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Isolation and characterization of mutant animal cell line defective in alkyl-dihydroxyacetonephosphate synthase: Localization and transport of plasmalogens to post-Golgi compartments

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ABSTRACT

We herein isolated plasmalogen-deficient Chinese hamster ovary (CHO) mutant, ZPEG251, with a phenotype of normal import of peroxisomal matrix and membrane proteins. In ZPEG251, plasmenylethanolamine (PlsEtn) was severely reduced. Complementation analysis by expression of genes responsible for the plasmalogen biogenesis suggested that alkyl-dihydroxyacetonephosphate synthase (ADAPS), catalyzing the second step of plasmalogen biogenesis, was deficient in ZPEG251. ADAPS mRNA was barely detectable as verified by Northern blot and reverse transcription-PCR analyses. Defect of ADAPS expression was also assessed by immunoblot. As a step toward delineating functional roles of PlsEtn, we investigated its subcellular localization. PlsEtn was localized to post-Golgi compartments and enriched in detergent-resistant membranes. Transport of PlsEtn to post-Golgi compartments was apparently affected by lowering cellular ATP, but not by inhibitors of microtubule assembly and vesicular transport. Partitioning of cholesterol and sphingomyelin, a typical feature of lipid rafts, was not impaired in plasmalogen-deficient cells, including peroxisome assembly-defective mutants, hence suggesting that PlsEtn was not essential for lipid-raft architecture in CHO cells.

1. Introduction

Peroxisome functions in unique biochemical pathways including various lipid metabolism such as synthesis of plasmalogens and docosahexaenoic acid and β-oxidation of very long chain fatty acids that are poorly oxidized in mitochondria. Plasmalogens are a major subclass of ethanolamine and choline phospholipids in which a long chain fatty alcohol is linked at *sn*-1 position through a vinyl ether bond. The first two reactions in plasmalogen biosynthesis are catalyzed by dihydroxyacetonephosphate acyltransferase (DHAPAT) and alkyl-dihydroxyacetonephosphate synthase (ADAPS) in peroxisomes [1]. Physiological importance of plasmalogens is inferred from the findings that plasmalogens are severely deficient in specimens from patients with various degenerative diseases and genetic disorders such as Zellweger syndrome and rhizomelic chondrodysplasia punctata (RCDP) [2]. However, functional roles and subcellular localization of plasmalogens remain elusive.

Several potential functions of plasmalogens have been proposed. Plasmalogens likely play a role as an antioxidant, based on the observation that plasmalogen-deficient Chinese hamster ovary (CHO) cells were less resistant to UV-induced oxidative stress [3,4]. Restoration of the plasmalogen content rescued such phenotypes of the cell mutants [3,4]. Plasmalogens are also required for transport of LDL-derived cholesterol from cell surface and/or endocytic compartments to endoplasmic reticulum (ER) [5]. Plasmalogens are relatively enriched in lipid-raft fractions [6,7]. Association of flotillin-1 and F3/contactin to lipid-raft microdomain is reduced in the brain of DHAPAT-knockout mice [8]. Subcellular distribution of plasmalogens is not fully defined, while several reports describe its presence in synaptic vesicles [9,10] and secretory granules [11].

In CHO cells, 11% of the total phospholipids are plasmalogens, primarily ethanolamine plasmalogen called plasmenylethanolamine (PlsEtn) [12], excluding plasmenylcholine [13]. In the present study, we isolated and characterized CHO cell mutant ZPEG251 defective in plasmalogen synthesis, addressing transport of plasmalogens. We also

Abbreviations: AOx, acyl-CoA oxidase; ADAPS, alkyl-dihydroxyacetonephosphate synthase; BFA, brefeldin A; β-MCD, β-methyl cyclodextrin; CHO, Chinese hamster ovary; DHAPAT, dihydroxyacetonephosphate acyltransferase; DRM, detergent-resistant membranes; EGFP, "enhanced" green fluorescent protein; ER, endoplasmic reticulum; HB, homogenizing buffer; lyso-PE, 2-acylglycerophosphoethanolamine; HG, *sn*-1-hexadecylglycerol; NDGA, nordihydroguaiaretic acid; P450R, cytochrome P450 reductase; P9OH/UV, 9-(1′-pyrene)nonanol/ultraviolet; PE, phosphatidylethanolamine; PlsEtn, plasmenylethanolamine; PM, plasma membranes; PNS, post-nuclear supernatant; PTS1 and PTS2, peroxisome targeting signal types 1 and 2; RT, reverse transcription; SM, sphingomyelin; TCA, trichloroacetic acid; TLC, thin-layer chromatography; TfR, transferrin receptor

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compared lipid raft micro-domain between normal and plasmalogen-deficient cells.

2. Materials and methods

2.1. Biochemicals

Restriction enzymes and DNA-modifying enzymes were purchased from Nippon Gene (Tokyo, Japan) and Takara (Tokyo, Japan). Fetal bovine serum and Ham's F-12 medium were from Invitrogen (Carlsbad, CA). We used rabbit antibodies to PTS1 [14], thiolase [15], Pex14p [16], acyl-CoA oxidase (AOx) [15], 70-kDa peroxisomal integral membrane protein [15], and malate dehydrogenase [17]. Antibodies against green fluorescent protein (GFP), cytochrome P450 reductase (P450R), Fyn, Gαq, H-Ras, caveolin, and Na^+/K^+-ATPase α-subunit were purchased from Santa Cruz (Santa Cruz, CA). Antibodies to Rab11 and flotillin-2 were from BD Transduction Laboratories (San Jose, CA). Antibody to transferrin receptor (TfR) was from Zymed (San Francisco, CA). Anti-Syntaxin 6 antibody was from Stressgen (Ann Arbor, MI). PlsEtn purified from bovine brain and chemically synthesized PlsEtn, 1-*O*-1′-(*Z*)-octadecenyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine were purchased from Doosan Serdary Research Laboratories (Kyungki-Do, Korea) and Avanti Polar Lipids (Birmingham, AL), respectively. Other standard lipids, β-methyl cyclodextrin (β-MCD), and saponin were purchased from Sigma (St. Louis, MO). Anti-mouse ADAPS antiserum was raised in rabbits by injection of its C-terminal peptide, GC-KSVKEYVDPSNIFGNRNLL, coupled to keyhole limpet hemocyanin [18].

2.2. ATP assay

Intracellular level of ATP was determined with an ELTIN ATP assay system, a Bioluminescence Detection kit for ATP (Promega, Madison, WI), according to the manufacturer's instruction.

2.3. Cell culture, selection of plasmalogen-deficient CHO cell mutant, and DNA transfection

CHO cells were cultured in Ham's F-12 medium supplemented with 10% fetal bovine serum under 5% CO_2/95% air [15]. Mutagenesis was performed by exposure of TKaEG2 cells [19] to 3-chloro-7-methoxy-9-(3-[chloroethyl]-amino propylamino)-acridine dihydrochloride (ICR191; Sigma) at 0.4 μg/ml for 8 h [20]. Cell colonies resistant to the treatment with 9-(1′-pyrene)nonanol/ultraviolet (P9OH/UV) but showing morphologically normal peroxisome assembly were isolated and analyzed for their plasmalogen levels as described below. For supplementation of PlsEtn, cells were cultured for 48 h in the presence of 10 μM *sn*-1-hexadecylglycerol (HG) [3]. Bovine brain PlsEtn and 1-*O*-1′-(*Z*)-octadecenyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine were separately dried up in tubes, resuspended in F-12 medium by sonication, and likewise added every 24 h to the culture medium at 46 μM. DNA transfection was performed as described [21].

2.4. Lipid extraction

Cells were metabolically labeled with ^{14}C-ethanolamine (Amersham Biosciences, Tokyo, Japan) [1] for 5 h and harvested after washing with PBS. Suspended cells were treated with 5% of trichloroacetic acid (TCA) for 30 min at room temperature and precipitated. Lipids were extracted according to Bligh and Dyer [22]. ^{14}C-labeled lipids were resolved on thin-layer chromatography (TLC) plates (silica gel 60, Merck KgaA, Darmstadt, Germany) using chloroform/methanol/acetic acid (65/25/10) and quantified by phosphorimaging using FLA-5000 Imaging Analyzer (Fuji Film. Tokyo, Japan). For cholesterol, extracted lipids were resolved in hexane/diethyl ether/acetic acid (80/20/1.5) and detected by iodine vapor [23]. For lipid analysis of subcellular fractions, lipids were extracted, re-suspended in PBS containing 1% defatted BSA, and re-extracted after TCA treatment.

2.5. Subcellular fractionation

CHO-K1 cells grown to a confluent stage in a 10-cm culture dish were metabolically labeled with ^{14}C-ethanolamine for 18 h. Cells were homogenized with a homogenizing buffer (HB, 0.25 M sucrose/1 mM EDTA in 10 mM HEPES-KOH, pH 7.4) by passing through a 23-G needle 20 times. Post-nuclear supernatant (PNS) fraction was adjusted to 20 or 12.5% (w/v) iodixanol with OptiPrep (60% iodixanol) [24] (Invitrogen) and centrifuged for 3 h at 186,000×*g* or 348,000×*g*, respectively, in an NVTi65 rotor (Beckman Instruments, Fullerton, CA). Ten 1 ml-fractions were collected from the bottom of tubes.

2.6. Treatment of cells with cholesterol-affecting agents

CHO-K1 cells were washed three times with phosphate-buffered saline and treated with either 10 mM β-MCD for 1 h at 37 °C in F12 minus FCS or 0.4% saponin in phosphate-buffered saline for 1 h at 4 °C.

2.7. Flotation

Cells were metabolically labeled with ^{14}C-ethanoamine or ^{14}C-acetate (Amersham) [25,26] for 18 h, harvested and resuspended in 300 μl of HB. Two hundred seventy microliters of cell homogenate was treated with 30 μl of 10% Triton X-100 on ice for 30 min and was adjusted to 40% iodixanol with 600 μl of OptiPrep. The sample (700 μl) in TLS55 centrifuge tubes (Beckman) was overlaid with 1.2 ml of 30% iodixanol/HB and 0.1 ml of 10 mM HEPES-KOH, pH 7.4, and centrifuged at 55,000 rpm (259,000×g) for 2 h. Two 1-ml fractions were collected from the top. Detergent-free rafts were prepared as follows. Confluent cells in one 10-cm dish were scraped into HB/Tris buffer (0.25 M sucrose and 20 mM Tris–HCl, pH 7.8) containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ [27]. Cells were pelleted and homogenized with HB/Tris buffer as above. PNS faction was adjusted to 30% iodixanol with Optiprep, overlaid with 0.4 ml of 20% iodixanol/HB/Tris, 0.9 ml of 5% iodixanol/HB/Tris, and 0.1 ml of HB/Tris, and centrifuged at 55,000 rpm for 1 h in a TLS55 rotor. Five 400-μl fractions were collected from the top.

2.8. Cloning of ADAPS from CHO cells

Chinese hamster (*Cl*) *ADAPS* was cloned as follows. Total RNA was prepared from wild-type CHO-K1 cells using an RNeasy kit (Qiagen, Hilden, Germany) and first strand cDNA was obtained by reverse transcription (RT). A 398-bp fragment was amplified using primers designed from human ADAPS-cDNA sequence [28], a sense ADAPSFw1 (5′-TTATGAAATGGAATGGATGGG-3′) and an antisense ADAPSRv1 (5′-CACAATCTTAACTACATCATCATG-3′). Based on the sequence of PCR fragment, we prepared another specific primer set for *ClADAPS*, *Cl*ADAPSFwA (5′-CTATAATGATTCCAAGTTCTTCTTC-3′) and *Cl*ADAPSRvA (5′-AACAACTATATCAGGAATCCGTTCC-3′). Approximately 2.0×10^5 independent colonies of cDNA library from wild-type CHO-K1 cells in pSPORT [14] were divided into small pools. Each pool was verified for *ClADAPS* by PCR with a set of primers, *Cl*ADAPSFwA and *Cl*ADAPSRvA. The positive pool was further divided into smaller pools until a single clone of *Cl*ADAPS was isolated. Nucleotide sequencing of both strands was done by a Dye-terminator DNA sequence kit (Applied Biosystems, Foster City, CA).

2.9. Mutation and mRNA-level analysis

ADAPS in ZPEG251 was assessed as follows. Total RNA was obtained from ZPEG251 as described above. RT-PCR was done with a pair of *Cl*ADAPS5′non (5′-CCACGCGTCCGGGCGGAAGCC-3′) and *Cl*ADAPSRvA, and *Cl*ADAPSFwA and *Cl*ADAPS3′non (5′-CTGGTATAACCATAGCTGATGACT-3′), to cover a full-length *ADAPS* open reading frame. The nucleotide sequence of the PCR products cloned in T-easy vector (Promega) was determined as above.

For Northern blotting, the blot of total RNA from wild-type CHO-K1 and ZPEG251 was hybridized with α-^{32}P-dCTP-labeled PCR products that had been amplified with a pair of *Cl*ADAPSFwB and *Cl*ADAPSRvB (nucleotide residues 931–1560) of *ClADAPS*. RT-PCR was performed with primers for *ADAPS*, *Cl*ADAP1748Fw: AACCGAGGCAGCTGCTAGAGATG and *Cl*ADAP1904Rv: CTGGGGTCCACATATTCCTTGACAG covering 157-bp sequence, and those for 227-bp long actin DNA as a control, sense primer: AAGATGACCCAGATCATG and antisense primer: AGGATCTTCATGAGGTAG.

2.10. Construction of ADAPS-HA_2

ClADAPS-HA_2 was amplified by PCR using primers Hind/*Cl*ADAPS5′Fw (5′-CCCAAGCTTGCCACCATGGCGGAGGCGGCGGGCGA-3′) and *Cl*ADAPS-HA_2/XbaRV (5′-GCTCTAGATTAATAATCGGGCACATCGTAGGGGTACGCATAATCGGGCACATCGTAGGGGTACGCCAAAAGGTTTCTGTTTCCAAAGATG-3′). PCR product was digested with *Hin*dIII and *Xba*I and cloned into the *Hin*dIII–*Xba*I site of pcDNA3.1Zeo (Invitrogen).

3. Results

3.1. Isolation and characterization of plasmalogen-deficient cell mutant

We mutagenized parent TKaEG2 cells [19], the wild-type CHO-K1 stably expressing both rat Pex2p and enhanced green fluorescent protein N-terminally tagged with peroxisome targeting signal type 2 (PTS2-EGFP), and searched for P9OH/UV-resistant cell colonies showing morphologically normal PTS2-EGFP import to peroxisomes. Of several P9OH/UV-resistant colonies, one cell clone named ZPEG251 showed peroxisomal localization of PTS2-EGFP and PTS1 proteins as well as peroxisomal membrane protein, Pex14p (Fig. 1A). Moreover, the conversion of 75-kDa AOx-A component to 53-kDa B- and 22-kDa C-components and the processing of 44-kDa precursor to 41-kDa mature form of 3-ketoacyl-CoA thiolase (thiolase) were evident in both CHO-K1 [15] and ZPEG251 cells, while only A-component of AOx and the thiolase precursor were detected in well-characterized peroxisome-defective CHO mutant, *pex2* Z65, as reported [15] (Fig. 1B). Such cellular phenotypes suggested that plasmalogen biosynthesis, but not the assembly of peroxisomes, was impaired in ZPEG251, similar to plasmalogen-deficient cell mutant NZel-1 isolated by Nagan et al. [1].