Topography and Membrane Association of Lecithin: Retinol Acyltransferase

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Fatty acid retinyl esters are the storage form of vitamin A (all-trans-retinol) and serve as metabolic intermediates in the formation of the visual chromophore 11-cis-retinol. Lecithin:retinol acyltransferase (LRAT), the main enzyme responsible for retinyl ester formation, acts by transferring an acyl group from the sn-1 position of phosphatidylcholine to retinol. To define the membrane association and localization of LRAT, we produced an LRAT-specific monoclonal antibody, which we used to study enzyme partition under different experimental conditions. Furthermore, we examined the membrane topology of LRAT through an N-linked glycosylation scanning approach and protease protection assays. We show that LRAT is localized to the membrane of the endoplasmic reticulum (ER) and assumes a single membrane-spanning topology with an N-terminal cytoplasmic/C-terminal luminal orientation. In eukaryotic cells, the C-terminal transmembrane domain is essential for the activity and ER membrane targeting of LRAT. In contrast, the N-terminal hydrophobic region is not required for ER membrane targeting or enzymatic activity, and its amino acid sequence is not conserved in other species examined. We present experimental evidence of the topology and subcellular localization of LRAT, a critical enzyme in vitamin A metabolism.

The metabolism of vitamin A (all-trans-retinol) leads to the formation of all-trans-retinoic acid and 11-cis-retinal derivatives that play crucial roles in such processes as development, immunity, and vision (1–6). Recent studies have highlighted the importance of lecithin:retinol acyltransferase (LRAT) in the absorption and retention of retinol in intracellular stores (7). LRAT catalyzes the synthesis of retinyl esters, thereby drawing retinol from the circulation to storage depots such as the lipid droplets of hepatic stellate cells and the retinosomes found in the retinal pigment epithelium (RPE) (8, 9). Mutations in the LRAT gene lead to early-onset severe retinal dystrophy (10). Disruption of the Lrat gene in Lrat−/− mice produces a severe impairment in retinol uptake and storage capacity. As a result, Lrat−/− mice are blind (11) and more susceptible to vitamin A deficiency than their wild-type (WT) counterparts (12). Lrat−/− mice possess only trace amounts of retinyl esters in most tissues (11, 12), yet, surprisingly, the levels of retinyl esters in their adipose tissue are elevated compared with those in WT mice (12). Adipose stores of retinyl esters could depend on the activity of acyl-CoA:retinol acyltransferase (12).

LRAT plays a pivotal role in determining retinol availability, which in turn affects the levels of all-trans-retinoic acid, a potent regulator of the expression of many genes via retinoic acid receptors (13). Expression of LRAT in the liver is influenced by multiple factors, including vitamin A status (14), retinoic acid (15), other synthetic retinoic acid receptor activators (16), and peroxisome proliferator-activated receptor β/δ agonists (17). Thus, regulation of LRAT activity provides a homeostatic mechanism whereby circulating retinol levels are kept relatively constant despite fluctuations in the dietary intake of retinol (18).

Properties of the LRAT-catalyzed reaction have been investigated using homogenates or microsomal fractions from the liver or RPE (19, 20). These early studies showed that LRAT is most likely a membrane protein that transfers acyl groups from the sn-1 position of phospholipids to retinol via an acyl-enzyme intermediate (21, 22). Although both LRAT and acyl-CoA:retinol acyltransferase retinol-esterifying activities are present in liver homogenates, they can be distinguished because LRAT prefers retinol bound to cellular retinol-binding protein I or II and phosphatidylcholine as substrates, whereas acyl-CoA:retinol acyltransferase uses free retinol and fatty acyl-CoA as substrates (21, 23, 24). The cloning of full-length LRAT cDNA by Ruiz et al. (25) allowed investigation of the contribution of different residues to the acyltransferase reaction. On the basis of site-directed mutagenesis and acyltransferase assays, Cys161, Tyr154, and His60 were proposed to form a catalytic triad that is directly involved in the LRAT-catalyzed esterification reaction (26). Deletion of the putative N- and C-terminal transmembrane domains of LRAT leads to production of active enzyme when expressed in bacteria (27). These results argue that the N and C termini are not necessary for the enzymatic activity of LRAT. On the basis of hydrophobic analysis, LRAT was predicted to have its catalytic domain in the cytoplasm and to
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anchor to the membrane via its putative N- and C-terminal transmembrane domains (28).

Here, we present experimental evidence that LRAT is a single membrane-spanning protein with an N-terminal domain that faces the cytoplasm. Elimination of the C- but not N-terminal hydrophobic domain led to a large reduction in acyltransferase activity and mislocalization of the protein when expressed in eukaryotic cells. This study provides a basis for understanding the subcellular localization and function of LRAT in eukaryotic cells.

MATERIALS AND METHODS

Expression of Mutant LRAT Proteins—The mouse full-length WT LRAT cDNA cloned in pCR-Blunt-II-TOPO (Invitrogen) served as a template for amplification and mutagenesis reactions. Truncation mutants of LRAT were constructed as follows. N-terminal LRAT (lacking the putative C-terminal transmembrane domain; corresponding to Met1–Ser195) was amplified with primers 5′-CACCATGAGAAGACCCATGCTGGA and 5′-TCAACTGTTCTCTGATCACGAATGA; tLRAT (lacking both the putative N- and C-terminal transmembrane domains; corresponding to Gly35–Ser195) was amplified with primers 5′-CACATGGGGAAGAACCGTCCCTATGA and 5′-CACA; and for LRAT-Rho, primer 5′-CACGCCCGTCTTGTTGGAGAAAGGCACGTAGAAGTT-

The juxtamembrane domain; corresponding to Met1–Ser195) was amplified with primers 5′-CACATGGGGAAGAACCGTCCCTATGA and 5′-CTCGAGTTCGGTCTAGGGATTTG- ACTAGGA; for LRAT2 (corresponding to Gly89–Gly129), primers 5′-GGATCCAGAGGTGTTGCTTCCAAACAGC and 5′-CTCGAGTTCGGTCTAGGGATTTG- ACTAGGA; for LRAT3 (corresponding to Val141–Glu179), primers 5′-GGATCCAGAGGTGTTGCTTCCAAACAGC and 5′-CTCGAGTTCGGTCTAGGGATTTG- ACTAGGA; for LRAT4 (corresponding to Gly208–Ser249), primers 5′-GGATCCAGAGGTGTTGCTTCCAAACAGC and 5′-CTCGAGTTCGGTCTAGGGATTTG- ACTAGGA; for LRAT5 (corresponding to Gly286–Ser327), primers 5′-GGATCCAGAGGTGTTGCTTCCAAACAGC and 5′-CTCGAGTTCGGTCTAGGGATTTG- ACTAGGA. PCR products were first cloned into the pCR-Blunt-II-TOPO (Invitrogen) and then subcloned into the EcoRI site of pcDNA4/TO under the control of a tetracycline-inducible promoter. For site-directed mutagenesis, we used the QuikChange mutagenesis kit (Stratagene) according to the manufacturer’s protocol. The fragment coding for tLRAT (corresponding to Glyv35–Ser195) was also subcloned into the EcoRI site of the bacterial expression vector pET-30b(+) (Novagen) in-frame with an N-terminal hexahistidine tag and transformed for expression in bacterial BL21(DE3) cells (Invitrogen).

Mapping the Epitope of an Anti-LRAT Monoclonal Antibody—An anti-mouse LRAT monoclonal antibody was raised against a bacterially expressed His-tagged fragment of mouse LRAT corresponding to Gln89–Glu129 as described previously (11). The following primers were used to amplify the sequence of LRAT and to further subdivide the antigenic region of LRAT into three overlapping fragments: for LRAT1 (corresponding to Gln89–Gly129), primers 5′-GGATCCAGAGGTGTTGCTTCCAAACAGC and 5′-CTCGAGTTCGGTCTAGGGATTTG- ACTAGGA; for LRAT2 (corresponding to Gly119–Gly150), primers 5′-GGATCCAGAGGTGTTGCTTCCAAACAGC and 5′-CTCGAGTTCGGTCTAGGGATTTG- ACTAGGA; for LRAT3 (corresponding to Val141–Glu179), primers 5′-GGATCCAGAGGTGTTGCTTCCAAACAGC and 5′-CTCGAGTTCGGTCTAGGGATTTG- ACTAGGA. PCR products were cloned into the BamHI-Xhol sites of the pGEX-4T-2 vector (GE Healthcare) downstream of and in-frame with the glutathione S-transferase (GST) open reading frame. The GST-LRAT fusion constructs were transformed for expression into bacterial BL21RP cells. Expression of GST fused to LRAT1, -2, or -3 was induced with isopropyl β-D-thiogalactopyranoside and purified by glutathione affinity chromatography. Expression of purified proteins was examined by SDS-PAGE and Coomassie Blue staining. The presence of the epitope was assayed by SDS-PAGE and immunoblotting of bacterial lysates from cells expressing the GST-LRAT fragment proteins using the anti-LRAT monoclonal antibody.

Solubilization Studies of LRAT—A suspension of bovine RPE microsomes (~400 μg of total protein) (31) containing endogenous LRAT was diluted three times with 50 mM Bis-Tris propane (pH 7.4). The appropriate concentration of salt or detergent was achieved by the addition of stock solutions of NaCl, CHAPS, n-dodecyl β-D-maltopyranoside, SDS, and 1,2-dihexanoyl-sn-glycero-3-phosphocholine (DHPC). The final volumes of these mixtures were fixed at 1 ml. Samples were incubated for 20 min at 4 °C and then centrifuged at 150,000 × g for 1 h at 4 °C. Supernatants were examined directly for enzymatic activity of LRAT, and pellets were resuspended in 1 ml of the given detergent solution prior to enzyme assays. The presence of the RPE-specific 65-kDa protein RPE65 and LRAT proteins was verified by immunoblot analysis using the anti-LRAT monoclonal antibody (11) and an anti-RPE65 polyclonal antibody (a gift from Dr. J. Saari, University of Washington).

Protease Protection Assays—Aliquots of RPE microsomes (50 μg of protein) (31) were resuspended in Bis-Tris propane (pH 7.5) supplemented with 0.6 or 2.4 units/ml proteinase K (Sigma) in the presence or absence of 1% Triton X-100 (total reaction volume of 30 μl). Samples were incubated at room temperature for 30 min. Proteolysis was terminated by the addition of phenylmethylsulfonyl fluoride at a final concentra-
tion of 5 mM. Samples were boiled for 5 min in 6× SDS-PAGE loading buffer, separated by SDS-PAGE, and examined by immunoblotting using the anti-LRAT monoclonal antibody or a rabbit anti-calreticulin polyclonal antibody (Sigma).

**Endoglycosidase F and Tunicamycin Treatment and Immunoblot Analysis**—Expression WT LRAT, I42N-LRAT, D128N-LRAT, Rho-LRAT, or LRAT-Rho containing N-linked glycosylation signals in T-Rex-293 cells was induced with tetracycline added 24 h before analysis. For tunicamycin-treated cells, we incubated cells with 1 μg/ml tunicamycin 3 h before and throughout the tetracycline induction. Tunicamycin-treated and untreated controls were examined by SDS-PAGE and immunoblotting with the anti-LRAT monoclonal antibody. Where indicated, the lysate of LRAT-expressing cells was divided in two equal fractions, one for endoglycosidase F treatment and one for control treatment. Proteins were briefly denatured for 15 min at 95 °C in 0.05% SDS and then cooled and supplemented with 1% Nonidet P-40, 50 mM sodium phosphate (pH 7.5), and 500 units/reaction endoglycosidase F (New England Biolabs) for treated samples; endoglycosidase F was omitted in control samples. The deglycosylation reaction was allowed to proceed for 2 h at 37 °C with shaking, following which samples were treated with 1% SDS and analyzed by SDS-PAGE and immunoblotting with the anti-LRAT monoclonal antibody.

**LRAT Activity Assays**—LRAT activity was assayed *in vitro* using 20 μl of RPE microsomes (31), 100 μl of homogenates from COS-7 cells transfected with various LRAT constructs, or 100 μl of bacterial lysate expressing mouse tLRAT. Reactions were carried out in Bis-Tris propane (pH 7.5) supplemented with 5 mM DHP, 1 mM dithiothreitol, 1% bovine serum albumin, and 20 μM all-trans-retinol. Activity assays of RPE fractions or bacterial lysates contained all-trans-retinol at a final concentration of 20 μM delivered in N,N-dimethylformamide. Total volumes of the reaction mixtures were fixed at 200 μl. Reactions were carried out at 37 °C for 15 min and then stopped by the addition of 300 μl of methanol. Retinoids were extracted with 300 μl of hexane. Alternatively, $K_m$ and $V_{max}$ values for WT LRAT and its truncation mutants expressed in COS-7 cells were derived from steady-state kinetic measurements. Reactions were performed using 10 μg of protein from homogenates of transfected COS-7 in Bis-Tris propane supplemented with 1% bovine serum albumin. Reactions were initiated by the addition of all-trans-retinol diluted in N,N-dimethylformamide. To determine saturation of enzyme with substrate, we used seven different final concentrations ranging from 0.1 to 4 μM. Product formation was measured during initial reaction velocity conditions. Reactions were carried out at 30 °C for 2 min and then quenched by the addition of 100 μl of methanol. Retinoids were extracted with 200 μl of hexane and analyzed on a Hewlett-Packard 1100 series HPLC system equipped with a diode array detector and a Beckman Ultrasphere Si normal-phase column (5 μm, 4.5 × 250 mm) (32). The initial velocity data were analyzed using the Michaelis-Menten equation by nonlinear regression analysis. $K_m$ and $V_{max}$ values were calculated based on Lineweaver-Burk plots derived from data obtained from three independent experiments.

**RESULTS**

**LRAT Is an Endoplasmic Reticulum (ER)-localized Membrane Protein**—Hydropathy analysis of the sequence of mouse LRAT indicated that there is only one region at the C terminus of the protein that has high probability of being a transmembrane domain (Fig. 1A). The previously postulated N-terminal transmembrane domain (28, 33) has a much lower probability of spanning the membrane because it contains several charged residues within its sequence (Fig. 1B, italic letters). The orientation of transmembrane domains can be predicted from the net charge difference between the two regions flanking the
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A.

![Predicted TM domains](image)

**FIGURE 1.** Prediction of the topology of LRAT. **A**, a hydropathy plot of mouse LRAT sequence analyzed using the Goldman-Engelman-Steitz algorithm (56) and processed by the TopPred II program (available at bioweb.pasteur.fr/seqanal/interfaces/toppred.html) (57). Possible transmembrane (TM) domains are indicated. **B**, sequence alignment of mouse (Mus) and human (Hum) LRAT polypeptides. The N-terminal hydrophobic domain that has a low probability of being a transmembrane domain is shown in an open box. Charged residues are shown in italics. The C-terminal sequence that has a high probability of being a transmembrane domain is shown in a shaded box. Secondary structure predictions are indicated under the sequences: α-helix element (H), β-strand element (E), and turn element (-). Predictions are based on knn-predictor program of neural network analysis (www.cmpharm.ucsf.edu/~nomi/knnpredict.html) (58). The NCEFH domain found in several unrelated proteins is also shown. Gly190 and Ser190 (N- and C-terminal residues of mouse tLRAT, respectively) and Ile42 (mutated to N-linked glycosylation acceptor sites) are shown in boldface.

B.

**FIGURE 2.** Characterization of the epitope recognized by the anti-LRAT monoclonal antibody. The fragment of LRAT used to produce the anti-LRAT monoclonal antibody (Gln89-Glu179) was further subdivided into three smaller overlapping fragments, i.e. LRAT1 (corresponding to Gln89-Gly129), LRAT2 (corresponding to Gly119-Gly150), and LRAT3 (corresponding to Val141–Glu179). A, LRAT1–3 were fused to the C terminus of GST, expressed in bacteria as recombinant GST fusion proteins, and examined by SDS-PAGE and Coomassie Blue staining (left panel). **B**, GST lane, recombinant GST; lane 1, recombinant GST-LRAT1; lane 2, recombinant GST-LRAT2; lane 3, recombinant GST-LRAT3. The fusion proteins were examined by SDS-PAGE and immunoblotting using the anti-LRAT monoclonal antibody (right panel). The apparent molecular mass of the migrating band is indicated. Lane 1, recombinant GST-LRAT1; lane 2, recombinant GST-LRAT2; lane 3, recombinant GST-LRAT3. The specificity of the anti-LRAT monoclonal antibody was verified by immunohistochemical staining (green) of RPE cells from WT and Lrat−/− mice. Nuclei were stained with Hoescht 33342 dye (Invitrogen). Scale bar = 20 μm.

LRAT was proposed to be an ER-localized protein based on the detection of LRAT activity in subcellular fractions isolated from different types of hepatic cells (37) and purified RPE microsomes (38) and the detection of LRAT protein by immunoblotting of microsomes derived from LRAT-transfected cells (25). Here, we provide further evidence for the ER localization of LRAT by immunofluorescence microscopy. We studied the localization of LRAT protein in T-Rex-293 cells expressing LRAT protein under the control of a tetracycline-inducible promoter. Immunohistochemical staining of LRAT-expressing cells exposed to the anti-LRAT monoclonal antibody and an anti-BiP antibody directed against the ER resident protein BiP showed that the two proteins co-localized to the ER compartment (Fig. 3).

The strength of the interaction of LRAT with the ER membrane was examined by subjecting purified bovine RPE microsomes to various treatments. We compared the effects of various reagents on the solubility of LRAT and another ER resident protein from the RPE, viz. RPE65. The solubility of LRAT was verified by immunoblotting (Fig. 4A) and by acyltransferase
In addition, alkaline extraction did not facilitate LRAT solubilization, as LRAT that was extracted with water or NaCl did not have very well (Fig. 4, reagents 1 and 2). CHAPS, n-dodecyl β-D-maltopyranoside, and SDS significantly solubilized the enzyme. The solubility conferred upon LRAT by these detergents came at the expense of inhibition of its activity, seen as a decrease in the total activity of the homogenate (Fig. 4B, reagents 3–5 versus reagents 1 and 2). DHPC (Fig. 4B, reagent 6) is both a detergent and substrate for LRAT (39). Even though DHPC was not as efficient in solubilizing LRAT, it retained the activity of soluble LRAT very well (Fig. 4, A and B, reagent 6). There was a small fraction of LRAT extracted from microsomes after treatment with water or NaCl that remained soluble during ultracentrifugation (Fig. 4A, lanes 1 and 2). However, the fraction of LRAT that was extracted with water or NaCl did not have acyltransferase activity (Fig. 4B, reagents 1 and 2, gray bars). In addition, alkaline extraction did not facilitate LRAT solubilization (data not shown).

The Putative C-terminal Transmembrane Domain Is Necessary for the Activity and Correct Membrane Targeting of LRAT—Previous analysis of the sequence of LRAT predicted two possible transmembrane domains (28), neither of which is necessary for the activity of bacterially produced truncated human LRAT (tLRAT) encompassing residues 31–196 (27). We investigated the role of the putative N- and C-terminal transmembrane domains of mouse tLRAT spanning residues 35–196. Mouse tLRAT protein expressed in bacterial cells exhibited robust acyltransferase activity, indicating that its catalytic domain is located in the region encompassing residues 35–195 (data not shown). This observation suggests that the putative transmembrane domains might have eukaryote cell-specific roles and could be involved in membrane targeting. We studied the targeting of WT LRAT and its truncation mutants expressed in COS-7 eukaryotic cells, which have lower endogenous LRAT activity compared with HEK-293 cells. Of the three truncation mutants examined, one retained only the putative C-terminal transmembrane domain (Ctm LRAT) of mouse LRAT. Another retained the putative N-terminal transmembrane domain (Ntm LRAT), whereas tLRAT lacked both putative transmembrane domains (Fig. 5B). We showed that WT LRAT expressed in T-REx-293 cells co-localized with the ER marker BiP (Fig. 3). Immunohistochemical staining and confocal microscopy analysis showed that full-length WT LRAT and Ctm LRAT expressed in COS-7 cells co-localized with the ER marker calreticulin, whereas neither Ntm LRAT nor tLRAT localized to the ER (Fig. 5A). Instead, Ntm LRAT and tLRAT localized to small cytoplasmic structures that were distinct from the ER and dispersed throughout the cell. Localization of tLRAT to a specific cellular compartment is probably due to self-aggregation of this domain, which requires strong detergents for solubilization even when expressed in bacteria (data not shown) (27). From the above experiments, we conclude that the C-terminal domain is necessary for the ER targeting and/or retention of LRAT. Next, we investigated whether aberrant targeting has any effect on the activity of LRAT. Considering the activity of bacterially expressed human and mouse tLRAT, the catalytic domain was present in all truncated forms of LRAT examined here. Based on the $K_m$ and $V_{max}$ values for the various forms of LRAT, we found that only WT LRAT and Ctm LRAT exhibited robust and comparable acyltransferase activity when expressed in eukaryotic cells, whereas Ntm LRAT and tLRAT had no activity (Fig. 5C). How the aberrant localizations of tLRAT and Ntm LRAT affect their enzymatic functions is unclear, although our experiments reveal a eukaryote cell-specific role for the C-terminal transmembrane domain in making this enzyme structurally active. We conclude that the putative C-terminal transmembrane domain of LRAT is necessary for the activity or folding of LRAT in eukaryotic cells in addition to or as a result of its role in targeting the protein to the ER membrane.

Membrane Topology of LRAT—We employed a protease protection assay to determine the membrane orientation of LRAT. This assay is based on the fact that the domain of a protein located within the membrane lumen of the microsome will be protected from proteolytic digestion by exogenous protease. Upon the addition of detergent, the protected domain should become accessible to proteolytic degradation. We isolated RPE...
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![Image](50x471 to 407x734)

**FIGURE 5. Characterization of the truncation mutants of LRAT.** A, the subcellular localization of LRAT truncation mutants was determined by immunohistochemical staining and confocal microscopy. COS-7 cells were transiently transfected with full-length WT LRAT and its truncation mutants. LRAT proteins were detected by the anti-LRAT monoclonal antibody (green), and calreticulin was detected by the anti-calreticulin polyclonal antibody (red). Scale bar = 10 μm. These are representative images of the LRAT-transfected cells investigated (n > 50). B, the truncation mutants studied included Ctm LRAT (lacking the putative N-terminal transmembrane domain of LRAT), Ntm LRAT (lacking the putative C-terminal transmembrane domain of LRAT), and tLRAT (lacking both the putative N- and C-terminal transmembrane domains of LRAT). C, the actin cytoskeleton activity of WT LRAT and its truncation mutants was assayed in vitro with all-trans-retinol used as the substrate. K_m and V_max values were calculated based on the data obtained from three independent experiments. NA, no enzymatic activity.

Microsomes and treated them with proteinase K in the presence or absence of 1% Triton X-100. We immunoblotted the treated membranes with the anti-LRAT monoclonal antibody or the anti-calreticulin polyclonal antibody. The luminal protein calreticulin was protected from digestion by exogenous proteinase in the absence of detergent (Fig. 6A, lower panel). This indicates that the RPE microsomal membranes were intact and correctly sided. The epitope recognized by the anti-LRAT monoclonal antibody encompasses a region within the central hydrophilic domain of LRAT (Fig. 2). Exogenous proteinase K cleaved LRAT as evidenced by the appearance of a lower molecular mass fragment recognized by the anti-LRAT monoclonal antibody (Fig. 6A, upper panel, second and third lanes). The protease digestion pattern of LRAT was not affected by the presence of Triton X-100 (Fig. 6A, upper panel, fifth and sixth lanes). In contrast, calreticulin was completely degraded by proteinase K in the presence of the detergent (Fig. 6A, lower panel, fifth and sixth lanes). Therefore, the protease resistance manifested by the hydrophilic core of LRAT (Fig. 6A, upper panel, fifth and sixth lanes) was not due to the membrane-restricted access of proteinase K. Similar results were obtained using exogenously added trypsin (data not shown). Our results suggest that part of LRAT is found within the cytoplasm, thus providing a good estimate of the membrane orientation of LRAT. However, the interpretation of the digestion pattern is affected by the partial protease resistance of the central hydrophilic core of the LRAT protein. Thus, we sought another method to further test the topological nature of LRAT.

To better determine the topology of LRAT, we employed an N-linked glycosylation scanning approach. This assay is based on the fact that the domain of LRAT found in the lumen of the ER should be accessible to N-linked glycosylation. We monitored the glycosylation status of recombinant LRAT proteins engineered to contain N-linked glycosylation acceptor sites (NX(S/T) consensus sequence) (40) in either the N or C terminus or within the central hydrophilic domain. To avoid possible sequence-based bias, we used site-directed mutagenesis to create two different point mutants containing NX(S/T) consensus sites within the central hydrophilic domain, viz. 142N-LRAT and D128N-LRAT. To study the glycosylation of the N- and C-terminal hydrophilic segments, we engineered fusion proteins of LRAT with the first 21 amino acid residues of bovine rhodopsin. These mutant LRAT proteins are depicted in Fig. 6B. The N-terminal fragment of rhodopsin contains two well-characterized N-linked glycosylation sites at positions 2 and 15 within sequence MNFGEGPNFYVPFSKGTGVR (with the Asn acceptor residues italicized and the NX(S/T) consensus sites underlined). We expressed the recombinant proteins in T-Rex-293 cells, analyzed the LRAT proteins by SDS-PAGE and immunoblotting, and established the glycosylation status of the different LRAT mutants based on the slower electrophoretic mobility of the glycoprotein versus the unglycosylated polypeptide. The only LRAT mutant that became glycosylated was the LRAT-Rho fusion protein as demonstrated by the presence of two slower migrating species of LRAT-Rho in comparison with Rho-LRAT, which has the same polypeptide mass and composition as LRAT-Rho (Fig. 6, C and D). The slower migrating species of LRAT-Rho were converted to the faster migrating LRAT-Rho unglycosylated polypeptide by treatment with endoglycosidase F, an enzyme that cleaves the N-linked glycans from glycoproteins (Fig. 6C). Incubation of LRAT-Rho cells with the glycosylation inhibitor tunicamycin prior to and during induction of protein expression with tetracycline led to the absence of the two slower migrating forms of LRAT-Rho (Fig. 6D). Therefore, both N-linked glycosylation acceptor sites in LRAT-Rho were accessible and recognized by oligosaccharyltransferase. These results demonstrate that the C terminus of LRAT is localized within the lumen of the ER. There was no shift in the migration of either 142N-LRAT or D128N-LRAT versus WT LRAT, which does not contain any potential...
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The localization of LRAT needs to be considered in the context of its function. It has been shown that, in the RPE, retinyl esters are associated with retinosomes (8). Retinosomes are distinct from other subcellular organelles such as the ER, Golgi, lysosomes, and mitochondria (8). We studied the activity and localization of LRAT-expressing transfected cells incubated with either all-trans-retinol or oleic acid (Fig. 7). The addition of all-trans-retinol resulted in formation of retinosomes in cultured cells. Retinosome-like fluorescent particles formed only in the presence of all-trans-retinol, but not oleic acid (Fig. 7, A and B versus C and D). Retinosomes could be visualized based on their autofluorescence (Fig. 7A) and using the neutral lipid fluorescent dye Nile red (Fig. 7B) (41). However, Nile red also allowed visualization of lipid bodies formed solely by supplementation with oleic acid (Fig. 7D). We did not observe any effect of all-trans-retinol addition on the localization of LRAT, which remained ER-associated. Of note is that the T-REX-293 cells we used for these experiments do not express detectable levels of RPE65, a protein proposed in some studies to be a retinyl ester-binding/transport protein (42, 43), yet we were still able to detect retinosome formation.

The N Terminus of LRAT Is Not Conserved in All Vertebrates—Analysis of the N-terminal domains of the known and putative vertebrate homologs of LRAT showed a low degree of conservation. We obtained sequences of known LRAT proteins and putative homologs from bovine, dog, opossum, frog (Xenopus laevis and Xenopus tropicalis), zebrafish (Danio rerio), and pufferfish (Tetraodon nigroviridis and Fugu rubripes) by searching the genome data base for proteins related to human LRAT. We built an alignment of these proteins using the program T-Coffee and the matrix BLOSUM62 with gap penalties of 11 for existence and 1 for extension. Supplemental Fig. 1, which shows the result of the alignment of the N termini of the LRAT proteins, clearly demonstrates that the stretch of hydrophobic residues observed in the mammalian LRAT proteins is not conserved in fish. In fact, the most conserved feature in the N termini of all LRAT proteins is the charged Gln-Lys pair and the bulky Phe25 residue. Based on these observations, together with our results showing that LRAT assumes a C-terminally anchored topology and the observation that deletion of the N terminus has no effect on the activity of LRAT, we conclude that the cytoplasmic N terminus of LRAT is not conserved and that it is not involved in catalysis or membrane

N-linked glycosylation acceptor sites. Treatment of proteins with endoglycosidase F or incubation of cells with tunicamycin had no effect on the migration of the Rho-LRAT, I42N-LRAT, or D128N-LRAT proteins (Fig. 6, C and D), demonstrating that the acceptor sites of I42N-LRAT, D128N-LRAT, and Rho-LRAT are not found within the lumen of the ER. D128N-LRAT was less well recognized by the anti-LRAT monoclonal antibody because of the position of the mutated residue within the mapped epitope region (Fig. 2). Faster migrating proteins that are most likely proteolytic products of LRAT can be seen as immunoreactive bands smaller than 25 kDa in several lanes. The N-terminal region of rhodopsin (fragment 1–21) was readily glycosylated when present in the N terminus of rhodopsin or fused to the C terminus of LRAT, but was not glycosylated when fused to the N terminus of LRAT to produce Rho-LRAT. These results demonstrate that the N-terminal hydrophobic region does not span the ER membrane and is either buried within the protein core or peripherally associated with the membrane. These findings are consistent with the predicted single membrane-spanning topology of LRAT with an N-terminal cytoplasmic/C-terminal luminal orientation (see Fig. 8).
anchoring of LRAT. Therefore, we propose that LRAT has a single membrane-spanning topology with an N-terminal cytoplasmic/C-terminal luminal orientation, as depicted in Fig. 8.

**DISCUSSION**

By virtue of the hydrophobic nature of their substrates, many retinoid- and steroid-processing enzymes are associated with intracellular membranes. The membrane milieu provides a better environment for retinoids and allows enzymes to channel substrates along their metabolic pathways. In the case of LRAT, this association also allows the enzyme access to the ester substrate at the sn-1 position of phospholipids. The retinol substrate of LRAT is obtained from the cytoplasm via cellular retinol-binding protein I or II (44). The results presented here indicate that the N terminus and putative catalytic domain of LRAT are both located in the cytoplasm, whereas the C-terminal transmembrane domain serves to anchor this protein to the membrane of the ER (Fig. 8). The LRAT topology described here suggests that its C-terminal transmembrane region serves a dual role of as a targeting signal and membrane anchor (signal-anchor) domain. The proximity of the signal-anchor domain of LRAT to its C terminus argues that translation and membrane translocation of the LRAT polypeptide are not coupled events and that LRAT is targeted to the ER membrane following synthesis in a post-translational fashion, similar to other tail-anchored proteins (Refs. 45 and 46; reviewed in Ref. 47).

To study the membrane topology of LRAT, we employed both a protease protection assay and an N-linked glycosylation scanning approach. The protease protection assay demonstrated that membrane-embedded LRAT is accessible to exogenous protease; thus, part of LRAT is found in the cytoplasm. We found that the central hydrophilic domain of LRAT that contains the putative catalytic domain and the epitope recognized by the anti-LRAT monoclonal antibody displays resistance to proteinase K. The same domain was shown previously to be involved in dimerization of LRAT in vitro, as was demonstrated in the case of bacterially expressed LRAT (28). Presumably, a compact higher order structure could prevent the access of proteinase K and impart protease resistance to the core of LRAT, as observed in our study. The membrane orientation of LRAT was established using an N-linked glycosylation scanning approach. The subcellular location of the central hydrophilic domain was studied using two point mutants of LRAT (I42N and D128N), creating N-linked glycosylation acceptor sites within the central hydrophilic domain. The subcellular location of the N and C termini was studied using recombinant fusion proteins of the first 21 amino acid residues of rhodopsin fused to the N or C terminus of LRAT. The extension of the N- and C-terminal regions was necessary because of the proximity of the putative transmembrane domains to the N or C terminus of LRAT. A minimum distance of 12–14 amino acid residues is required between the luminal end of the transmembrane domain and the N-linked glycosylation acceptor site (48). These limitations were circumvented by placing the acceptor site farther from the membrane by use of a fusion protein. Another advantage is that the N terminus of rhodopsin does not interfere with translocation of rhodopsin across the ER, so it is suitable as a reporter of membrane translocation.

The topology and membrane orientation presented here are in good agreement with the subcellular localization of the retinol and ester substrates. Our structural analysis indicated that the C-terminal transmembrane domain of LRAT is necessary for the ER localization and activity of this enzyme in eukaryotic cells. The N-terminal hydrophobic stretch of LRAT does not span the membrane of the ER and is not required for ER membrane targeting. Truncation of the N-terminal domain leads to production of active enzyme as shown for Ctm LRAT. Moreover, the N-terminal domain shows a low degree of sequence conservation in other species examined. These results suggest a different role for the N-terminal hydrophobic region of LRAT in eukaryotes. One possibility is that the N-terminal hydrophobic domain of LRAT is involved in interacting with accessory or regulatory proteins in a species-specific manner. Such regulatory interactions might include cellular retinol-binding protein accessory proteins, which are known to present substrate (23, 49) and even directly modulate the acyltransferase activity of LRAT (44). More studies are necessary to establish the role of the N-terminal domain of LRAT in vivo.

Exactly how retinyl esters are transported from the ER to retinosomes or lipid droplets of hepatic stellate cells remains an open question. Retinyl esters produced by LRAT at the cyto-

![FIGURE 7. Formation of retinyl ester-containing vesicles in cell culture.](Image 61x510 to 397x733)
plasmic face of the ER membrane are transported to retinosomes both in vivo in the RPE cell layer and, as shown here, to similar structures in cultured LRAT-expressing cells. Interestingly, the hepatic stellate cells of Lrat/H11002/H11002 mice lack the large lipid-containing droplets that normally store retinyl esters (12). The composition of lipid droplets of hepatic stellate cells includes many other lipid classes in addition to retinyl esters, yet the absence of LRAT affects the formation of these specialized structures (12). Retinosomes found in the RPE have been shown to be distinct from other subcellular organelles and to include proteins (such as adipose differentiation-related protein) that are involved in lipid metabolism (8). It is feasible that newly synthesized retinyl esters accumulate between the two leaflets of the ER and eventually bud off into the cytoplasm encapsulated by the cytoplasmic leaflet of the ER membrane, a possibility considered for the transport of triglycerides (9). This model would allow lipid droplets to recruit phospholipids and peripheral membrane proteins associated with the cytoplasmic leaflet of the ER. Possibly, small G-proteins mediate vesicle trafficking between the ER and lipid storage structures. A recent study shows that several Rab proteins are localized proximal to the lipid bodies; among these G-proteins, heterologously expressed Rab18 is observed at the surface of lipid bodies (50). Mobilization of the retinyl ester pool is another aspect of retinoid metabolism that is not fully understood. It is not clear whether retinyl-ester hydrolase enzymes have direct access to retinyl esters in retinosomes or whether retinyl esters have to be transported to another cellular compartment prior to their hydrolysis. More studies are necessary to establish the transport mechanism for retinyl esters between the ER and hepatic stellate cell lipid droplets or retinosomes. The role of the different cellular compartments in the metabolism of retinoids and the transport proteins involved in this process represent a very interesting and important aspect of retinoid metabolism.

The RPE65 protein is currently the only protein known to associate with retinyl esters (42, 43). It has been reported that RPE65 is involved in the isomerization reaction that produces 11-cis-retinol and that retinyl esters are the preferred substrates for this reaction (51–53). It has also been proposed that palmitoylation of RPE65 by LRAT regulates its subcellular localization and its ligand binding selectivity versus retinol or retinyl esters (54). We have shown here that microsome-derived RPE65 associates in a stable fashion with the ER membrane, and we have shown previously that RPE65 does not co-localize with retinyl ester-containing retinosomes (8). The RPE65 protein was not detectable in the LRAT-expressing T-REx-293 cells described here and is also absent in hepatic stellate cells. Additionally, movement of retinyl esters from the ER to lipid droplets is not affected in Rpe65/H11002/H11002 mice, which accumulate high levels of retinyl esters in large droplets (8, 55). Therefore, other carrier proteins/mechanisms must be involved in the transport of retinyl esters from the ER to lipid droplets or retinosomes. It is, however, still possible that RPE65 is involved in the efflux of retinyl esters from retinosomes to the ER in the RPE (8, 55) through its retinyl-ester hydrolase activity and partial solubility. Consequently, the cytoplasmic location of the catalytic domain of LRAT would allow RPE65 immediate access to the retinyl esters stored in the ER membrane.
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to the newly synthesized retinyl esters for rapid regeneration of 11-cis-retinol.

In this study, we have provided experimental evidence of the association of LRAT with the ER membrane, and we have described its membrane topology. The C-terminally anchored topology allows LRAT to interact with other proteins and factors found on the cytoplasmic side of the ER membrane. Such factors include the substrate retinol, accessory binding proteins, other enzymes, and regulatory proteins. The results of this study should help uncover any associated enzymes or regulatory proteins, and they are relevant to understanding the mechanism of the LRAT-catalyzed reaction and the formation and storage of retinyl esters.

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