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The topology of the triacylglycerol synthesizing enzyme Lro1 indicates that neutral lipids can be produced within the luminal compartment of the endoplasmatic reticulum

Implications for the biogenesis of lipid droplets

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Eukaryotes store metabolic energy in form of neutral lipids, which are deposited within a dedicated organelle, termed lipid droplet (LD). While neutral lipids are synthesized by ER localized integral membrane proteins, the fate of these lipids after their synthesis and the mechanism resulting in their accumulation in LDs are not well understood. We have recently shown that LDs are functionally connected to the ER membrane allowing for a bidirectional and energy-independent transport of integral membrane proteins and possibly lipids between the two compartments during lipogenesis or lipolysis. To further characterize the nature of this connection, we investigated the topology of triacylglycerol (TAG) formation. Here we show that the active site residues of the TAG biosynthetic enzyme in yeast, Lro1, a homolog of the lecithin cholesterol acyltransferase (LCAT)-related proteins, are located within the ER luminal domain of the enzyme, suggesting that TAG formed by Lro1 is initially present in the ER luminal leaflets of the ER membrane. The topology of TAG formed by Lro1 thus contrasts that of the second TAG biosynthetic enzyme, Dga1, which has a cytosolic acyl-CoA binding domain and thus is likely to catalyze TAG formation in the cytosolic leaflet of the ER membrane. Since TAG formed by either Dga1 or Lro1 can be efficiently packed into LDs we conclude that neutral lipids from both the cytosolic as well as the luminal leaflet of the ER membrane can be concentrated and packed into LDs.

Key words: lipid droplets, endoplasmatic reticulum, triacylglycerol, steryl esters, lipoproteins, organelle biogenesis, *Saccharomyces cerevisiae*

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In eukaryotic cells an excess of free fatty acids and sterols (cholesterol in humans, ergosterol in yeast) is converted to neutral lipids, notably triacylglycerols (TAG) and steryl esters (STE), which are deposited into a dedicated storage compartment, called lipid droplet (LD). LDs are composed of a core of neutral lipids that is surrounded by an unusual phospholipid monolayer onto which LD-localized proteins appear to specifically associate. Some of these LD-localized proteins appear to fulfill structural functions such as the perilipins in mammals and oleosins in plants while others have well established functions in lipid synthesis or breakdown of neutral lipids, including lipases and acyltransferases.[1-5]

The prevailing hypothesis of LD biogenesis postulates that neutral lipids accumulate within the hydrophobic core of the ER membrane, where the lipid droplet grows and finally buds into the cytoplasm, surrounded by a membrane monolayer that is derived from the former cytoplasmic leaflet of the ER membrane.[6] Despite the apparently wide acceptance of this model, evidence to support this hypothesis is rare and controversial.[7,8] Inclusion of neutral lipids within the ER bilayer, or LDs budding from the ER into the cytosol, for example, have never been unequivocally observed.

LDs in *Saccharomyces cerevisiae* serve the same function and are structurally related to those of mammalian cells and plants.[9] Neutral lipids synthesis and thus LD formation in yeast is under control

of four enzymes, two of which produce TAG and two make STE.[10] The acyl-CoA:sterol acyltransferases (ACATs), Are1 or Are2, are polytopic ER membrane proteins that synthesize STE. TAG, on the other hand, can be produced either by Lro1 or Dga1. Dga1 catalyzes the acyl-CoA-dependent synthesis of TAG from diacylglycerol.[11,12] Dga1 has a hairpin like topology with both its N- and C-termini facing the cytosol, and a conserved lipid binding region which maps to the first transmembrane domain, suggesting that Dga1 accepts cytosolic acyl-CoA and synthesizes TAG from membrane-embedded substrate, diacylglycerol, into the cytosolic leaflet of the ER membrane.[13,14] The second TAG biosynthetic enzyme, Lro1, has homology to lecithin cholesterol acyltransferase (LCAT)-related proteins and catalyzes TAG formation through the CoA-independent transesterification of a fatty acid from phospholipids to diacylglycerol.[15,16] Synthesis and storage of neutral lipids is dispensable for the viability of *S. cerevisiae* because a *dga1*Δ *lro1*Δ *are1*Δ *are2*Δ quadruple mutant is viable and lacks detectable LDs.[17]

By using a genetic system that allows for the controlled induction of neutral lipid formation and hence LD biogenesis, we have recently reported that in cells which have no LDs, otherwise LD localized proteins are homogenously distributed within the ER membrane.[18] Upon induction of LD formation, these proteins begin to concentrate over discrete regions within the ER membrane where neutral lipids appear to accumulate, indicating that nascent LDs are indeed formed at the ER membrane. Photobleaching (FRAP) experiments combined with immunoelectron microscopy, revealed that integral membrane proteins, which are biosynthetically inserted into the ER membrane, are transported from the ER to LDs in a pathway that is independent of vesicle formation and fusion and that is not blocked upon energy-depletion or at low temperature, suggesting a passive, possible diffusion-driven mechanism for the biosynthetic transport of integral membrane proteins from the ER to LDs.[18] These data thus indicate that LDs are functionally connected to the ER membrane, allowing the bidirectional transport of integral membrane proteins and possibly lipids between the two compartments as required during lipogenesis or lipolysis. At a morphological level, this connection between the ER and LDs may be established via a membrane bridge that allows the diffusion of integral membrane proteins and lipids between the two compartments, alternatively, LDs may not form independent cytosolic structures as is frequently postulated, but they may either be enwrapped by the ER membrane and even form a domain within the ER that is enriched in neutral lipids and proteins that associate with these neutral lipid-rich domains, as has been proposed based on freeze fracture data, which reported membrane continuity between the ER and the surface of LDs.[19] To investigate the relation between neutral lipid formation and LD structure in more detail we were interested in determining the topology of the TAG biosynthetic enzyme, Lro1. Here we show that the active site residues of Lro1 are located within the ER luminal domain of the enzyme suggesting that TAG formed by the action of Lro1 is initially present within the luminal leaflet of the ER membrane. The two TAG biosynthetic enzymes, Dga1 and Lro1 thus differ with respect to their topological orientation and precise site at which TAG is formed, Dga1 into the cytosolic leaflet of the ER membrane and Lro1 into the ER luminal leaflet of the ER membrane.

Analysis of the number and position of potential transmembrane domains of Lro1 indicates that the enzyme contains one predicted transmembrane domain located between amino acids 80 and 100. To determine whether Lro1 is indeed an integral membrane protein and to examine the membrane topology of the enzyme, functional N- and C-terminally epitope-tagged versions of Lro1 were generated. Functionality of these tagged versions of the enzyme was assessed by examining TAG formation in cells that express the tagged enzyme as the only source to catalyze TAG formation (data not shown). Subcellular fractionation revealed that Lro1 is enriched in a 13k microsomal fraction and thus that the protein is membrane associated, consistent with previous characterizations of its enzymatic activity (**Fig. 1A**).[15,16] Extraction of microsomal membranes with salt or exposure to alkaline pH did not render the enzyme soluble (**Fig. 1B**). Lro1 was solubilized upon detergent treatment of microsomes only, indicating that Lro1 is an integral membrane protein, as is Wbp1, a component of the oligosaccharyl transferase complex, used as a control (**Fig. 1B**). Protease protection experiments in the presence and absence of detergent revealed that the N-terminus of Lro1 is protease accessible whereas the C-terminus is protected from protease digestion, indicating Lro1 has an odd number of transmembrane domains (**Fig. 1C**). Lro1 contains 9 potential N-linked glycosylation sites, all of which are located C-terminal to the transmembrane domain. At least one of these sites is indeed glycosylated as revealed by treatment with endoglycosidase H, indicating an ER luminal orientation of the C-terminal part of Lro1 (**Fig. 1D**). Lro1 belongs to the superfamily of hydrolytic enzymes having an α/β hydrolase-fold with a catalytic mechanism that is similar to that of serine proteases and lipases, with a catalytic triad consisting of a Ser, His and an acidic amino acid. Based on structural and functional homology with several lipases, the active site residues of Lro1 were predicted and they correspond to S205, D567 and H617 of the yeast enzyme.[20,21] Based on these structural considerations and the fact that the C-terminal domain of Lro1 must be localized in the ER lumen, where N-glycosylation occurs, we propose a model for the topology of Lro1 with a single transmembrane domain and a large ER luminal domain containing the active site residues within an α/β hydrolase fold (**Fig. 1E**). Consistent with our finding that the active site of the enzyme is localized in the ER lumen, Lro1 can be converted into a soluble enzymatically active and secreted protein by removal of the transmembrane region.[22]

It is interesting to note that the biogenesis of LDs from ER luminally produced TAG, as is indicated by the membrane topology of Lro1, is topologically equivalent to the biogenesis of lipoprotein particles, a well defined pathway for the packaging and secretion of neutral lipid containing globules from the liver. Similar to LDs, very low-density lipoproteins (VLDLs) are also composed of a

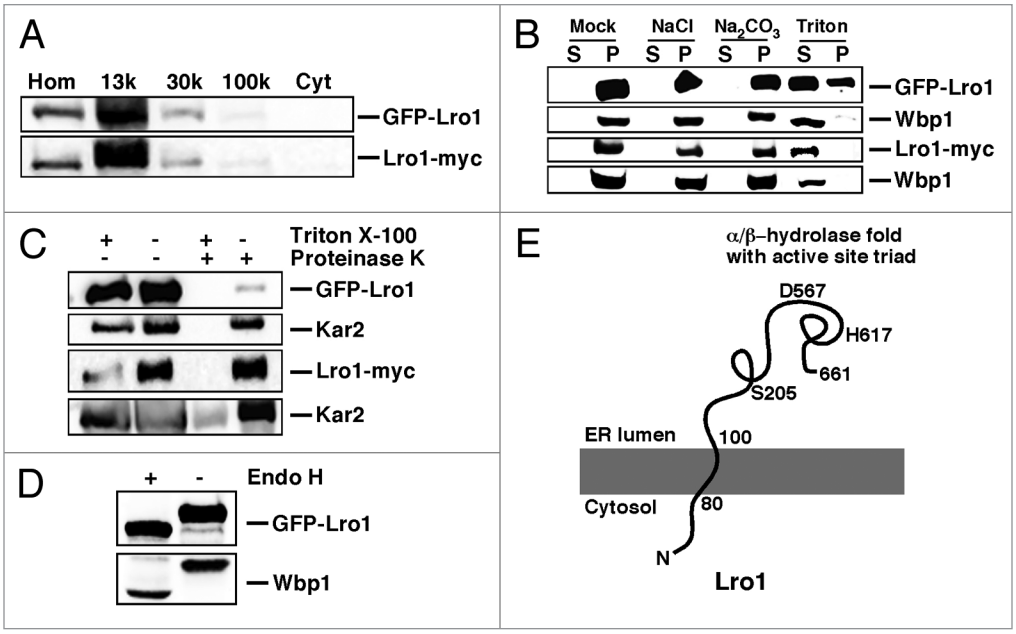

Figure 1. Membrane topology of Lro1. (A) Differential fractionation of epitope-tagged versions of Lro1. Homogenates from cells expressing N- or C-terminally tagged versions of Lro1 were differentially fractionated by centrifugation at the relative centrifugal force (*g*) indicated. Equal amounts of proteins were separated and detected with antibodies against myc or GFP. Hom, homogenate, Cyt, cytosol. (B) Lro1 is an integral membrane protein. Equal amounts of proteins from the 13k microsomal fraction were incubated with buffer (Mock), 1 M NaCl, 0.1 M Na_2CO_3 or 1% Triton X-100 for 30 min, samples were centrifuged at 13k for 15 min and proteins in the pellet (P) and supernatant (S) were analyzed by western. The integral ER membrane protein and subunit of the oligosaccharyl transferase complex, Wbp1 served as control. (C) Protease protection assays. Microsomes were incubated with proteinase K (9 μg/ml) in the presence or absence of detergent (0.1% Triton X-100), proteins were separated by SDS-PAGE and probed with antibodies against GFP or myc. Detection of the ER luminal chaperone Kar2 served as a control for membrane integrity. (D) Endoglycosidase H (Endo H) treatment of Lro1. Cell homogenates were incubated with (+) or without (-) endo H, proteins were TCA precipitated, separated and probed with antibodies against GFP or the glycosylated Wbp1. (E) Proposed model for the topology of Lro1. Cytosolic location of the N-termini and position of putative transmembrane domain in Lro1 is indicated. Putative residues of the catalytic triad of Lro1 are indicated and numbered (S205, D567, H617).

hydrophobic core of TAG and STE that is surrounded by a phospholipid monolayer and one apolipoprotein (apo) B protein. Assembly and maturation of VLDLs is generally believed to include two lipidation steps, the first of which occurs in the ER lumen and involves the addition of small amounts of neutral lipids to apoB-100 during its translocation across the ER membrane to form a partially lipidated, pre-VLDL particle.[23,24] The topology of this first lipidation step is assigned to the ER lumen and requires an ER resident protein, microsomal triglyceride transfer protein (MTP), indicating that in this system, TAG is available from within the ER lumen.[25]

Recent molecular dynamics simulations indicate that TAG sequesters within a model bilayer membrane and spontaneously aggregates within the center of the bilayer. These TAG aggregates appear to remain stable on the microsecond scale, giving the bilayer a blister-like appearance.[26] In this model, TAG could probably enter the neutral lipid aggregate from either side of the bilayer, which would effectively neutralize any difference in the topology of its original synthesis.

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