocalization with LDs under these conditions. In contrast, PNPLA7-ΔR-EGFP showed punctate and semi-ringlike enrichments in close proximity to LDs, indicating a possible interaction of this truncation mutant with LDs. This phenomenon was even more pronounced for PNPLA7-C-EGFP, which presented as numerous ring-shaped structures surrounding cellular LDs (Fig. 5A). Moreover, PNPLA7-C-EGFP colocalized with ectopically expressed PLIN2-mCherry, a bona fide LD protein, suggesting that it indeed associates with the LD surface (30, 31) (Fig. 5B).

To further study the interactions of PNPLA7 protein domains with LDs, we fractionated transfected COS-7 cells by ultracentrifugation and assessed the abundance of each PNPLA7 variant in the LD fraction by immunoblotting. Consistent with the microscopy data, ectopically expressed PNPLA7-C-EGFP was markedly enriched in the LD fraction, resembling the distribution of endogenous PLIN2 (Fig. 5C). Low levels of PNPLA7-EGFP, PNPLA7-ΔTM-EGFP, and PNPLA7-ΔR-EGFP were also detected in the LD fraction. However, the abundance of these proteins was much lower as compared with PNPLA7-C-EGFP.

To identify the minimal domain required for LD targeting of PNPLA7-C-EGFP, we further truncated the protein and assessed the ability of these mutants to colocalize with LDs. Schematic drawings of the truncations and their effects on LD targeting are summarized in Fig. 6A. Mutants containing truncations at the C terminus retained the ability to localize to LDs, suggesting that the patatin-like phospholipase domain is largely dispensable for LD targeting. In contrast, even minor trunca-

Figure 4. Functional contribution of PNPLA7 protein domains to ER targeting. A, domain architecture of PNPLA7 variants used in this experiment. B, subcellular distribution of PNPLA7 variants. COS-7 cells were cotransfected with PNPLA7-EGFP or PNPLA7-EGFP truncation mutants and a recombinant DsRed2-tagged marker of the ER and analyzed by confocal fluorescence microscopy. Scale bars, 10 μm. C, subcellular distribution of PNPLA7 variants in soluble and membrane fractions. COS-7 cells were transfected with PNPLA7-EGFP or PNPLA7-EGFP truncation mutants, separated into cytosol and membranes by ultracentrifugation, and analyzed by immunoblotting using an antibody against GFP. S, soluble; P, pellet.
tions at the N terminus of the C domain compromised LD targeting. As shown in Fig. 6, A and B, a stretch between amino acids 681 and 967 was sufficient for LD targeting, whereas further N- or C-terminal truncations abolished LD association of the C domain. Thus, a stretch of 287 amino acids is essential for the interactions between the C domain of PNPLA7 and LDs.

Because the N-terminal region contains three putative CBDs (Fig. 7A), we next asked whether the presence of cNMP analogs would affect the subcellular distribution of PNPLA7. Incubation of cells with 8-CPT-cAMP provoked a subcellular redistribution of ectopically expressed PNPLA7-EGFP, which manifested as ring- and semi-ringlike enrichments of the protein close to the LD surface on top of its reticulate ER-like distribution (Fig. 7B, inset). A similar response was elicited by 8-CPT-cGMP (data not shown). In contrast to PNPLA7-EGFP, PNPLA7-N-EGFP was not enriched at LDs under identical conditions. Furthermore, deletion of CBD3 but not CBD1 or CBD2 abolished LD interactions of PNPLA7-EGFP (Fig. 7, A and B). In summary, these data show that the C domain localizes to cellular LDs and suggest an involvement of both the C domain and CBD3 in LD interactions of PNPLA7 in response to elevated cNMP levels. Noteworthy, the specific C18:1 LPC hydrolase activity of PNPLA7 was largely unchanged upon incubation of cells with FAs and/or cNMPs (Fig. 7C) or starvation (data not shown), suggesting that alterations in subcellular distribution do not alter PNPLA7 activity.

The C domain mediates catalytic activity of PNPLA7

We finally aimed to understand how specific protein domains regulate the enzymatic function of PNPLA7. To do so, we expressed PNPLA7-EGFP or truncated PNPLA7-EGFP mutant proteins in COS-7 cells and compared cellularlysolubilized phospholipase activities. Immunoblotting revealed similar expression levels of PNPLA7-EGFP and truncated versions of the protein (Fig. 8A). As shown in Fig. 8B, ectopic expression of PNPLA7-EGFP or PNPLA7-C-EGFP increased cellular LPC hydrolase activity to a similar extent (2.1–2.3-fold) as compared with EGFP-expressing controls, suggesting that the C domain maintains full catalytic competence also in the absence of TM and R domains. The specific activities of PNPLA7-H9004 TM-EGFP and PNPLA7-H9004 R-EGFP were 3.0- and 3.3-fold higher than the activity of EGFP-expressing controls, thereby moderately exceeding the activity of PNPLA7-EGFP. In contrast, expression of PNPLA7-N-EGFP failed to increase cellular lysophospholipase activity, which is consistent with a requirement of the C domain for substrate hydrolysis (Fig. 8B). To assess whether the catalytic C domain is functional also in intact cells, we generated COS-7 cells stably expressing PNPLA7-C-EGFP or EGFP, respectively, and performed lipid analyses. Expression of PNPLA7-C-EGFP reduced cellular levels of LPC by 42% com-
pared with EGFP-expressing control cells but did not affect cellular levels of PC, PE, PS, LPE, and LPS (Fig. 8 C). Thus, the C domain is both required and sufficient to mediate catalytic activity of PNPLA7 in mammalian cells.

Discussion

In this study, we assessed the role of PNPLA7 in cellular lipid metabolism and analyzed the structure–function relationships between domain architecture, subcellular distribution, and enzymatic activity of the protein. Ectopic expression of PNPLA7 markedly increased cellular hydrolase activities toward lysophospholipids including LPC, LPE, and LPS. In contrast, phospholipids with two acyl chains were poor substrates for PNPLA7, suggesting a preference of the enzyme for mono- over diacylglycerolphospholipids. In line with this observation, stable expression of PNPLA7 in COS-7 cells decreased cellular levels of the monoacylglycerolphospholipid LPC but not of diacylglycerolphospholipids such as PC, PE, and PS. Despite increased LPE hydrolase activities, the cellular levels of these lysophospholipid classes were also not altered upon PNPLA7 expression, arguing for a preference of PNPLA7 for LPC in a cellular context. Moreover, stable reduction of PNPLA7 expression by RNAi decreased cellular LPC hydrolase activity in AML12 cells, further supporting an enzymatic function of PNPLA7 as LPC hydrolase. PNPLA7 is closely related to PNPLA6, a protein involved in axon maintenance and brain function, which has been previously shown to regulate hydro-
[The natural text content is too long to be quoted here. The full content is available in the provided image.]
ing Technology (Danvers, MA). antisera against the R domains of murine PNPLA7 and PNPLA6 were generated by repetitive immunizations of rabbits with the peptides CSVPPLSNH-GEVDELRSQSGSNT and PAGDPVKPTSEAPAPL-LSRC, respectively, which were coupled to keyhole limpet hemocyanin as carrier (PitChem, Graz, Austria). An ECL™ sheep anti-mouse IgG antibody (NA931) linked to horseradish peroxidase (HRP) was purchased from GE Healthcare. An HRP-linked goat anti-rabbit IgG antibody (PI-1000) was purchased from Vector Laboratories (Burlingame, CA). An HRP-linked goat anti-guinea pig IgG antibody (6090-04) was purchased from Southern Biotechnology (Birmingham, AL). A Rhodamine Red™, X-linked goat anti-mouse IgG antibody was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA), and DyLight® 488-conjugated goat anti-rabbit IgG antibody was obtained from Thermo Fisher Scientific.

Protein sequence analysis

Protein domains were analyzed using the protein family database (Pfam), an online database containing collections of protein domains and families (38). TM domains were predicted using Hidden Markov Models (39).

Plasmids and cloning of recombinant proteins

The vectors pDsRed2-ER, pmCherry-N1, and pEGFP-N3 were obtained from Clontech. pEGFP-N1 containing the coding sequence (cds) of human PLIN2 was a kind gift from Stefan Höning (Cologne, Germany). To generate a plasmid encoding PLIN2-mCherry, the cds of PLIN2 was amplified using the primers 5'-GCG AAT TCG CCA TGG CAT CCG TGT CAG TGT A-3' and 5'-CCG AAC ATT TTT CAA CAT GTA C-3', and the PCR product was digested with EcoRI and Agel and ligated into the multiple cloning site of pmCherry-N1. The cds of PNPLA7 was amplified by PCR using cDNA of murine cardiac muscle as template and the primers 5'-TTT TCG AGG CCA TGG AGG AGC AGT CCC AGT CC-3' (F1) and 5'-GCG AAT TCG AGG AAG GAT GTT CCA GTC TTG G-3' (R1). PNPLA7 truncation mutants were generated by PCR using the primers 5'-TTT TCG AGG CCA TGG AGG AGC AGT CCC AGT CC-3' (F1) and 5'-GCG AAT TCG AGG AAG GAT GTT CCA GTC TTG G-3' (R1), and the PCR product was digested with EcoRI and NotI and inserted into the multiple cloning site of pEGFP-N3. The resulting plasmids were used to generate lentiviral expression vectors, pEGFP-N3 constructs encoding the open reading frames for EGFP, PNPLA7-Egfp, and PNPLA7-C-Egfp were digested with Xhol and NotI, and the resulting fragments were inserted into the multiple cloning site of pLX IRES Puro (Clontech). Mission® lentiviral pLKO.1 vectors encoding scrambled shRNA or shRNAs targeting murine PNPLA7 were obtained from Sigma-Aldrich. Constructs used for RNAi experiments targeted the sequences CCAAGAG-GATTTCGCGCTTATA (shRNA1) and CATGTCCTTGT-CAGGCTATAT (shRNA2) of the PNPLA7 cds.

Cell culture and transfection

Neuro-2a cells (ATCC CCL-131) were maintained in minimum essential medium supplemented with 2 mM l-glutamine, 1 mM sodium pyruvate, non-essential amino acids, 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin. COS-7 cells (ATCC CRL-1651) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. AML12 cells (ATCC CRL-2254) were cultured in a 1:1 mixture of DMEM and Ham’s F-12 medium supplemented with 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.005 mg/ml insulin, 0.005 mg/ml transferrin, 5 mg/ml selenium, and 40 mg/ml dexamethasone. All cells were maintained at 37 °C, 95% humidity, and 5% CO2. Cells were transfected with Metafectene® (Biontex GmbH, Munich, Germany) according to the manufacturer’s instructions and used for experiments 24 h thereafter. Cells were treated with 400 μM oleic acid bound to bovine serum albumin (BSA; essentially fatty acid-free, Sigma-Aldrich), 1 mM 8-CPT-cAMP, or 1 mM 8-CPT-cGMP for 16 h or with Hanks’ buffered salt solution for 4 h before being processed for imaging or lipid hydrolyase assays.
Functional domains of PNPLA7

Generation of lentivirus and stable infection of COS-7 and AML12 cells

Lentiviral particles harboring pLVX IRES Puro vectors for the expression of EGFP-tagged proteins or pLKO.1 vectors for the expression of shRNAs were generated in HEK293T cells (ATCC CRM-3216) according to the manufacturer’s instructions (Clontech). Before transduction, COS-7 or AML12 cells were seeded into 6-well plates at a density of 300,000 cells/well. Cells were incubated for 24 h with lentivirus-containing supernatants in the presence of 8 μg/ml Polybrene. To select for stable expression, cells were maintained for 7 days in medium containing 2 μg/ml puromycin.

Lipid analysis

Total lipids of cell pellets (1 × 10⁶ cells) were extracted twice according to Folch et al. (40) using chloroform/methanol/water (2:1:0.6, v/v/v) containing 500 pmol of butylated hydroxytoluene, 1% acetic acid, and 100 pmol of internal standards (17:0/17:0 PC, 19:0/19:0 PC, 17:0/17:0 PE, 17:0/17:0 PS, and 17:0 LPC, Avanti Polar Lipids) per sample. Extraction was performed under constant shaking for 60 min at room temperature. After centrifugation at 1,000 g for 15 min, the lower organic phase was collected. 2.5 ml of chloroform was added to the remaining aqueous phase, and the second extraction was performed as described above. Combined organic phases of the double extraction were dried under a stream of nitrogen and resolved in 150 μl of methanol/2-propanol/water (6:3:1, v/v/v) for UPLC-triple quadrupole analysis. Chromatographic separation was modified after Knittel et al. (41) using an AQUITY UPLC system (Waters) equipped with a Kinetex EVO C18 column (2.1 × 50 mm, 1.7 μm; Phenomenex) starting with a 25-min gradient with 100% solvent A (MeOH/H₂O (1:1, v/v), 10 mM ammonium acetate, 0.1% formic acid). An EVOQ Elite™ triple quadrupole mass spectrometer (Bruker) equipped with an electrospray ionization source was used for detection. Lipid species were analyzed by selected reaction monitoring (PC: MH+ to m/z 184, 25 eV; LPC: MH+ to m/z 184, 22 eV; PE: MH+ to −m/z 141, 20 eV; LPE: MH+ to −m/z 141, 17 eV; PS: MH+ to −m/z 185, 20 eV; LPS: MH+ to −m/z 185, 17 eV). Data acquisition was done by MS Workstation (Bruker). Data were normalized for recovery and extraction and for ionization efficiency by calculating analyte/internal standard ratios.

Subcellular fractionation

COS-7 cells were washed with phosphate-buffered saline (PBS), scraped from tissue culture plates, and collected by brief centrifugation. For the preparation of membrane and cytosol, cells were homogenized in 10 mM HEPES, 0.25 M sucrose, 1 mM EDTA, pH 7.4 (buffer A), by passing 30 times through a 26-gauge needle on ice. PNSs were obtained by centrifugation at 4 °C and 1,000 × g for 10 min. PNS was further fractionated into cytosol and membrane fractions by ultracentrifugation at 4 °C and 100,000 × g for 60 min. For the isolation of lipid droplets, cells were homogenized on ice with a Dounce homogenizer in 20 mM Tris/HCl, 1 mM EDTA, pH 7.4, and lipid droplets were isolated by ultracentrifugation using a discontinuous sucrose gradient according to Brasnaemle and Wolins (42) with minor modifications. Protein concentrations of cell extracts were determined with the Bio-Rad Protein Assay kit according to the manufacturer’s instructions (Bio-Rad) using BSA as standard. Alternatively, protein concentrations were determined with the Pierce® BCA™ Protein Assay kit according to the manufacturer’s protocol (Thermo Scientific).

Membrane extraction and proteinase K protection assays

Membrane fractions of COS-7 cells were resuspended in buffer A and incubated in the presence of 1 mM NaCl, 0.1 mM Na₂CO₃, or 1% Triton X-100 for 30 min on ice. Afterward, soluble and insoluble components were separated by centrifugation at 4 °C and 100,000 × g for 30 min and subjected to immunoblotting. For membrane protection assays, isolated membrane fractions were incubated in the presence of 0–10 μg/ml proteinase K in the absence or presence of 1% Triton X-100 for 15 min at room temperature. The reactions were stopped by the addition of PMSF at a final concentration of 2 mM, further incubated for 5 min, and subjected to immunoblotting.

Immunoblotting

Samples were mixed with Laemmli buffer, denatured for 5 min, subjected to SDS-PAGE, and electroblotted onto PVDF membranes. Membranes were incubated for 1 h with 20 mM Tris/HCl, 150 mM NaCl, 0.1% Tween 20, pH 7.5 (TBST), containing 10% milk to block unspecific binding sites. Primary and secondary antibodies were diluted in TBST containing 5% milk and incubated with the membrane for 1 h at room temperature followed by extensive washing with TBST.

RNA extraction and RT-qPCR

Cells were harvested and snap frozen in liquid nitrogen. RNA was extracted using the TRizol® reagent (Invitrogen™, Life Technologies) according to the manufacturer’s instructions. RT-qPCR was performed as described previously (43) using the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Life Technologies) and the Maxima™ SYBR Green/ROX PCR reaction mixture (Thermo Scientific Fermentas, Waltham, MA). The following primers were used: Pnpla7 fw, 5’-CGT TTG TTC CAA CGA CCA CC-3’; Pnpla7 rv, 5’-TCT GCT AGT GCC CTG AGG AT-3’; 36B4 fw, 5’-GCT TCA TTG TGG GAG CAG ACA-3’; 36B4 rv, 5’-CAT GGT GTT CTT GCC CAT CAG-3’. Relative mRNA levels were quantified according to the ΔΔCt method using 36B4 as a reference gene.

Lipid hydrolyase assays

Protein samples for lipid hydrolyase assays were prepared by sonication of COS-7 or AML12 cells in 0.25 M sucrose, 1 mM EDTA, 1 mM DTT containing 20 μg/ml leupeptin, 2 μg/ml antipain, and 1 μg/ml pepstatin (solution A) followed by centrifugation at 4 °C and 1,000 × g. Lipid substrates were prepared by sonication of glycerophospholipids in 200 mM Bistris propane buffer, pH 7.7, containing 10 mM CHAPS, 1 mM EDTA, and 600 mM NaCl. Unless otherwise indicated, substrates contained lysophospholipids at final concentrations of 3 mM or diacylglycerophospholipids at final concentrations of 1 mM. Assays were started by the addition of 50 μl of substrate to 50-μl samples in solution A and incubated under steady shaking at 37 °C in a water bath. Substrate hydrolysis is expressed as release of...
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Immunostaining and confocal fluorescence microscopy

COS-7 cells were seeded in chambers mounted onto coverslips (Sarstedt, Nümbrecht, Germany) and transfected as described above. Cells expressing EGFP-tagged proteins were fixed with 4% paraformaldehyde for 20 min at room temperature, and LDLs were counterstained using HCS LipidTOX Deep Red. Cells expressing HA-PNPLA7 were fixed with 4% paraformaldehyde for 20 min at room temperature and permeabilized for 15 min with PBS containing either 0.1% Triton X-100 or 0.0005% digitonin. Cells were washed three times with PBS and incubated at room temperature with PBS containing 1% BSA for 1 h to block unspecific binding. Primary antibodies were diluted in blocking solution and incubated with cells overnight at 4°C. Cells were washed with PBS and incubated with secondary antibodies diluted in blocking solution at room temperature for 1 h followed by extensive washing with PBS. Cells were imaged using a Leica SP5 confocal microscope equipped with a Leica HCX 63× 1.4 numerical aperture oil immersion objective. EGFP and DyLight 488-conjugated antibodies were excited at 488 nm, and emission was detected between 500 and 530 nm. DsRed2, mCherry, and Rhodamine Red were excited at 561 nm, and emission was detected between 580 and 610 nm. HCS LipidTOX Deep Red was excited at 633 nm, and emission was detected between 650 and 700 nm.

Statistical analysis

All measurements were performed in triplicates. Data are presented as means ± S.D. Statistical significance was determined by the Student’s unpaired t test. Group differences were considered statistically significant for p < 0.05 (*).
Functional domains of PNPLA7

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