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Reorganization of cellular retinol-binding protein type 1 and lecithin:retinol acyltransferase during retinyl ester biosynthesis

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Abstract

Background—Cellular retinol-binding protein, type 1 (Crpb1), chaperones retinyl ester (RE) biosynthesis catalyzed by lecithin:retinol acyltransferase (LRAT).

Methods—We monitored the subcellular loci of LRAT and Crpb1 before and during RE biosynthesis, and compared the results to diacylglycerol:acyltransferase type 2 (DGAT2) during triacylglycerol biosynthesis in three cell lines: COS7, CHO and HepG2.

Results—Before initiation of RE biosynthesis, LRAT distributed throughout the endoplasmic reticulum (ER), similar to DGAT2, and Crpb1 localized with mitochondria associated membranes (MAM), surrounded by LRAT. Upon initiating RE biosynthesis in cells transfected with low amounts of vector to simulate physiological expression levels, Crpb1 remained with MAM, and both Crpb1 and MAM re-localized with LRAT. LRAT formed rings around the growing lipid droplets. LRAT activity was higher in these rings relative to the general ER. LRAT-containing rings colocalized with the lipid-droplet surface proteins, desnutrin/adipose triglyceride lipase and perilipin 2. Colocalization with lipid droplets required the 38 N-terminal amino acid residues of LRAT, and specifically K36 and R38. Formation of rings around the growing lipid droplets did not require functional microtubules.

General significance—These data indicate a relationship between LRAT and Crpb1 during RE biosynthesis in which MAM-associated Crpb1 and LRAT colocalize, and both surround the growing RE-containing lipid droplet. The N-terminus of LRAT, especially K36 and R38, are essential to colocalization with the lipid droplet.

Keywords

acyltransferase; lipid droplet; retinol; retinyl ester; vitamin A

1. Introduction

Vertebrates require vitamin A (retinol) for reproduction, growth, vision and to regulate many essential physiological processes, such as nervous and immune system function, intermediary metabolism, and epithelial differentiation [1-6]. Retinol supports the biosynthesis of retinoic acid (RA)¹, which controls transcription and translation *via*

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activating nuclear receptors [7-9]. Retinol not used immediately to support vision or to produce RA is stored as retinyl esters (RE), predominantly in the liver, but also in adipose, lung, and in other vitamin A target tissues [10, 11]. Retinol in most tissues occurs bound to the high-affinity, ligand-specific cellular retinol binding-protein, type 1 (Crpb1) [12, 13]. The holo-Crpb1 complex serves as substrate for RE biosynthesis catalyzed by lecithin:retinol acyltransferase (LRAT), and RA synthesis catalyzed by short-chain dehydrogenases/reductases [14-16]. Crpb1 isolates with cytosol, whereas LRAT isolates with microsomes upon tissue homogenization and differential centrifugation [13, 17, 18]. Nevertheless, recombinant holo-Crpb1 crosslinks with endogenous LRAT, consistent with physical proximity and ability of LRAT to access Crpb1-bound retinol [19].

In contrast to holo-Crpb1, apo-Crpb1 inhibits RE synthesis by LRAT and stimulates RE hydrolysis [20, 21]. These data suggest that the relative amounts of holo-Crpb1 and apo-Crpb1 affect flux of retinol between RE formation vs. RE hydrolysis.

LRAT catalyzes RE synthesis by transferring an acyl group from the SN1 position of lecithin to retinol [22-24]. *LRAT*-null mice fed lab chow thrive, but do not form RE in liver and many other tissues, demonstrating that LRAT serves as the major retinol acyltransferase *in vivo* and perhaps the only one in liver [25, 26]. Acyl-CoA:retinol acyltransferases, which do not recognize holo-Crpb1 as substrate, also biosynthesize RE [27]: one such acyl transferase has been identified as diacylglycerol:acyltransferase type 1 (DGAT1) [28, 29]. DGAT1, and perhaps others, seem important to RE synthesis in select loci, such as skin, mammary gland and retinal pigment epithelia [30-32]. In contrast, LRAT has wider expression loci [33].

RE are stored in lipid droplets, intracellular organelles more widely known for storing triacylglycerol (TG) and cholesterol esters [34-36]. Two models have been proposed for lipid droplet formation. In one, ER-resident acyltransferases deposit esters between the monolayers of the ER membrane [37]. As esters accumulate, the bulge forces the monolayers to separate, and the lipid droplet pushes from the ER into the cytoplasm, coated by a monolayer of phospholipids derived from the ER membrane. Larger lipid droplets form in the cytoplasm through fusion between smaller lipid droplets. This model excludes acyltransferases and other transmembrane proteins from the lipid droplet, because the droplet cannot accommodate their transmembrane domains [38]. The second model postulates that lipid droplets are formed external but adjacent to the ER at specialized sites [39]. In this model, “both ER membranes lie external to and follow the contour of the lipid droplet, enclosing it” similar to “an egg cup (the ER) holding an egg (the lipid droplet)”. Evidence for the second model was developed using the relatively new FRIL technique, *i.e.* electron microscopic examination of freeze-fractured lipid droplets treated to retain a single layer of molecules in their native positions. The second model allows for acyltransferases and other membrane proteins to bind with lipid droplets [40].

The present study sought to provide further insight into the spatial relationship between LRAT and Crpb1, and their relationships to lipid droplets during RE synthesis, and to compare the process of RE synthesis to TG synthesis catalyzed by DGAT2, the major TG biosynthetic enzyme in eukaryotes [41]. We found that RE biosynthesis does not require Crpb1, but in the presence of Crpb1, LRAT and Crpb1 juxtapose before RE biosynthesis, but localize to lipid droplets during RE biosynthesis, and that Crpb1 colocalizes with mitochondria associated membranes (MAM). These data provide further evidence for a Crpb1-LRAT interaction during RE biosynthesis, in which Crpb1 facilitates RE formation, and provide further insight into the process of RE biosynthesis.

2. Materials and methods

2.1 Materials

Rabbit anti-calnexin (Cat No. SPA-865D) was purchased from Stressgen. Guinea pig anti-perilipin 2 (Cat #RDI-PROGP40) was purchased from Fitzgerald Industries International. Secondary antibodies were purchased from Invitrogen: Alexa 647 donkey anti-rabbit IgG; Alexa 546 donkey anti-rabbit IgG; Alexa 647 goat anti-guinea pig IgG; Alexa 546 donkey anti-mouse IgG. Mouse anti-GM130 and anti- α -tubulin were gifts from Carolyn Bertozzi and Eva Nogales, respectively.

2.2 Cell culture

COS7 cells were cultured in DMEM, HepG2 cells were cultured in MEM, and CHO cells were cultured in F12 medium, each with 10% fetal bovine serum. A solution of oleic acid bound to bovine serum albumin was prepared according to the protocol in *Current Protocols in Cell Biology* [42]. Retinol-free media were prepared by irradiating commercial media under a UV lamp less than 5 cm from the media at 302 nm (6 watt) for 2 min. Irradiated media were analyzed by high-performance liquid chromatography to confirm elimination of retinol [43]. Acyltransferase substrate incubations were done for 5 hr with 5 μ M retinol or 400 μ M oleic acid, unless noted otherwise.

2.3 Expression constructs and transfection

The expression vectors used for fusion proteins included pGFP2-N2 (LRAT, DGAT2, CRBP1), pEGFP-C1 (LRAT) and pDsRed-N1 (ATGL, CRBP1). RFP was linked to the N-terminal of perilipin 2 in pcDNA3. Primers are described in Table 1. For transient expression, cells were plated onto 12 mm glass cover slips in 24-well plates. On the second day, cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol, except for the plasmid quantity. The quantities of plasmids used per well for each experiment are indicated in the figure legends. Cells were analyzed one day post transfection. For stable expression, CHO cells were transfected with LRAT-GFP and underwent G418 selection for one month.

2.4 Immunofluorescence microscopy

Cells were fixed with 4% paraformaldehyde for 15 min at room temperature and washed three times with phosphate-buffered saline (5 min each). Fixed cells were permeabilized with 0.1% Triton X-100 for 5 min. For antibody staining, cells were blocked with blocking buffer with 10% donkey serum in phosphate-buffered saline for 1 hr at room temperature. Primary antibodies were diluted in blocking buffer and incubated overnight at 4°C (calnexin, 1:500; perilipin 2, 1:100). Secondary antibodies were diluted in blocking buffer and incubated at room temperature for 1 hr. Lipid droplets were stained with Nile Red. Nuclei were stained with DAPI (Invitrogen). Live mitochondria staining was done according to the manufacturer's protocol using MitoTracker Red CMXRos or MitoTracker Deep Red 633 (Invitrogen). Images were taken with a Carl Zeiss LSM 510 Meta laser scanning confocal microscope with a 100x objective. RE autofluorescence was detected with a standard method for retinoids using an excitation of 350 nm and emission from 610 to 700 nm, wavelengths of low GFP emission [44, 45]. Bars represent 5 μ m, unless noted otherwise. The presence of RE were confirmed by high-performance liquid chromatography [43].

3. Results

3.1 LRAT localizes in rings that surround lipid droplets

We generated LRAT and DGAT2 constructs with GFP fusions at their C-termini to provide insight into mechanisms of incorporating newly synthesized RE into lipid droplets and to compare the outcome with TG incorporation into lipid droplets. LRAT transfected into COS7 and CHO cells with low amounts of DNA formed rings that surrounded RE-containing lipid droplets generated by incubating cells with retinol (Fig. 1A, B). DGAT2 adopted a similar ring pattern that surrounded TG in COS7 cells (Fig. 1C, D) and HepG2 cells (Fig. 1F), when oleate was present. Both LRAT and DGAT2 concentrated in rings surrounding lipid droplets upon incubation with their substrates (Fig. 1D, E). With lower acyltransferase expression levels, achieved by transfecting lower amounts of constructs, ~50% of COS7 cells contained LRAT rings, and 80-90% contained DGAT2 rings.

Rings varied among cells, but increased in numbers and sizes as incubation with substrates progressed. DGAT2 remained with the ER network after 1.5 hr incubation with oleic acid (Fig. 2). Rings began to emerge by 2.5 hr. In LRAT expressing cells, sizes and numbers of lipid droplets also increased with incubation time.

3.2 Rings forms predominantly at low acyltransferase expression levels

We observed consistently that rings formed in cells with lower LRAT expression (Fig. 3A, Table 2). In addition, cells with rings showed more intense retinoid autofluorescence than cells without rings, regardless of the level of LRAT expression, consistent with more efficient RE formation (Fig. 3B).

3.3 Acyltransferases emerge from the ER

We used calnexin, a luminal-projecting ER membrane protein, to determine the extent and time of ring association with the ER. Large differences in the numbers and sizes of lipid droplets were consistent with rings at different stages of lipid droplet formation. Two distinct cell populations were apparent. In the first, LRAT and DGAT2 localized to both rings and non-ring structures. Calnexin associated with all non-ring structures, but only with ~30-40% of rings (Fig. 4A, 5A). Remaining rings either did not overlap with calnexin or showed only partial overlap (Fig. 4B, 5B). In the second group of cells, LRAT and DGAT2 occurred only in rings. In this group, only ~10% of the cells had calnexin in the rings (Fig. 4C, 5C). This suggests a progression of LRAT and DGAT from the ER, to rings emerging from the ER, to rings independent of the ER.

3.4 Cytoplasm-originating lipid droplet surface proteins localize to rings

We determined the loci of two lipid droplet surface proteins in cells with rings to confirm association of LRAT and DGAT2 rings with lipid droplets. Desnutrin/ATGL, a TG/RE hydrolase, localizes to lipid droplet surfaces [46]. The PAT family lipid droplet binding-protein perilipin 2 (also known as ADRP) binds to single phospholipid layers, once lipid droplets are formed [47, 48]. Perilipin 2 is expressed widely in non-adipose tissues and protects lipid droplets from hydrolases, thereby promoting lipid accumulation. Both perilipin 2 and ATGL co-localized with LRAT and DGAT2 during RE and TG biosyntheses on lipid droplet surfaces (Fig. 6). These data show that the acyltransferases form rings that include lipid droplet surface proteins, which surround the surfaces of lipid droplets.

3.5 Lipid droplets recruit acyltransferases independently of homologous substrate

To determine whether a heterologous substrate affects acyltransferase recruitment by lipid droplets, we expressed LRAT-GFP with DGAT2, or DGAT2-GFP with LRAT and

incubated with oleic acid or retinol. In LRAT and DGAT2 expressing cells, rings formed around lipid droplets after either retinol or oleic acid incubation (Fig. 7). When treated with retinol, DGAT2-GFP surrounded RE containing lipid droplets. When treated with oleic acid, LRAT-GFP surrounded TG containing lipid droplets. TG lipid droplets and acyltransferase-containing rings did not form in cells transfected with DGAT2 in the absence of oleic acid incubation (data not shown). These data indicate that lipid droplets do not require homologous substrate to recruit acyltransferases to their surfaces.

3.6 Crbp1 colocalizes with mitochondria-associated membranes (MAM)

We constructed a Crbp1-RFP fusion protein to determine the loci of Crbp1 relative to LRAT, because Crbp1 delivers retinol to LRAT for esterification [14, 15]. Crbp1 co-localized with mitotracker, the mitochondria/MAM marker, independently of LRAT expression (Fig. 8A). Although the image was taken with retinol in the medium (RE were not formed because LRAT was not present), Crbp1 colocalized with mitochondria/MAM in the absence of retinol, as well (not shown). When LRAT-GFP was expressed in the absence of Crbp1, it formed rings around MAM (Fig. 8B). Mitochondria/MAM-filled rings appeared in about 60-70% cells with rings, whereas the remaining rings were filled with lipid droplets without mitochondria/MAM. Coexpressing LRAT and Crbp1 in the absence of retinol, revealed LRAT surrounding Crbp1 (Fig. 8C). After incubation with retinol, two patterns of LRAT expression emerged relative to Crbp1, which were not mutually exclusive in individual cells. In one, LRAT surrounded regions of Crbp1 colocalization with mitochondria/MAM, i.e. there was little change in the relationship between LRAT and Crbp1 relative to the absence of retinol incubation, except for the formation of RE, which colocalized with Crbp1 (Fig 8D). In this case, rings were filled with Crbp1-RFP and mitochondria/MAM and retinoid autofluorescence was modest within rings. In the other pattern, LRAT localized in the rings around RE droplets (Fig. 8E). Crbp1 localized in and around the rings, but remained colocalized with the mitochondria/MAM marker. After 5 hr incubation with retinol, ~70% of the LRAT and Crbp1 double expressing cells had mitochondria/MAM-filled rings. We did not detect retinol autofluorescence in any region without LRAT expression (Fig. 8D, E). In contrast to LRAT, DGAT2 did not surround mitochondria/MAM after incubation with its substrate (Fig. 8F). These data show that Crbp1 colocalizes to mitochondria/MAM, regardless of the presence of retinol or LRAT, and associates with lipid droplets and LRAT during RE biosynthesis.

3.7 Rings form independently of microtubules

Ring formation seems to involve localized ER reorganization. ER morphology changes by sliding along the microtubule network or extending along polymerizing microtubules [49, 50]. Disrupting microtubule-dependent motor proteins or disassembling microtubules reduces ER sub-compartment movement [51]. Lipid droplets move along microtubules through microtubule dependent motor proteins, such as dynein. Knocking down dynein and kinesin proteins alters sizes of lipid droplets [52]. Therefore, we visualized the microtubule network during lipid droplet formation. Initially, DGAT2 expression overlapped with microtubules in the absence of oleic acid treatment (Fig. 9). During oleic acid incubation, DGAT2 migrated to regions outside of the microtubule network. Treatment with nocodazole, a microtubule disrupter, did not change the numbers or sizes of rings formed, indicating that a fully functional microtubule network is not essential to ring formation (Fig. 10).

3.8 Lipid droplet association requires the N-terminus of LRAT

We tested the contribution of the LRAT N-terminus to lipid droplet association with a fusion of the N-terminal transmembrane and cytosolic domains, residues 1 to 195, to GFP (LRAT-N-GFP). LRAT-N-GFP localized with mitochondria/MAM in the absence of lipid synthesis (Fig 11A). After incubating with oleic acid to induce lipid droplet formation, LRAT-N-GFP

translocated to lipid droplets (Fig. 11B, C). The net charge of residues that flank transmembrane regions of eukaryotic membrane proteins contributes to determining topology [53-55]. Several charged amino acid residues flank the N-terminus hydrophobic region of LRAT (Fig 12A). We tested whether these charged residues affect lipid droplet localization. A fusion of the N-terminal hydrophobic region, amino acid residues 1 to 38, to GFP (LRAT-L-GFP), which included the flanking positive amino acids, targeted to lipid droplets (Fig 12B). The N-terminal hydrophobic region without the flanking positive amino acid residues (LRAT-S-GFP) did not direct GFP to lipid droplets (Fig. 12C).

4. Discussion

LRAT rings formed predominantly in cells with low LRAT expression, achieved by limiting the amount of transfection vector (Fig. 3). This would limit the amount of LRAT produced, in contrast to transfecting with higher amounts of vector, as is more usually the case. Higher and non-physiological concentrations of LRAT produced by transfecting with higher amounts of vector result in LRAT “clumps” consistent with aggregation. Several lines of evidence support this deduction: transfection with the usual amounts of vector cause over expression; LRAT formed aggregates instead of distributing evenly when higher vector amounts were used (Fig. 1D); LRAT oligomerizes when concentrated [18]. Thus, it is reasonable to expect that transfecting with lower amounts of LRAT would represent a physiological presence of LRAT compared to transfecting with larger quantities of vector.

Immunofluorescence confocal microscopy indicated that Crbp1 colocalizes with the mitochondria and/or the MAM. MAM are specialized compartments of the ER that interact tightly with mitochondria [56, 57]. Because many labs have confirmed that Crbp1 isolates with the cytosolic fraction upon differential centrifugation, it is probable that Crbp1 is not an integral mitochondrial protein [12, 13, 58, 59]. It is reasonable to conclude that homogenization solubilizes a loosely associated Crbp1 from the MAM and transfers it to the cytosolic fraction, isolated upon differential centrifugation. These data are consistent with Crbp1 function in RE biosynthesis, because MAM are enriched in enzymes that catalyze lipid synthesis and localize near lipid droplets [56, 57].

Holo-Crbp1 occurs as the most abundant intracellular form of non-esterified retinol, at least in liver, despite capacity for membranes to sequester far more retinol than occurs in cells during normal vitamin A nutriture [12]. RE formation is coupled to cellular retinol uptake [60]. In the absence of acyl ester biosynthesis, our data showed that LRAT surrounded Crbp1. During acyl ester biosynthesis, two patterns emerged with respect to Crbp1 and LRAT. In one, Crbp1 remained in the LRAT-enclosed spaces, and in the other Crbp1 concentrated at the outer surfaces of LRAT-enclosed lipid droplets. A model of RE biosynthesis can be constructed from the progression of these rearrangements of LRAT and Crbp1 during lipid droplet formation. Initially, in the absence of retinol and lack of RE biosynthesis, LRAT and Crbp1 partially overlap. Upon introduction of retinol, LRAT and Crbp1 colocalize, and both surround lipid droplets during RE biosynthesis, as Crbp1 remains in contact with the MAM. These data are consistent with a physical relationship between LRAT and Crbp1 in which LRAT accesses retinol bound to Crbp1, with both proteins on the surfaces of the emerging and enlarging lipid droplets. Previous data that revealed crosslinking of holo-Crbp1 with LRAT support this model [19]. Kinetic analysis also supports ability of LRAT to interact with Crbp1, because a Michaelis-Menten (*i.e.* enzyme/substrate) relationship has been demonstrated between holo-Crbp1 and LRAT [14, 15]. Moreover, RE formation *in vitro* occurs in the presence of holo-Crbp1 at rates faster than can be supported by the nM concentrations of unbound retinol in equilibrium with holo-Crbp1. These low concentrations of unbound retinol result from the high affinity of Crbp1 for retinol (k_d value 0.1 to 3 nM) [61, 62].

Crpb1 sequesters retinol inside a β -clam structure, with the entire retinoid structure isolated from the cellular milieu [59]. Ability of LRAT to function through interaction with retinol bound to Crpb1 supports the notion that Crpb1 serves as a retinol chaperone, *i.e.* promotes efficient use of the scarce, but essential pro-autacoid, by allowing access only to enzymes that recognize Crpb1 and then retinol. This concept does not postulate that LRAT requires retinol bound to Crpb1, but rather postulates a microenvironment in which LRAT interacts with holo-Crbp1 in context of membrane composition and structure to allow retinol transfer, without requiring retinol diffusion throughout the cell. In absence of the chaperone, detrimental (non-productive) reactions may occur, normally restricted by the chaperone, as illustrated by the rapid elimination of retinoids from livers of mice lacking Crbp1 (*Rbp1*-null mice) [63].

Association of LRAT with lipid droplets was confirmed by its colocalization with two lipid droplet associated proteins, perilipin 2 and ATGL, and by comparing it to the diacylglycerol acyltransferase DGAT2. LRAT adopted subcellular loci dynamics similar to DGAT2. Both enzymes concentrate around the enlarging lipid droplets when cells are exposed to substrates that promote lipid droplet biogenesis [64]. We also showed that LRAT and DGAT2 differ in loci during acyl ester formation. DGAT2 occurs as an ER resident acyltransferase that concentrates in MAM before and during ester formation, in contrast to LRAT [64].

LRAT has a transmembrane domain at its C-terminal that serves as a targeting signal and membrane anchor domain that fixes it in the ER membrane with most of the enzyme, including its active site projecting into the cytoplasm [65]. The N-terminus hydrophobic domain does not contribute to enzyme activity or to establishing topology. Here we demonstrated that the N-terminal hydrophobic domain in the absence of the remaining residues can anchor GFP in the MAM before ester synthesis—a situation that does not occur with the entire protein. During lipid synthesis, the N-terminus of LRAT locates GFP to lipid droplets, just as occurs with intact LRAT, in the presence of positively charged amino acid residues 36 and 38. These data establish that the N-terminal 38 residues of LRAT are both necessary and sufficient to anchor LRAT to lipid droplets, and indicate that LRAT relocation to the surfaces of lipid droplets during ester synthesis is a “built-in” function of the enzyme. Fig. 13 portrays a model of this process. The model indicates transfer of LRAT from the ER to the surface of lipid droplets, consistent with occurrence of acyltransferases derived from the ER/MAM on lipid droplet surfaces [37, 38]. Alternatively, because ER/MAM remain in close proximity to lipid droplets, it is intriguing to question whether LRAT spans the space between the MAM/ER and lipid droplets, anchored by its C-terminus to the MAM/ER and by its N-terminus to the surface of the lipid droplet. Future work will distinguish these two possibilities.

Both liver stellate cells and hepatocytes express Crbp1, encoded by *Rbp1*, and both express retinoid metabolizing enzymes, although stellate cells store most liver RE [34-36]. Stellate cells of *Rbp1*-null mice, however, have fewer and smaller lipid droplets compared to wild type mice [63]. Liver retinyl palmitate, the quantitatively major RE, and retinol are ~50% lower in 4-week-old *Rbp1*-null mice than wild type. *Rbp1*-null mice also eliminate total liver retinoids 6-fold faster than wild type mice. These data showed that Crbp1 arrests rapid elimination of RE, but did not address whether RE formation requires Crbp1. The current data show that intact cells do not require Crbp1 to generate RE, consistent with a previous report that LRAT functions both in the absence and presence of Crbp1 with approximately the same maximum velocity [21].

A working model postulates that during biosynthesis TG accumulate between the leaflets of the ER membrane, before budding off to form a lipid droplet. Lipid droplet growth then occurs through homotypic fusion of smaller droplets [38]. Electron micrographs, however,

have indicated that lipid droplets associate tightly with the ER membrane, but are not necessarily internal to the membrane [66]. Sheets of ER membrane surround the growing lipid droplet, cradling it like an “eggcup”. In this model, direct transfer occurs of lipids from acyl transferases to growing droplets. The positioning of LRAT and DGAT2 in rings around lipid droplets indicates that newly synthesized RE and TG have opportunity to incorporate directly into the droplets. Interestingly, we have observed that the fraction of LRAT around droplets has disproportionately high enzyme activity: a similar phenomenon was reported for DGAT2 [64].

5. Conclusions

Crpb1 localizes with MAM in intact cells, whereas LRAT distributes throughout the ER. During RE biosynthesis, LRAT and MAM-associated Crpb1 colocalize, and both surround the growing lipid droplet. The N-terminus of LRAT, especially residues K36 and R38, is essential to its colocalization with the lipid droplet.

Acknowledgments

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Highlights

LRAT and Crbp1 co-localize during retinyl ester biosynthesis

LRAT and Crbp1 surround lipid droplets during retinyl ester formation

Crbp1 localizes in intact cells with mitochondrial associated membranes

The N-terminal thirty-eight amino acid residues direct LRAT to lipid droplets during acyl ester biosynthesis

Fig. 1.

LRAT-GFP and DGAT2-GFP form rings that surround lipid droplets upon incubating cells with retinol or oleic acid. (A) COS7 cells transfected with 0.05 μg LRAT-GFP were incubated with retinol. Left to right: nuclei (blue); LRAT-GFP (green); lipid droplets (red); merge. (B) CHO cells transfected with 0.05 μg LRAT-GFP were incubated with retinol. Left to right: RE autofluorescence (yellow); LRAT-GFP (green); merge. (C) COS7 cells transfected with 0.1 μg DGAT2-GFP were incubated with oleic acid. Left to right: lipid droplets (red); DGAT2-GFP (green); nuclei (blue); merge. (D) COS7 cells transfected with 0.05 μg LRAT-GFP (green): left, aggregates formed before retinol treatment (white arrows); right, rings formed after incubation with retinol. (E) COS7 cells transfected with 0.1 μg DGAT2-GFP (green): left, a network pattern formed in the absence of oleic acid; right, rings formed after oleic acid treatment. (F) Rings in HepG2 cells. HepG2 cells were transfected

with 0.3 μg DGAT2-GFP and incubated with oleic acid. Left to right: nuclei (blue); DGAT2-GFP (green); lipid droplets (red); merge. Bars represent 5 μm .

Fig. 2.

Time course of ring formation in DGAT2 and LRAT expressing cells. (A) COS7 cells were transfected with 0.1 μg DGAT2-GFP (green) and incubated with oleic acid for the times indicated. Rings emerged at 2.5 hr. Bars represent 5 μm . (B) Quantification of lipid droplets with times of oleic acid incubation. Eight DGAT2-GFP expressing cells were selected randomly from each of 3 slides. (C) Quantification of RE lipid droplets in LRAT expressing cells. CHO cells were transfected with LRAT-GFP and incubated with retinol. One hundred cells on each of three slides were scanned for RE-containing droplets (autofluorescence) with diameters >1.5 μm . Left: the average diameter of lipid droplets with diameters >1.5 μm . Right: the number of cells with RE lipid droplet diameters >1.5 μm . Results are mean values $\pm$ SE of measurements from three individual slides.

Fig. 3. Rings form during low-level LRAT expression and associate with robust RE synthesis. (A) A CHO cell clone stably expressing LRAT-GFP (green) with low LRAT-GFP expression formed rings (white arrow). (B) Cells with high LRAT-GFP expression (note the LRAT aggregates) did not form rings after 5 hr incubation with retinol; nucleus (blue). (C-D) CHO cells with rings had high RE auto-fluorescence. (C) RE auto-fluorescence (yellow); (D) LRAT-GFP (green); (E) merge. Bars represent 5 μ m.

Fig. 4. Positions of LRAT-GFP-containing rings with respect to the ER. COS7 cells transfected with 0.05 μg LRAT-GFP were incubated with retinol. Yellow arrows indicate colocalization of rings and calnexin at lipid droplet peripheries. (A) Cells are shown with LRAT-GFP in both rings and non-ring structures. In these cells, LRAT-GFP colocalizes with the ER. Left to right: nucleus (blue); LRAT-GFP (green); calnexin (purple); lipid droplets (red); merge. (B) Cells are shown with LRAT-GFP mostly in rings. In these cells, LRAT-GFP concentrates in specific sections of the ER. Left to right: nuclei (blue); LRAT-GFP (green); calnexin (purple); lipid droplets (red); merge. (C) An example of the ER forming a semi-ring

colocalizing with LRAT-GFP: left to right: nucleus (blue); LRAT-GFP (green); calnexin (red); merge. Bars represent 5 μm .

Fig. 5. Positions of DGAT2-GFP-containing rings with respect to the ER. COS7 cells were transfected with 0.1 μ g DGAT2-GFP and treated with oleic acid. Yellow arrows indicate colocalization of rings and calnexin at peripheries of lipid droplets. (A) Cells with DGAT2-GFP in rings and non-ring structures, with DGAT2-GFP colocalizing with the ER. Left to right: nuclei (blue); DGAT2-GFP (green); calnexin (purple); lipid droplets (red); merge. (B) Cells with DGAT2-GFP in rings and non-ring structures, with DGAT2-GFP colocalizing only partially with the ER. Left to right: nuclei (blue); DGAT2-GFP (green); calnexin (purple); lipid droplets (red); merge. Boxes indicate rings showing partial ER colocalization. (C) DGAT2-GFP forms rings separated from the ER network. Left to right: nuclei (blue);

DGAT2-GFP (green); calnexin (red); merge. The majority of DGAT2-GFP localized to rings. Bars represent 5 μ m.

Fig. 6. Colocalization of LRAT-GFP and DGAT-GFP with perilipin 2-RFP and ATGL-RFP. (A) COS7 cells were co-transfected with 0.1 μ g LRAT-GFP and 0.4 μ g perilipin 2-RFP and were incubated with retinol. Left to right: nuclei (blue); LRAT-GFP (green); perilipin 2-RFP (red); RE autofluorescence (yellow); merge. (B) COS7 cells were co-transfected with 0.2 μ g DGAT2-GFP and 0.4 μ g perilipin 2-RFP and were treated with oleic acid. Left to right: nucleus (blue); DGAT2-GFP (green); perilipin 2-RFP (purple); lipid droplets (red); merge. (C) COS7 cells were co-transfected with 0.1 μ g LRAT-GFP and 0.4 μ g ATGL-RFP and were incubated with retinol. Left to right: nuclei (blue); LRAT-GFP (green); ATGL-RFP (red); merge. (D) COS7 cells were co-transfected with 0.2 μ g DGAT2-GFP and 0.5 μ g

ATGL-RFP and were incubated with oleic acid. Left to right: nuclei (blue); DGAT2-GFP (green); ATGL-RFP (red); merge. Bars represent 5 μm .

Fig. 7.

Lipid droplet rings recruit acyltransferases, regardless of acyl group composition. (A) COS7 cells were transfected with 0.1 μg DGAT2-GFP and 0.1 μg LRAT and incubated with retinol. Left to right: RE autofluorescence (red); DGAT2-GFP (green); merge. (B) COS7 cells were transfected with 0.1 μg LRAT-GFP and 0.1 μg DGAT2 and incubated with oleic acid. Left to right: LRAT-GFP (green); lipid droplets (red); merge. Bars represent 5 μm.

Fig. 8. Localization of LRAT with Crbp1 and MAM/mitochondria. (A) Crbp1-GFP colocalizes with mitotracker. COS7 cells were transfected with 0.8 μ g Crbp1-GFP. Cells were incubated with retinol. Left to right: RE autofluorescence (yellow); Crbp1 (green); Mitotracker (red); merge. (B) LRAT-GFP forms rings around MAM during retinol incubation in the absence of Crbp1. COS7 cells were transfected with 0.1 μ g LRAT-GFP and treated with retinol. Left to right: nuclei (blue); LRAT-GFP (green); mitotracker (red); merge. (C) LRAT-GFP surrounds Crbp1-RFP in the absence of retinol. COS7 cells were co-transfected with 0.1 μ g LRAT-GFP and 0.8 μ g Crbp1-RFP and were not treated with retinol. Left to right: LRAT-GFP (green); Crbp1-RFP (red); merge. (D) After incubation

with retinol, one pattern showed LRAT-GFP forming rings that surrounded Crbp1-RFP and MAM (white arrows). COS7 cells were co-transfected with 0.1 μ g LRAT-GFP and 0.8 μ g Crbp1-RFP and treated with retinol. Left to right: LRAT-GFP (green); Crbp1-RFP (red); RE autofluorescence (blue); merge of panels 1-3; mitotracker (purple); merge of Crbp1-RFP and mitotracker. (E) After incubation with retinol, a second pattern showed LRAT-GFP forming rings that surround lipid droplets (white arrows) and colocalized (partially) with Crbp1 and MAM. COS7 cells were co-transfected with LRAT-GFP and Crbp1-RFP and treated with retinol as in D. Left to right: LRAT-GFP (green); Crbp1-RFP (red); RE autofluorescence (blue); merge of panels 1-3; mitotracker (purple); merge of Crbp1-RFP and mitotracker. (F) DGAT2-GFP does not form rings around MAM after oleic acid incubation. COS7 cells were transfected with 0.1 μ g DGAT2-GFP and treated with oleic acid. Left to right: nuclei (blue), DGAT2-GFP (green); mitochondria (red); merge. Bars represent 5 μ m.

Fig. 9.

Time course of DGAT2-GFP association with the microtubule network during lipid droplet formation. Oleic acid incubation for (A) 0 hr, (B) 1.5 hr, (C) 2.5 hr. The yellow arrow points to a ring no longer associated with the microtubule network. COS7 cells were transfected with 0.1 μg DGAT2-GFP. The microtubule network was stained with anti α -tubulin and a secondary antibody conjugated with Alexa 647. Left to right: DGAT2-GFP (green); microtubules (red); nuclei (blue); merge. Bars represent 5 μm .

Fig. 10.

Disrupting the microtubule network did not prevent ring formation. COS7 cells were transfected with 0.1 μ g DGAT2-GFP and incubated with 1 μ g/ml nocodazole at 4 °C for 15 min to disrupt microtubules. Cells were then incubated with oleic acid and 1 μ g/ml nocodazole. Microtubules were stained as described in the legend of Fig. 9. Left to right: lipid droplets (red); DGAT2-GFP (green); microtubules (purple); nuclei (blue); merge. (A) Vehicle control with lipid droplets stained. (B) Nocodazole-treated cells with lipid droplets stained. Bars represent 5 μ m.

Fig. 11.

The N-terminal hydrophobic region of LRAT directs localization to lipid droplets. COS7 cells were transfected with 0.1 μ g of LRAT-N-GFP, *i.e.* the N-terminal 195 amino acid residues fused to the N-terminus of GFP. (A) LRAT-N-GFP localizes with mitotracker in medium without oleate. Left to right: nucleus (blue); LRAT-N-GFP (green); mitotracker (red); merge of all. (B) LRAT-N-GFP dissociates partially from mitochondria/MAM after treatment overnight with 400 μ M oleic acid. Left to right: nucleus (blue); LRAT-N-GFP (green); mitotracker (red); merge of all signals. (C) LRAT-N-GFP associates with lipid droplets after oleic acid treatment. Left to right: nucleus (blue); LRAT-N-GFP (green); lipid droplets (red); merge.

Fig. 12.

Importance of positively charged residues to function of the LRAT N-terminus. (A) Two constructs of the N-terminus of LRAT were fused to the N-terminus of GFP. The long form, LRAT-L-GFP, retained the positively charged residues at positions 36 and 38. The short form, LRAT-S-GFP, lacked the last seven residues of the longer form, including the two positively charged residues. (B) LRAT-L-GFP, the long form of the N-terminus of LRAT with the two positively charged residues, targeted to lipid droplets. (C) LRAT-S-GFP, the LRAT N-terminus lacking the terminal positively charged residues, did not associate with lipid droplets. Left to right: nucleus (blue); LRAT-GFP (green); lipid droplets (red); merge of all three. COS7 cells were transfected with 0.1 μ g LRAT-GFP constructs and treated with 400 μ M oleic acid overnight.

Fig. 13.

A model of LRAT and Crbp1 reorganization during RE biosynthesis. In the absence of RE formation, Crbp1 colocalizes with MAM (Fig. 8A), and LRAT colocalizes with the ER marker calnexin and surrounds Crbp1 in the MAM (Fig. 8B, C). During incubation with retinol, LRAT, expressed at lower levels to avoid aggregation, forms rings that increase in numbers and sizes with incubation time and surround RE-containing lipid droplets (Fig. 1A, B; Fig. 2C; Fig. 3). Droplets had relatively intense retinoid autofluorescence, consistent with relatively efficient RE formation (Fig. 3B). LRAT started in the ER, and during lipid droplet formation progressed to rings emerging from the ER, and to rings seemingly independent of the ER (Fig. 4). Crbp1 remained in the MAM, and both Crbp1 and MAM colocalize around lipid droplets with LRAT (Fig. 8D, E). LRAT and Crbp1 also colocalize with the lipid droplet surface proteins perilipin 2 and ATGL during RE biosynthesis (Fig. 6). LRAT required residues K36 and R38 in its N-terminus to translocate to lipid droplets (Fig. 11, 12).

TABLE 1

Primers used.

ORF	Forward primer	Reverse primer	GI No.	size (bp)
ATGL	5'-ATAAGCTTATGTTCCCGAGGGAGACC-3'	5'-TGGAATCCAGATACTGGCAGATGCTAC-3'	40352881	1.4
RFP	5'-ATGGATCCATGGCCTCTCCGAGAAC-3'	5'-TAGATATCCAGGAACAGGTGGTGGC-3'	55724874	678
perilipin 2	5'-GCGATATCGCAGCAGCAGTAGTGGATC-3'	5'-AACTCGAGTTACTGAGCTTTGACCTCAGACTGCTGGAC-3'	32451986	1300
Crp1	5'-ATGAATTCATGCCTGTGGACTTCA-3'	5'-CAGGATCCTGTGTACTTTCTTGAACAC-3'	77416367	408
DGAT2-GFP	5'-ATGAATTCATGAAGACCTCATCGCCGC-3'	5'-ACGGATCCGTTACCTCCAGCACCTCAG-3'		2000
DGAT2	5'-ATGGATCCATGAAGACCTCATCGCCGC-3'	5'-GAGAATTCTCAGTTCACCTCCAGCACCTCA-3'	27693971	1200
LRAT-GFP	5'-TAGAATTCATGAAGAACCAATGCTGGAAGCT-3'	5'-GCGGATCCGCCAGACATCATCCACAAG-3'		1500
LRAT	5'-ATGGATCCATGAAGAACCAATGCTG-3'	5'-GAGAATTCTCAGCCAGACATCATCCACAAG-3'	170650594	696
LRAT-I-GFP	5'-ATGAATTCATGAAGAACCAATGCTGGAAG-3'	5'-CAGGATCCTGTGCCTCTCCCGGG-3'		
LRAT-s-GFP	5'-ATGAATTCATGAAGAACCAATGCTGGAAG-3'	5'-GTGGATCCACGGTTCTTCCCTGTGCCTC-3'		

Table 2
Effects of LRAT and Crbp1 expression levels on ring formation

CHO cells stably-transfected with LRAT or LRAT and Crbp1 were incubated with retinol to induce RE biosynthesis. High and low LRAT-GFP expression were scored by visual screening. High- expressing cells contained bright fluorescence, corresponding to an intensity level <200 arbitrary units, quantified by image J. Low-expressing cells showed dim fluorescence, corresponding to intensity levels <200 units. Data are means $\pm$ SE of 3 transfections for each condition, each with 89-144 cells.

Crbp1	% Cells with high LRAT (% with rings)	% Cells with low LRAT (% with rings)
–	39 $\pm$ 3.5 (0)	61 $\pm$ 3.5 (83)
+	40 $\pm$ 1.2 (0)	58 $\pm$ 1.2 (85)