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Research paper

Identification mouse patatin-like phospholipase domain containing protein 1 as a skin-specific and membrane-associated protein

Ping-An Chang *, Li-Ping Han, Lan-Xi Sun, Fei-Fei Huang

Key Laboratory of Molecular Biology, College of Bio-information, Chongqing University of Posts and Telecommunications, Chongqing 400065, PR China

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ABSTRACT

Patatin-like phospholipase domain containing protein 1 (PNPLA1) mutations have been identified to be associated with autosomal recessive congenital ichthyosis (ARCI) in recent years. However, its molecular characters have not been achieved until now. In the current study, the full length coding cDNA sequence of mouse PNPLA1 (mPNPLA1) was identified firstly. There were several putative transmembrane domains (TMDs) in mPNPLA1 by bioinformation analysis. mPNPLA1 was further found to be expressed exclusively in the membrane fraction in mammalian cells. However, it did not colocalized with the endoplasmic reticulum (ER) or lipid droplets (LDs). Moreover, the mRNA levels of mPNPLA1 was detected to be highly expressed in the skin, while very weak or even less in other mouse tissues by quantitative PCR. In addition, based on experiments with inhibitors and inducer of protein degradation pathways, mPNPLA1 was demonstrated to be degraded by macroautophagy, but not by the proteasome. These results indicated PNPLA1 was a skin-specific and membrane-associated protein for the first time, suggesting that it may mainly play a role in the skin.

1. Introduction

Autosomal recessive congenital ichthyosis (ARCI) is a group of skin syndromes characterized with rare, heterogeneous keratinization disorders affecting cornification (Rodríguez-Pazos et al., 2013). ARCI includes several forms of non-syndromic ichthyosis. Harlequin ichthyosis (HI, OMIM #242500), lamellar ichthyosis (LI, OMIM #242300), and congenital ichthyosiform erythroderma (CIE, OMIM #242100) are the main skin phenotypes (Sugiura and Akiyama, 2015). Significant progress has been made toward understanding the pathomechanisms and underlying genetic defects of ARCI. Since 1995, transglutaminase (TGase)-1 (TGM1) mutations were initially identified as the cause of LI, nine known genes are now causally associated with ARCI in human patients, including *patatin-like phospholipase domain protein 1* (PNPLA1) gene (Sugiura and Akiyama, 2015).

PNPLA1 was identified as a novel ARCI-causing gene in human and dog in 2012 (Grall et al., 2012). All affected individuals with PNPLA1 mutations were born as collodion babies (CBs) and the impairment seemed to be congenital and severe at birth (Grall et al., 2012). Recently, several novel PNPLA1 gene mutations were identified in a Spanish family and several unrelated consanguineous Pakistani families with ARCI (Fachal et al., 2014; Ahmad et al., 2015; Lee et al., 2016). In addition to ARCI, genetic variation in the adiponutrin family suggested that PNPLA1 may exhibit a modest effect on obesity in children and adolescents (Johansson et al., 2009).

Mammalian PNPLA protein family has been found to play critical roles in diverse aspects of lipid metabolism and signal pathway (Kienesberger et al., 2009). Nine PNPLA members were divided into three subgroups. PNPLA1 belongs to the first subgroup, which is also called the adiponutrin family (Lake et al., 2005; Kienesberger et al., 2009). In addition to PNPLA1, the adiponutrin family contains adipose triglyceride lipase (ATGL, PNPLA2), adiponutrin (PNPLA3), gene sequence-2 (GS2, PNPLA4) and GS2-like (PNPLA5). Except PNPLA1, other adiponutrin family proteins have been found to play key roles in lipid metabolism with diverse lipolytic and acyltransferase activities (Kienesberger et al., 2009; Baulande and Langlois, 2010). In contrast to other adiponutrin family members, human PNPLA1 gene was not observed to be expressed in a number of tissues (Lake et al., 2005). In contrast to the previous results, human PNPLA1 was observed to be expressed at very low levels in a number of tissues, notably in the

Abbreviations: ARCI, autosomal recessive congenital ichthyosis; ATGL, adipose triglyceride lipase; BAT, brown adipose tissue; CBs, collodion babies; CHX, cycloheximide; CIE, congenital ichthyosiform erythroderma; CM, cardiac muscle; CMA, chaperone-mediated autophagy; DMEM, Dulbecco's modified Eagle's medium; ECL, enhanced chemiluminescence; ER, endoplasmic reticulum; GFP, green fluorescence protein; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; GS2, gene sequence-2; HI, harlequin ichthyosis; LD, lipid droplet; LI, lamellar ichthyosis; 3-MA, 3-methyladenine; PBS, phosphate-buffered saline; PCR, Polymerase chain reaction; PNPLA, patatin-like phospholipase domain containing; 36B4, ribosomal protein large, P0; RT-PCR, Reverse transcription-PCR; Q-PCR, quantitative-PCR; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SM, skeletal muscle; TMD, transmembrane domain; WAT, white adipose tissue.

* Corresponding author.
E-mail address: changpa@cqupt.edu.cn (P.-A. Chang).

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digestive system, spleen and bone marrow recently (Wilson et al., 2006; Chang et al., 2013). Immunofluorescence showed that the human PNPLA1 existed in the epidermis and stronger expression in the granular layer (Grall et al., 2012).

PNPLA1 appears to play a role in glycerophospholipids synthesis, which is of prime importance in the keratinization process and terminal differentiation of keratinocytes (Grall et al., 2012). Until now, little is known the characters and function of PNPLA1. Here, mouse PNPLA1 (mPNPLA1) is found to have several putative TMDs and then certified to be a membrane-associated protein. However, it does not colocalize with the endoplasmic reticulum (ER) or lipid droplets (LDs). Further, mPNPLA1 is highly expressed in the skin. Moreover, it is demonstrated to be degraded by macroautophagy, but not by the proteasome. These results characterized mPNPLA1 for the first time and provided insights into the structure and function of PNPLA1.

2. Materials and methods

2.1. Materials

African green monkey kidney fibroblast-like cell line COS-7 was purchased from the Cell Center of Chinese Academy of Medical Sciences (Beijing, China). Plasmid pEGFP-N3 and pDsRed2-ER were from Clontech (Palo Alto, CA, USA). TRIzol® reagent, SuperScriptTM III First-Strand Synthesis System for RT-PCR, the transfection reagent Lipofectamine 2000 and HCS LipidTOX™ Deep Red neutral lipid stain were purchased from Invitrogen Life Technologies (Groningen, The Netherlands). SYBR® RT-PCR Kit and Ex Taq™ HS DNA polymerase, *Xho* I and *Eco*R I, pMD19-T were purchased from Takara (Dalian, China). Cell culture reagents, cycloheximide (CHX), MG132, 3-methyladenine (3-MA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Mouse anti-GFP, anti-actin and anti-GAPDH monoclonal antibodies, goat anti-mouse IgG HRP were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Enhanced chemiluminescence (ECL) reagents were obtained from Pierce Biotechnology (Rockford, IL, USA).

2.2. Molecular cloning of mouse PNPLA1 coding cDNA sequence

Total RNA of mouse skin was isolated using TRIzol® reagent and then was reversely transcribed to cDNA with Oligo $(dT)_{20}$ as the primer according to the manual of SuperScript™ III First-Strand Synthesis System for RT-PCR. According to the predicted mPNPLA1 gene sequence (GenBank accession no. NM_001034885.3), two specific forward and reverse primers, 5′ATGGACGAACAGGTGTTCAAAGGA3′ and 5′TTAGGA GTTCTGGCCACTCA CTCCCT3′, were designed to amplify mPNPLA1 coding sequence. The PCR reaction volume is 50 μl, which contains 5 μl 10 × PCR buffer, 4 μl 2.5 mM dNTP mixture, 2 μl synthesized cDNA template, 1 μl 20 μM forward and reverse primers, 0.5 μl Ex Taq™ HS DNA polymerase and 36.5 μl sterilized H_2O. The sequence was amplified by the following program: 95 °C for 4 min to pre-denature cDNA template; then 40 amplification cycles of 95 °C for 15 s, 60 °C for 25 s; 72 °C for 2 min; last 72 °C for 10 min to extend. The PCR products were cloned into T-vector and sequenced.

2.3. Computational sequence analyses

The amino acid sequence of mPNPLA1 was deduced from the obtained cDNA sequence using DNAman. The sequences of mPNPLA1 and human PNPLA1 (hPNPLA1, GenBank accession no. ACJ70710.1) were aligned using ClustalW (www.ebi.ac.uk/clustalw). Protein domains were analyzed using the protein family database (PFAM), an online database containing collections of protein domains and families (http://pfam.xfam.org/, Finn et al., 2016). A hydropathy plot of mPNPLA1 was generated by the method of Kyte and Doolittle using a window parameter of 19 amino acids (Kyte and Doolittle, 1982). Bioinformatic on-line programs: HMMTOP (http://www.enzim.hu/hmmtop/) (Tusnády and Simon, 1998), TMpred (http://embnet.vital-it.ch/software/TMPRED_form.html) (Hofmann and Stoffel, 1993), TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/) and TopPred (http://mobyle.pasteur.fr/cgi-bin/portal.py?#forms::toppred) were used to predict transmembrane domain (TMD).

2.4. Real-time quantitative-PCR

Total RNA was isolated from adult mouse 17 tissues using TRIzol® reagent and then reversely transcribed to cDNA with random primers. SYBR® real-time quantitative-PCR (Q-PCR) was performed by using MX3000P Real-Time PCR System (Stratagene, La Jolla, CA, USA) with *ribosomal protein*, *large*, *P0* (*Rplp0*, *36B4*), as an internal control. Two specific primers, 5′TCTGCGGGATTGAGATGGAGA 3′ and 5′CACATCGTACAAAAACTGTCGC 3′, were designed for the expression analysis of mPNPLA1 gene. The forward and reverse primers for *36B4* were 5′AGATTCGGGATATGCTGTTGGC 3′ and 5′ TCGGGTCCTAGACCAGTGTTC3′, respectively. Relative expression was determined by the Ct method and expressed as fold change with log scale. Three biological replicates were conducted for each tissue and each biological replicate was technically repeated three times.

2.5. Plasmid construction

For generation of mPNPLA1 fused with the GFP tag at the C-terminus, the forward primer containing an internal *Xho* I site, Kozak consensus (underlined), and a translation start condon (bold), 5′ TTCT CGAGGCC**ATG**GACGAACAGGTGTTCAA3′; was paired with the specific reverse primers, 5′ GCGAATTCGAGGAGTTCTGGCCACTCACTC 3′ with an *Eco*R I site to remove the original stop codon to amplify the coding sequences of mPNPLA1. PCR fragment was cloned into the *Xho* I/*Eco*R I sites of pEGFP-N3 to make pmPNPLA1-GFP, which was certified by DNA sequencing.

2.6. Cell culture, transfection and treatment

COS-7 cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 μg/ml each penicillin and streptomycin. Incubations were carried out at 37 °C in a humidified atmosphere of 5% CO_2/95% air. The cells were maintained in the logarithmic phase of growth and sub-cultured at 3–4 days intervals. The plasmid, pEGFP-N3 and pmPNPLA1-GFP, DNA was transfected into COS-7 cells using Lipofectamine 2000. After transfection for 24 h, 20 μM MG132 dissolved in DMSO and 10 mM 3-MA dissolved in water were added for 18 h to inhibit the protein degradation by the proteasome and autophagy respectively (Long et al., 2009; Chang et al., 2009). DMSO and water treatment was the control. At the same time, 30 μM CHX was added to the cells to avoid the confounding effects of ongoing protein synthesis. For starvation experiments, in the presence of 30 μM CHX transfected COS-7 cells were cultured in Hanks' solution for 6 h (Long et al., 2009).

2.7. Preparation of cell extracts and western blotting

COS-7 cells were collected by trypsinization and washed twice with phosphate buffered saline (PBS) after 48 h transfection or cell treatment. Cells were disrupted on ice in lysis buffer (0.25 M sucrose, 1 mM EDTA, 1 mM dithiothreitol, 20 μg/ml leupeptin, 2 μg/ml antipain, 1 μg/ml pepstatin, pH 7.0) by sonication. Nuclei and cell debris were removed by centrifugation at 1000*g* at 4 °C for 5 min. The supernatant was the total extraction. The total extraction was then centrifuged at 100,000*g* at 4 °C for 1 h to obtain membrane (pellet) and cytosolic (supernatant) fractions. The membrane fractions were resuspended in lysis buffer. The protein expression in cell extracts or fractions obtained was detected by western blotting as previous description (Chang et al., 2013).The blots were stripped and reprobed for anti-actin or anti-GAPDH antibody. The