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Differentially localized acyl-CoA synthetase 4 isoenzymes mediate the metabolic channeling of fatty acids towards phosphatidylinositol

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ABSTRACT

The acyl-CoA synthetase 4 (ACSL4) has been implicated in carcinogenesis and neuronal development. Acyl-CoA synthetases are essential enzymes of lipid metabolism, and ACSL4 is distinguished by its preference for arachidonic acid. Two human ACSL4 isoforms arising from differential splicing were analyzed by ectopic expression in COS cells. We found that the ACSL4_v1 variant localized to the inner side of the plasma membrane including microvilli, and was also present in the cytosol. ACSL4_v2 contains an additional N-terminal hydrophobic region; this isoform was located at the endoplasmic reticulum and on lipid droplets. A third isoform was designed de novo by appending a mitochondrial targeting signal. All three ACSL4 variants showed the same specific enzyme activity. Overexpression of the isoenzymes increased cellular uptake of arachidonate to the same degree, indicating that the metabolic trapping of fatty acids is independent of the subcellular localization. Remarkably, phospholipid metabolism was changed by ACSL4 expression. Labeling with arachidonate showed that the amount of newly synthesized phosphatidylinositol was increased by all three ACSL4 isoenzymes but not by ACSL1. This was dependent on the expression level and the localization of the ACSL4 isoform. We conclude that in our model system exogenous fatty acids are channeled preferentially towards phosphatidylinositol by ACSL4 overexpression. The differential localization of the endogenous isoenzymes may provide compartment specific precursors of this anionic phospholipid important for many signaling processes.

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1. Introduction

Lipid metabolism allows the synthesis of membranes and the adaptation to nutrient availability by either storing or oxidizing lipids. For all these processes, the activation of fatty acids by the esterification with coenzyme A is an essential requirement. Fatty acyl-CoA synthetases (ACS)/fatty acid-CoA ligases (EC 6.2.1.3) are the enzymes catalyzing this reaction. There are thirteen mammalian ACS which accept long chain fatty acids as a substrate [1,2]. As expected from their importance for basic cellular functions, there is usually redundant expression of several ACS [3,4].

Long chain fatty acyl-CoA synthetase 4 (ACSL4) is ubiquitously expressed but especially abundant in the brain and the adrenal gland. It uses preferentially arachidonate and eicosapentaenoate but palmitate is also activated efficiently [5–7]. Differential splicing generates two mRNAs; ACSL4_v1 is the abundant transcript whereas ACSL4_v2 appears restricted to neurons [8–10]. ACSL4_v2 contains an earlier in-frame start codon which translates into an additional 41 amino acids. A point mutation affecting both transcripts is one of the genetic causesof non-specific X chromosome linked mental retardation [10,11]. ACSL4 has also been suggested to be involved in cancerogenesis, presumably by regulating the level of free arachidonate and thereby changing eicosanoid biosynthesis and apoptotic processes [12–14]. In the adrenal gland, ACSL4 plays a role in steroidogenesis [15].

Long chain ACS enzymes receive their fatty acid substrate from the membrane but release their fatty acyl-CoA product presumably to the cytosolic side [16,17]. Fatty acyl-CoA molecules are generally assumed to be equilibrated rapidly across the cellular environment [18,19]. Nevertheless, many observations indicate that the metabolic fate of activated fatty acids depends on which ACS did the synthesis. This has led to the hypothesis that ACSL enzymes are channeling fatty acids into specific metabolic pathways [19,20]. However, the molecular mechanism behind fatty acid channeling is not known.

The metabolism of fatty acids has enormous implications for widespread and serious lipid-associated diseases like diabetes type 2 and atherosclerosis. Here, we followed the idea that the subcellular localization of ACSL enzymes is relevant for the metabolic channeling of fatty acids [19,21]. Our strategy was to use a gain-of-function approach to examine the metabolic consequences of ACSL4 targeted

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to two different subcellular compartments. After analyzing the localization by confocal microscopy and subcellular fractionation, we looked for metabolic changes by using radiolabeled fatty acids and thin layer chromatography.

2. Material and methods

2.1. Cloning of ACSL4 plasmids

2.1.1. ACSL4 (ACSL4_v1$_{FLAG}$)

RNA was isolated from human hepatoma HepG2 cells (ATCC HB-8065) and transcribed into cDNA. PCR with primers s-H3-A4 and a-A4-BhI yielded DNA of the expected size which was digested with HindIII and BamHI, and ligated into the tagging plasmid Murr1-FLAG based on the mammalian expression vector pcDNA3 (Invitrogen, Carlsbad, CA). Murr1-FLAG was cloned from Murr1-RFP [22] by inserting the paired oligo s-flag and a-flag coding for the FLAG epitope tag between BamHI and NotI. The final ACSL4 expression plasmid contains the complete open reading frame of the human ACSL4 transcript variant 1 (NCBI reference sequence NM_004458.2) followed by the epitope tagging cassette (ADPDYKDDDDK).

2.1.2. ACSL4-GFP (ACSL4_v1-GFP)

Plasmid ACSL4 was subcloned with HindIII and BamHI into pEGFP-N1 (Clontech, Mountain View, CA). The localization pattern by confocal microscopy was the same as for ACSL4_v1$_{FLAG}$.

2.1.3. GFP-ACSL4 (GFP-ACSL4_v1)

Plasmid ACSL4 was digested with HindIII and XhoI and subcloned into pEGFP-C3 (Clontech) cut with HindIII and SalI. GFP-ACSL4 was recovered with AgeI and NotI and subcloned into the retroviral vector pRIJ, which is a derivative of pQCXIP (BD Biosciences, Heidelberg, Germany) with a more convenient polylinker.

2.1.4. M-ACSL4 (ACSL4_v2$_{FLAG}$)

cDNA was prepared from the human neuroblastoma cell line SH-SY5Y (ATCC CRL-2266) and used for PCR with primers A4brain and a-A4-Bh1. The product was digested with HindIII and BamHI and ligated into Murr1-FLAG as above. The M-ACSL4 plasmid contains the full length cDNA of the human ACSL4 transcript variant 2 (NCBI NM_022977.2) followed by the epitope tagging cassette.

2.1.5. Tom20-A4 (tom20-ACSL4_v1$_{FLAG}$)

This plasmid encodes amino acids 1–30 of the human outer mitochondrial membrane receptor tom20 (NCBI NP_055580.1), followed by full length ACSL4_v1 and the FLAG epitope. It was derived by subcloning tom20-A4-GFP with SpeI and NotI, which in turn was obtained from ACSL4-GFP and tom20.pNice using XhoI and NotI. Tom20.pNice is a self-made mitochondrial tagging plasmid assembled with two paired oligos coding for the N-terminus of human tom20; these were assembled sequentially between NheI and BglII of pEGFP-N1.

Oligonucleotide sequences:

a-A4-BhI	ACGTGGATCCGCTTTGCCCCCATACATTCGTTC
a-flag	GGCCGCTTACTTATCGTCGTCATCCTTGTAATCTG
A4brain	ACGTAAGCTTACCATGAAACTTAAGCTAAATGTGC
s-flag	GATCCAGATTACAAGGATGACGACGATAAGTAAGC
s-H3-A4	GAATTCAAGCTTACCATGGCAAAGAGAATAAAAGC.

All ACSL4 cDNAs were identical to the corresponding database entries as verified by sequencing.

2.2. Cell culture, transfection and quantification of ACSL4 expression

COS-7 cells (ATCC CRL-1651) were grown in DMEM 4.5 g/l glucose supplemented with 10% FCS and glutamate (all reagents from Invitrogen, Carlsbad, CA). Cells were grown to 60% confluency in 6-well plates (10 cm^2/well) and transfected using 2.0 μg DNA and 10 μl FUGENE HD (Roche, Mannheim, Germany) for 4 h. The expression vector pcDNA3 (Stratagene, Santa Clara, CA) was used as control, and experiments started 24 h after transfection. Where indicated (+OA), the efficient formation of lipid droplets was induced by incubation with 180 μM oleate bound to 45 μM BSA for 18 h.

The differentially targeted ACSL4 variants were expressed with different efficiencies. Comparable expression was achieved by adjusting the transfection conditions. ACSL4: 1.0 μg of ACSL4 plasmid, 1.0 μg pcDNA3; M-ACSL4: 4.0 μg M-ACSL4 plasmid in 20 μl FUGENE HD, and 8 h of incubation time; and tom20-A4: 0.2 μg tom20-A4 plasmid, 1.8 μg pcDNA3. The expression level of FLAG epitope tagged rat ACSL1 [6,23] was similar to tom20-A4 as verified by Western blotting.

HuH-7 cells (JCRB 0403) were stably transduced with retroviral supernatant obtained by transfecting Phoenix-GP cells with GFP-ACSL4.pRIJ, followed by puromycin selection as described [24]. Control HuH-7 cells were transduced in parallel using an empty retroviral plasmid.

2.2.1. Relative quantification of ACSL4 expression

Mock treated total cell lysates were separated by 8% SDS-PAGE, and the Western blots developed with mouse anti-FLAG M2 antibodies (Sigma, St. Louis, MO) to detect the ACSL4 isoenzymes, and with mouse anti-β-actin (clone AC-15; Sigma) for internal calibration. Secondary antibodies were horseradish peroxidase-conjugated from Jackson ImmunoResearch, West Grove, PA. Multiple exposures of Roentgen films were used for densitometric analysis by ImageJ 1.37v software (http://rbs.info.nih.gov/iJ).

2.3. Immunofluorescence microscopy

The following plasmids coding for fluorescent fusion proteins were used as membrane markers: GFP-TMD contains the transmembrane domain of canine podocalyxin [25], and localizes preferentially to the plasma membrane [26]. Prominin-GFP is targeted to microvilli of the plasma membrane [27]. ER-RFP contains the full length sequence of human Sec61ß [28]. Pex-RFP features a C-terminal SKL targeting motif for peroxisomes [28]. Mito-GFP contains the N-terminal mitochondrial matrix targeting signal of ornithine carbamyl transferase [29]. GFP-FATP4 was used as an ER membrane marker protein [23] with high expression but no tendency for the formation of artificial karmallae/stacked ER cisternae [30].

Cells grown on 11 mm cover slips were fixed for 20 min with 4% paraformaldehyde in PBS, and either directly embedded in Mowiol 4-88 (Calbiochem, San Diego, CA), or processed for indirect immunofluorescence of ACSL4 isoenzymes. Fixed cells were permeabilized with 0.1% saponin and blocked with 0.5% gelatin and 0.5% BSA for 10 min. Incubation with mouse anti-FLAG M2 antibody was for 60 min. After washing with SG solution (0.01% saponin, 0.2% gelatin in PBS), secondary antibodies were applied for 60 min (donkey anti-mouse coupled to Cy3 or Cy2; Jackson ImmunoResearch). Neutral lipids were stained with a 1:1000 dilution of a saturated stock solution of BODIPY 493/503 (D3922, Invitrogen) in ethanol for 10 min at RT. Nuclei were stained with 0.5 μg/ml of Hoechst 33342.

Image acquisition was on a Leica TCS SP2 confocal microscope (Leica Microsystems, Wetzlar, Germany) equipped with a 63× oil immersion objective (NA 1.32) for all figures except Fig. 2G (Olympus BX41, 60× oil immersion Plan S Apo NA 1.35, F-view II CCD camera, cell^D software). During pre-scanning, brightness was adjusted so that (over)saturation was minimized. All images are single sections of a representative focal plane obtained sequentially for each fluorochrome with 4× line averaging. Figures were arranged and labeled with Adobe Photoshop and Illustrator (Adobe Systems Inc., Mountain View, CA).

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