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Identification and characterization of the ER/lipid droplet-targeting sequence in 17β-hydroxysteroid dehydrogenase type 11

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ABSTRACT

17β-Hydroxysteroid dehydrogenase type 11 (17βHSD11) is mostly localized on the endoplasmic reticulum (ER) membrane under normal conditions and redistributes to lipid droplets (LDs) when the formation of LDs is induced. In this study, confocal microscopy analyses of the subcellular localization of the mutated 17βHSD11 proteins in cells with or without LDs revealed that both an N-terminal hydrophobic sequence and an adjacent sequence that has a weak homology with the PAT motif are independently necessary and both parts together (28 amino acid residues in total) are sufficient for the dual localization of 17βHSD11. Mutation analyses suggest that the PAT-like motif in 17βHSD11 will not be functionally similar to the canonical PAT motif. Hsp60 was identified as a possibly interacting protein with the PAT-like motif, and biochemical and microscopic analyses suggest that Hsp60 may be partly, but not necessarily involved in recognition of the PAT-like part of the targeting sequence of 17βHSD11.

Lipid droplets (LDs)[1] are found in many cell types as intracellular organelles, composed primarily of a core of neutral lipids surrounded by a phospholipid/cholesterol monolayer [1,2] and many associated proteins [3]. The compositions and physiological roles of LDs are diverse among cell types: adipocytes store excess energy reserves as triacylglycerols in the cores of massive LDs and release them in a regulated manner; steroidogenic cells accumulate cholesterol esters in numerous small LDs for steroid hormone biosynthesis, and most cells use stored cholesterol esters for membrane cholesterols [1,3]; hepatocytes rapidly respond to changes in dietary conditions by inducing the formation of LDs [1]. The diversities in LD sizes, core contents and mechanisms controlling the flux of molecules into and out of LDs are maintained by the proteins embedded in or associated with the phospholipid/cholesterol monolayer surrounding the LD cores [4–6].

Many proteins, up to 35 at least, have been reported as being associated with LDs in various cell types and physiological conditions, mostly owing to the recent progress in proteomic analysis by mass spectrometry [7–10]. The most widely studied LD proteins are perilipin, adipocyte differentiation-related protein (ADRP), the tail-interacting protein (TIP47), and S3-12, collectively known as the PAT protein family sharing a conserved PAT motif [11]. In addition, we recently identified a new PAT family member named MLDP (myocardial LD protein) via a data base search followed by biochemical and microscopic analyses [4]. The PAT proteins share a common structural motif but their tissue and subcellular distributions are diverse: perilipin is expressed exclusively in adipocytes and steroidogenic cells and is involved in hormone-stimulated lipolysis [12–14], ADRP is expressed ubiquitously but depends on the total fat mass and functions in incorporation and accumulation of lipid [15,16], and TIP47 was first identified as a binding partner of the mannose 6-phosphate receptor in the *trans*-Golgi network [17] but recent work has shown that it is also localized on LDs [18,19]. Long chain acyl-CoA synthetase 3 was recently identified as a major LD-associated protein in human hepatoma cells [8] and its involvement in local synthesis of neutral lipids and LD formation was demonstrated [5,20]. In addition, several proteins including small GTPases, caveoline and phospholipase D, have been identified on LDs and their roles in LD formation and its interaction with other organelles have been explored [3,21–24].

Studies of the targeting signals in not only PAT proteins [4,25–30] but also other LD-associated proteins [2,31,32] have shown the lack of a common structural motif for LD-targeting. In most cases, the amino acid residues that are critical for targeting the LD-associated proteins are not localized in small regions but spread discontinuously throughout broader regions [4,25,27–29], and the importance of the total three-dimensional structure has been emphasized [4,19]. The differences in the targeting sequences and mechanisms among the LD-associated proteins are possibly intimately related to the diversity in their intracellular functions. Thus characterization of the targeting sequences and mechanisms

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[1] *Abbreviations used:* LDs, lipid droplets; ADRP, adipocyte differentiation-related protein; TIP47, the tail-interacting protein; MLDP, myocardial LD protein; 17βHSD11, 17β-hydroxysteroid dehydrogenase type 11; PPARα, proliferator-activated receptor α; CHO , Chinese hamster ovary; FBS, fetal bovine serum; OGDH, 2-oxoglutarate dehydrogenase; PMF, peptide mass fingerprinting; TG, triglyceride.

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of various types of LD-associated proteins is necessary to understand the diverse physiological roles of LDs in various cell types. However, as most types of cultured cells have the ability to generate LDs under conditions of elevated fatty acids, many studies have been focused on the LDs in less specialized cell types and these studies may have applicable limits for understanding the diverse physiological roles of LDs.

We have identified 17β-hydroxysteroid dehydrogenase type 11 (17βHSD11) as a new protein whose expression is largely regulated by peroxisome proliferator-activated receptor α (PPARα) in the mouse intestine and liver [33]. According to published observations by Fujimoto et al., 17βHSD11 is an LD-associated protein in human hepatoma cells [8], and we further investigated its tissue and subcellular distribution using mice and cultured cells under different physiological conditions [34]. 17βHSD11 is induced by administration of a PPARα agonist in intestinal epithelia and hepatocytes with heterogeneous localization in both the cytoplasm and vesicular structures. Microscopic studies using Chinese hamster ovary (CHO) cells expressing green fluorescence protein-tagged 17βHSD11 and subcellular fractionation studies of the liver homogenate showed that it is mostly localized in the ER under normal conditions whereas it is concentrated on the LDs when they are induced. Furthermore, a pulse-chase experiment suggested that 17βHSD11 redistributed to the LDs via the ER. Thus 17βHSD11 is an intestine and liver enriched protein that localizes on both the ER and LDs depending on physiological conditions.

We have been interested in the possibility that diet-derived or oxidized hydrophobic, potentially toxic molecules may also be included in the LD cores in intestine and liver cells and that 17βHSD11, as one of the enzymes associated with the LDs, may be involved in the metabolism of such molecules. As the first step in examining this possibility, we are studying the regulatory mechanisms for expression of 17βHSD11 and its redistribution from the ER to LDs according to physiological conditions. In this study, we used mutation analysis to identify the N-terminal region of 17βHSD11 as the LD-localizing signal. It is composed of two parts both of which are necessary and sufficient for redistribution of 17βHSD11 from the ER to LDs during the formation of LDs. Furthermore, Hsp60 was identified as a protein partly and indirectly involved in recognition of the latter part of the targeting sequence.

Materials and methods

Plasmid construction

Construction of plasmid for full-length 17βHSD11 with GFP at the C-terminus was described previously [34]. Various C-terminal truncation mutants of 17βHSD11 were generated by PCR using full-length cDNA as the template. The PCR primers used are listed in Table 1. The amplified fragments were digested with EcoRI and XhoI and cloned into pCGFP2 (a kind gift from Dr. Higashi in our laboratory). Amino acid substituted mutant plasmids (pCGFP2-17βHSD11-N35 (V23A, F26A)) were constructed using Mutan-Super Express Km Kit (Takara, Kyoto, Japan) by the inverted PCR method using the mutagenic oligonucleotide (Table 1) after subcloning the EcoRI–BamHI fragment from pCGFP2-17βHSD11-N35 into pKF18k-2 (pKF18-17βHSD11-N35). The PCR products were blunted using KOD polymerase (Toyobo, Osaka, Japan), phosphorylated with T4 polynucleotide kinase (Takara), ligated using Quick Ligation kit (Nippon Gene, Tokyo, Japan) and transformed into *Escherichia coli* JM109 competent cells. The mutated fragment was excised out from the plasmid DNA using EcoRI and XhoI and cloned back into pCGFP2 vector. Other amino acid substituted and small deletion mutants were constructed by the inverse PCR method as above using the primers listed in Table 1. GST-fused 17βHSD11 targeting sequence and its mutant plasmids were constructed by the PCR method using pKF18-17βHSD11-N35 or its mutant plasmids as template DNAs and the primers listed in Table 1. The amplified DNA fragments were cloned into pGEX4T-1 (GE Healthcare) after digestion with EcoRI and XhoI. The identities of all the constructs were confirmed by DNA sequencing.

Table 1

HSD11-GFP truncated constructs	
Left	
HSD11	5′-GGgaattcGTTTAGGACCGGGAACGAGAGC-3′
Right	
N21	5′-GGCctcgagAGACTCAATGCTG-3′
N28	5′-GGCctcgagGGGAATAAAAAGCTTG-3′
N35	5′-GGActcgagGGCGACAGATTTTTTC-3′
N50	5′-GGCctcgagTCTTCCAATCCCATGACCAGC-3′
N138	5′-GGCctcgagTTCAATCTGAGGGTCCTGCGT-3′
N232	5′-GGCctcgagCTTGGTGCTTGGGTTCTTGAT-3′
HSD11 N35(V23A,F26A)	
Mutagenic oligonucleotide	5′-GAGTCTCTTGCCAAGCTTGCTATTCCCAAG-3′
HSD11 N35(E20A,K24A)	
Left	5′-CTTGTCGCGCTTTTTATTCCCAAG-3′
Right	5′-GCTAGCAATGCTGAAGACGATG-3′
HSD11 N35Δ4-16	
Left	5′-TTCAGCATTGAGTCTCTTGTCAAGC-3′
Right	5′-ATACTTCATCCTTTTGGAGGCCC-3′
HSD11 N35Δ22,23	
Left	5′-AAGCTTTTTATTCCCAAGAAG-3′
Right	5′-AGACTCAATGCTGAAGACG-3′
HSD11 N35Δ22-28	
Left	5′-AAGAAGAAAAAATCTGTCGCC-3′
Right	5′-AGACTCAATGCTGAAGACG-3′
GST pull-down	
Left	5′-GCCgaattcATGAAGTATCTTCTTGACTTG-3′
Right	5′-GAActcgagTCAGGCGACAGATTTTTTC-3′

Cell culture and DNA transfection

CHO-K1 cells were maintained in F-12 Nutrient Mixture medium (Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS) at 37 °C and 5% CO_2. CHO-K1 cells constitutively expressing mouse 17βHSD11 tagged with Myc at the C-terminus [34] were cultured in the presence of 250 μg/mL G418 (Nacalai Tesque, Tokyo, Japan). Transient transfection of CHO-K1 cells was carried out using Plus Reagent and Lipofectamine (Invitrogen) according to the manufacturer's instructions. Briefly, 0.4 μg/well plasmid DNA was incubated with 0.8 μl/well Plus Reagent, 1.2 μl/well Lipofectamine Reagent and 250 μl of serum-free F-12 medium and the 60% confluent cells in a 24-well plate were exposed to this preincubated DNA–lipofectin complex. After exposure for 3 h, the cells were cultured in F-12 medium supplemented with 10% FBS with or without 150 μM oleic acid for an additional 21 h.

Western blotting

SDS–PAGE and Western blotting were performed as described [35]. Protein determination for the SDS–PAGE samples was by Dc Protein Assay (Bio-Rad). The primary antibodies used were as follows: mouse anti-human HSP60 monoclonal antibody SPA-806 (Stressgen, Victoria, Canada), anti-c-Myc mouse monoclonal antibody (Nacalai Tesque, Kyoto, Japan), anti-mouse 17βHSD11 rabbit polyclonal antibody [34] and anti-mouse mitochondrial 2-oxoglutarate dehydrogenase (OGDH) rabbit polyclonal antibody (kindly provided by Dr. K. Higashi in our laboratory).

Fluorescence imaging

Transiently transfected CHO cells cultured on poly-lysine-coated coverslips were washed with PBS and then fixed with 4%