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Characterization of the interaction of diacylglycerol acyltransferase-2 with the endoplasmic reticulum and lipid droplets

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ABSTRACT

Acyl CoA:diacylglycerol acyltransferase-2 (DGAT2) is an integral membrane protein that catalyzes the synthesis of triacylglycerol (TG). DGAT2 is present in the endoplasmic reticulum (ER) and also localizes to lipid droplets when cells are stimulated with oleate. Previous studies have shown that DGAT2 can interact with membranes and lipid droplets independently of its two transmembrane domains, suggesting the presence of an additional membrane binding domain. In order to identify additional membrane binding regions, we confirmed that DGAT2 has only two transmembrane domains and demonstrated that the loop connecting them is present in the ER lumen. Increasing the length of this short loop from 5 to 27 amino acids impaired the ability of DGAT2 to localize to lipid droplets. Using a mutagenesis approach, we were able to identify a stretch of amino acids that appears to have a role in binding DGAT2 to the ER membrane. Our results confirm that murine DGAT2 has only two transmembrane domains but also can interact with membranes via a previously unidentified helical domain containing its active site.

1. Introduction

Acyl coenzyme A:diacylglycerol acyltransferase (DGAT) catalyzes the synthesis of triacylglycerols (TG) using fatty acyl coenzyme A and 1,2-diacylglycerol as substrates. TG is the major storage form of energy in eukaryotic organisms and its increased accumulation in certain tissues is linked to several metabolic disorders, including obesity, diabetes, and cardiovascular disease.

DGAT activity is highest in tissues that have a large capacity for TG storage, such as white adipose tissue, mammary gland, small intestine, and liver [1]. Two entirely unrelated DGAT enzymes, DGAT1 and DGAT2, have been found to be responsible for TG synthesis in mammals [2–4]. These two enzymes have similar expression patterns yet appear to have distinct roles in TG metabolism [1–3,5,6]. In particular, studies *in vivo* in mice have shown that DGAT2 is essential for survival in the postnatal period, while DGAT1 is not [5,6].

Both DGAT1 and DGAT2 are integral membrane proteins of the endoplasmic reticulum (ER) [7,8]. DGAT2 and several other lipid synthesizing enzymes are enriched in a specialized ER subdomain termed mitochondrial-associated membranes (MAM) [7,9–12]. MAM is in physical contact with the outer mitochondrial membrane and this close apposition of membranes is believed to facilitate the intracellular transport of lipids and calcium exchange between the ER and mitochondria [10,13–15].

In addition to localizing to the ER, DGAT2, but not DGAT1, is found to be associated with lipid droplets when TG synthesis is stimulated by incubating cells with oleate [7,12,16,17]. Presumably, the interaction of DGAT2 with lipid droplets serves to catalyze localized TG synthesis to promote efficient lipid droplet expansion. Is DGAT2 present in lipid droplets, or in ER membranes in close proximity to the lipid droplet surface? Immunogold electron microscopy and subcellular fractionation experiments have provided evidence that a pool of DGAT2 is actually present on lipid droplets [16]. The presence of DGAT2 in lipid droplets raises an interesting topological problem. DGAT2 has two transmembrane domains between amino acids 66 and 115 that are thought to span the lipid bilayer of the ER while its N- and C-termini are exposed to the cytosol [18]. However, the outer layer of lipid droplets comprises phospholipid monolayer. It has been proposed that DGAT2 adopts a hairpin structure with its transmembrane domains merely embedded in the ER membrane rather than completely spanning the ER lipid

Abbreviations: ADRP, adipose differentiation-related protein; ALK16, alkynyl-palmitate; CoA, coenzyme A; DG, diacylglycerol; DGAT, acyl CoA:diacylglycerol acyltransferase; DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; HPHG, histidine–proline–histidine–glycine; HRP, horse radish peroxidase; HSP70, heat shock protein 70; MAM, mitochondrial-associated membranes; NEM, *N*-ethylmaleimide; PEG-mal, methoxypolyethylene glycol maleimide 5000; PBS, phosphate-buffered saline; TG, triacylglycerol; TMD, transmembrane domain

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bilayer. This orientation could allow DGAT2 to be loosely associated with the ER, facilitating its diffusion from the ER membrane to lipid droplets where it could interact with the phospholipid monolayer surrounding this organelle [19,20]. However, a DGAT2 mutant missing its transmembrane domains was still able to interact with both membranes and lipid droplets [12]. This suggested that other regions could tether DGAT2 to the ER membrane and lipid droplets.

In this report, we explored the nature of the interaction of DGAT2 with both membranes and lipid droplets to understand how this enzyme is able to promote the synthesis and storage of TG. Specifically, we remapped the topology of DGAT2 on membranes and lipid droplets utilizing a less disruptive approach whereby cysteine residues of DGAT2 were modified with thiol reactive polyethylene glycol. We also examined the role that the transmembrane domains have in the movement of DGAT2 from the ER to lipid droplets. Lastly, we attempted to identify additional regions of DGAT2 that tether the protein to membranes.

2. Materials and methods

2.1. Cell culture and transfection

HEK-293T and COS-7 cells (American Type Tissue Culture Collection) were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum in a 37 °C incubator with 5% CO_2. To stimulate TG synthesis and lipid droplet formation, cells were incubated with 0.5 mM oleate complexed to 0.67% fatty acid-free bovine serum albumin (molar ratio, 4.7:1) for 12 h. For transfections, 20 μg plasmid DNA in 430 μL of 0.15 M NaCl and 120 μL of 0.1% polyethylenimine (pH 7.0) were incubated for 10 min at room temperature and then added drop-wise to a 100-mm culture dish containing 10 mL of DMEM with 10% fetal bovine serum and cells at approximately 50% confluence. After 4 h, the media was removed and cells were washed and re-fed with fresh media. At 24–48 h post-transfection, cells were harvested and used for experiments.

2.2. Generation of DGAT2 mutants

N-terminal FLAG-tagged murine DGAT2 (FL-DGAT2), in the eukaryotic expression vector, pCDNA3.1, was used as a template for all mutagenesis reactions. Mutations were introduced into cDNA sequences by PCR using PfuUltra DNA polymerase (Stratagene). All plasmids were sequenced to confirm the presence of the desired mutations. Fluorescent fusion proteins, where full-length DGAT2 (mCherry/DGAT2) or the two transmembrane domains (TMD) from amino acids 55–124 (mCherry/TMD1 + 2) were joined, in-frame, to the C-terminus of the pmCherry-C1 mammalian expression vector (Clontech), were generated in a previous study [12].

2.3. Membrane preparation

Cells were washed twice with ice-cold phosphate-buffered saline (PBS), harvested by scraping, and collected by centrifugation (600 ×*g*). Cells were resuspended in 200 μL of PBS and disrupted by 20 passages through a 27-gauge needle. Cell debris and nuclei were pelleted by centrifugation at 1000 ×*g* for 2 min, and the supernatant was centrifuged at 100,000 ×*g* for 30 min at 4 °C. Membrane pellets were resuspended in PBS or 50 mM Tris–Cl (pH 7.4)/250 mM sucrose.

2.4. Alkali extraction of membrane proteins

First, 100 μg of total membrane protein was incubated in 200 μL PBS with or without 0.18 M sodium carbonate (pH 12) at 4 °C for 30 min. Samples were then centrifuged at 100,000 ×*g* for 30 min. Supernatants were collected and pellets resuspended in 200 μL of PBS. SDS-PAGE loading buffer was added to a 50-μL aliquot of the pellet and supernatant fractions. Samples were separated by SDS-PAGE and analyzed by immunoblotting.

2.5. Isolation of crude mitochondrial, microsomal, and cytosolic fractions

Cells were lysed in 400 μL PBS by 20–25 passages through a 27 gauge syringe needle. Cell lysates were centrifuged at 1000 ×*g* for 2 min to pellet un-lysed cells and nuclei. The supernatant was centrifuged at 10,300 ×*g* for 10 min to pellet crude mitochondria (mitochondria and MAM) [21]. The supernatant was then centrifuged at 100,000 ×*g* for 30 min at 4 °C to pellet microsomal membranes. The final supernatant was used as the cytosolic fraction. All membrane pellets were resuspended in 200 μL 50 mM Tris–Cl (pH 7.4)/250 mM sucrose.

2.6. Floating fat layer isolation

Cells were lysed in LD buffer (20 mM Tris–Cl (pH 7.4)/1 mM EDTA) by 20 passages through a 27-gauge syringe needle. Cellular debris and nuclei were removed by centrifugation at 1000 ×*g* for 2 min. The cell lysate was then centrifuged at 10,300 ×*g* for 10 min at 4 °C to pellet crude mitochondria, which were resuspended in LD buffer. The supernatant was adjusted to 20% sucrose and overlaid onto 1 mL 60% sucrose in LD buffer. Six milliliters of 5% sucrose in LD buffer was then added and the tube filled to the top with LD buffer (~5 mL). The tube was centrifuged at 200,000 ×*g* for 30 min at 4 °C (SW40 rotor). The fat layer at the top of the tube was removed to a 1.5 mL tube and brought to a final volume of 500 μL with LD buffer. Aliquots of membranes or isolated fat were separated by SDS-PAGE and analyzed by immunoblotting.

2.7. Cysteine accessibility assay

Crude mitochondrial membranes were isolated as described above, except that 2 mM DTT was included in the cell lysis buffer to reduce cysteine residues. Membrane pellets were then washed to remove DTT and resuspended in 50 mM Tris–Cl (pH 7.4)/250 mM sucrose prior to use.

Under non-denaturing conditions, 100 μg of membrane protein was incubated with or without 1 mM methoxypolyethylene glycol maleimide 5000 (PEG-mal; Nanocs) for 1 h on ice. In a separate reaction, free cysteines were first blocked with 5 mM *N*-ethylmaleimide (NEM) for 30 min. Membranes were washed to remove NEM and then incubated with 1 mM PEG-mal with 2% SDS for 20 min. For denaturing conditions, 100 μg of membrane protein was incubated with 1 mM PEG-mal with 2% SDS for 1 h at room temperature. In a separate reaction, membrane proteins were first pretreated with 5 mM NEM for 30 min. Membranes were washed to remove excess NEM, re-isolated by centrifugation, and resuspended in 50 mM Tris–Cl (pH 7.4)/250 mM sucrose. Samples were then incubated with 1 mM PEG-mal. All reactions were quenched with 10 mM DTT. Samples were separated by SDS-PAGE and immunoblotted with anti-FLAG.

2.8. DGAT activity assays

DGAT activity was determined by measuring the formation of *N*-[(7-nitro-2-1,3-benzoxadiazol-4-yl)-methyl]amino (NBD)-TG from NBD-palmitoyl-CoA [22]. The reaction contained 100 mM Tris–Cl (pH 7.5), 20 mM $MgCl_2$, 0.625 mg/mL BSA, 200 μM 1,2-dioleoylglycerol, 25 μM NBD-palmitoyl-CoA (Avanti Polar Lipids), and 50-100 μg of protein sample in a final volume of 200 μL. The assay was incubated at 37 °C for 10 min and was terminated by the addition of chloroform/methanol (2:1 v:v) followed by 800 μL of H_2O. Lipids were extracted and separated by thin layer chromatography in diethyl ether/hexane/methanol/acetic acid (55:45:5:1, v/v/v/v). NBD-TG was detected with a VersaDoc 4000 molecular imaging system (Bio-Rad Laboratories, Inc.) and fluorescence was quantified with Quantity One software (Bio-Rad).

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