Molecular and Biochemical Characterization of Lecithin Retinol Acyltransferase*  

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The enzyme responsible for conversion of all-trans-retinol into retinyl esters, the lecithin retinol acyltransferase (LRAT) has been characterized at the molecular level. The cDNA coding for this protein was cloned and its amino acid sequence deduced. LRAT is composed of a polypeptide of 230 amino acid residues with a calculated mass of 25.3 kDa. Tissue distribution analysis by Northern blot showed expression of a 5.0-kilobase transcript in the human retinal pigment epithelium as well as in other tissues that are known for their high LRAT activity and vitamin A processing. Affinity labeling experiments using specific compounds with high affinity for LRAT and monospecific polyclonal antibodies raised in rabbits against two peptide sequences for LRAT confirmed the molecular mass of LRAT as a 25-kDa protein. High performance liquid chromatography analysis of the reaction product formed by HEK-293 cells transfected with LRAT cDNA confirmed the ability of the transfected cells to convert [3H]all-trans-retinol into authentic [3H]all-trans-retinyl palmitate as chemically determined.

Vitamin A (retinol) is the substrate for the biosynthesis of several functional retinoids (retinol derivatives) which are essential for important biological processes such as, vision, reproduction, and development (for review, see Ref. 1). The small intestinal epithelium is the first site of interaction and processing of retinol after absorption from the diet. Within this epithelium, the final processing step involves the esterification of retinol with long chain fatty acids, primarily palmitic, and incorporation of the retinyl esters into the hydrophobic core of chylomicrons which are secreted into the lymph and ultimately enter the blood via the thoracic and other lymphatic ducts (2). Chylomicron remnants are taken up by the liver and the retinyl esters are stored until needed. Following de-esterification by the liver, retinol is complexed with a 21-kDa retinol-binding protein (RBP)1 and secreted into the circulation as a complex with transthyretin whereby it is distributed to other tissues (3). Both proteins are thought to transport and protect retinol from oxidation and/or isomerization during the distribution process. In the eye, and more specifically in the retinal pigment epithelium (RPE), this retinol complex interacts with the basal membrane of RPE cells probably via an RBP receptor (4, 5), where retinol is delivered into the cytoplasm to initiate a unique and highly specialized process for the RPE, the visual cycle (for review, see Refs. 6–8). During this important process, the retinol bound to a cellular retinol-binding protein (CRBP), is trans-esterified by an enzyme named lecithin retinyl acyltransferase (LRAT) (9–11) that transfers an acyl group from lecithin to retinol to generate all-trans-retinyl esters. All-trans-retinyl esters generated by the activity of LRAT are not only presumptive storage forms of vitamin A, but they are also substrates for an isomerohydrolase which transforms the esters into an intermediate 11-cis-retinol (12, 13). Due to a membrane-associated alcohol dehydrogenase (14, 15), 11-cis-retinol is then oxidized and converted into 11-cis-retinaldehyde which is the chromophore for rhodopsin and the cone photopigments. In the RPE, the esterification process of retinol by LRAT is critical for the continuation of the visual cycle since it supplies the processed substrate for the isomerization reaction. Although LRAT has been solubilized, partially purified and substantially studied with respect to its kinetic properties and substrate specificity (16, 17), it has never been characterized at the molecular level. An ordered ping-pong bi-bi mechanism in which lecithin first acylates the enzyme describes the kinetic mechanism of action of LRAT (17). The effects of potent competitive reversible and irreversible specific inhibitors for its enzymatic activity have been described (18, 19). One of these has been used as a probe to determine the putative mass of LRAT. Using [3H]all-trans-retinyl α-bromooacetate (RBA) which binds specifically to LRAT among partially purified proteins from bovine RPE, a product of about 25 kDa was labeled (20). In contrast, while using radiation inactivation analysis on intact microsomal membranes from rat liver the activity of LRAT yielded a target size averaging 52–56 kDa (21). In addition to RPE and liver, LRAT activity has also been reported in several other tissues such as, testis, intestine, and pancreas.

1 The abbreviations used are: CRBP, cellular retinol-binding protein; LRAT, lecithin retinol acyltransferase; RBA, all-trans-retinyl α-bromooacetate; BACMK, N-boc-l-biotinyl-11-aminoundecane chloromethyl ketone; RT-PCR, reverse transcription-polymerase chain reaction; HEK-293, human embryonic kidney 293 cells; RPE, retinal pigment epithelium; ECL, enhanced chemiluminescence; bP base pair(s); UTR, untranslated region; BSA, bovine serum albumin; DTT, dithiothreitol; HPLC, high performance liquid chromatography.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF071510.

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Characterization of Lecithin Retinol Acyltransferase

The LRAT activity was assayed as described under “Experimental Procedures.” Specific activity (units/mg) is expressed as nanomoles of [11,12-3H] retinyl ester formed in 1 min/1 mg.

| Steps                                  | Total protein | Specific activity | Total activity | Purification Yield |
|----------------------------------------|---------------|-------------------|----------------|--------------------|
| Detergent extract                      | 235.2         | 0.008             | 1.88           | 1.0                |
| DEAE-Sepharose                         | 56.6          | 0.032             | 1.81           | 4.0                |
| Thiopropyl Sepharose                   | 24.6          | 0.008             | 0.93           | 4.5                |
| Mono Q                                 | 2.2           | 0.216             | 0.48           | 27.0               |

(11, 22, 23), suggesting a role in essential biological processes. Because of the metabolic relevance of LRAT, particularly in the metabolism and storage of vitamin A, its molecular characterization is of vital importance.

In this study, we report on the biochemical properties of LRAT and the generation of specific polyclonal antibodies. Additionally, we describe its primary structure and verify its authenticity by the expression of LRAT in transformed mammalian cell lines.

EXPERIMENTAL PROCEDURES

Materials—Frozen bovine eye cups devoid of retinas were purchased from J. A. and W. L. Lawson Co., Lincoln, NE. [11,12-3H] All-trans-retinol (specific activity 31.4 Ci/mmol) was obtained from NEN Life Science Products Inc. [11,12-3H] Retinyl palmitate (0.9 m M) was obtained from NEN Life Science Products Inc. [11,12-3H] N-retinyl acetate was prepared using partially purified LRAT protein (0.52 mg), 0.1 M Tris-HCl, pH 8.0, 2 mM DTT, and BACMK (2 μM) in a final volume of 250 ml. Inhibition was measured at 10, 20, 30, 40, and 60 min by taking 10-μl aliquots of the incubation mixture and adding them to the assay mixture and the conversion of retinol to retinyl palmitate was analyzed as described in the LRAT activity assays section.

Affinity Labeling of LRAT and Peptide Analysis—Partially purified enzyme was preincubated with specific activity was 94 °C for 5 min. The enzymatic activity was inactivated at 42 °C for 15 min. The enzyme was preincubated as described earlier with cholesterol chloroacetate to block nonspecifically labeled proteolytic sites potentially labeled with BACMK (20).

The labeled product was detected by an ECL Western blotting assay (Amersham). The putative LRAT band was extracted from the gel, digested with trypsin, and the peptide analysis was performed by the Harvard Microsequencing Facility.

RNA Isolation from Human RPE Cells—RPE cells were dissected from explants of human eyes collected within 4 h (adult) and less than 1 h (fetal) postmortem. The poly(A)+ RNA fraction was extracted from RPE cells by using the Fast-Track System (Invitrogen).

Isolation and Characterization of Human LRAT cDNAs—Based on sequence data from the putative bovine LRAT protein corresponding to the N-terminal region and an internal peptide sequence (see “Results”), the following pair of degenerate primers were designed: 5′-ATGAARAAACNNATGCTNGARGC-3′ (forward) and 5′-DATNCCTRTGNGTNARRTG-3′ (reverse). RT-PCR experiments were performed on poly(A)+ RNA from human RPE cells, according to the vendor’s recommendations in the RNA-PCR kit (Perkin-Elmer). 200 ng of poly(A)+ RNA was reverse transcribed in a reaction mixture containing a final concentration of 5 mM MgCl2, 1 × PCR buffer, 1 mM dNTPs, 1 unit/μl of RNase inhibitor, 2.5 units/μl of reverse transcriptase, and 2.5 μl of degenerate reverse primer. The total 20-μl reaction was overlaid with 50 μl of mineral oil and incubated at 42 °C for 15 min. The viral particle was inactivated at 94 °C for 5 min. For PCR amplification, 1 μl of a mixture containing 2.5 μl of degenerate forward primer, 2 mM MgCl2, 1 × PCR buffer, 65.5 μl of sterilized water, and 2.5 units/100 μl of AmpliTaq DNA polymerase were added to the 20-μl reverse transcription reaction. Amplification was performed at 94 °C, 1 min; 60 °C, 1 min; and 72 °C, 2 min for 35 cycles, followed by 10 min incubation at 72 °C. By using the degenerate primers described above, a 189-bp fragment was amplified. A human fetal RPE cDNA library was constructed in the Uni-ZAP XR vector (Stratagene) by using poly(A)+ RNA from cultured fetal human RPE cells obtained from aborted fetuses of 15–24 weeks gestation and maintained and harvested as described earlier (24). All of the DNA inserts were unidirectionally placed between the unique EcoRI and XhoI restriction sites of the pBluescript vector. The cDNA library was screened with the 189-bp DNA fragment. Five positive cDNA clones out of 2.5 × 105 plaques were further characterized by sequencing both strands with the dideoxy chain termination method, using the Sequenase 2.0 system (U. S. Biochemical Corp.).

Northern Blot Analysis—Two μg of poly(A)+ RNA from human fetal and adult RPE cells were electrophoretically separated in a 1.2% agarose-formaldehyde gel and blotted onto a nylon membrane. Filter membranes were stained with 2 μg of poly(A)+ RNA from human fetal and adult RPE cells were electrophoretically separated in a 1.2% agarose-formaldehyde gel and blotted onto a nylon membrane. Filter membranes were stained with 2 μg of poly(A)+ RNA from human fetal and adult RPE cells.
Characterization of Lecithin Retinol Acyltransferase

Expression of LRAT in Human Embryonic Kidney-293 and 293T Cells (HEK-293 and HEK 293T)—A 1.0-kilobase EcoRI/HDNA fragment encoding for LRAT, which included 92 bp of 5’-UTR, 690 bp of coding sequence, and 254 bp of 3’-UTR was subcloned into the EcoRI site of the polylinker of the pcDNA3 vector (Invitrogen) where expression is driven by the human cytomegalovirus promoter. HEK-293 and a similar cell line HEK-293T cells (which carry the large T antigen from SV40 and therefore exhibit higher expression due to amplification of vectors such as the pcDNA3) were grown in 100-mm plates to 80% confluency. Transfection experiments were performed using LipofectAMINE (Life Technologies, Inc.) and 30 μg of DNA for LRAT/pcDNA3 construct or empty vector. Cells were collected 24, 48, and 72 h after transfection.

Polyclonal Anti-LRAT Antiserum and Western Blot Analysis—Two peptide sequences GAAGDKDRGNFSFYETSS and HLDLESQKQALL-NEEVARRAE corresponding to positions 28–44 and 126–146 of the LRAT polypeptide described in this work were selected for their antigenicity and accessibility (25, 26). Rabbits were immunized with a mixture of both peptides. Polyclonal antisera were generated by contract with Alpha Diagnostics International. Native human RPE cells stored at −80 °C after collection were analyzed. For the purpose of higher protein expression, HEK-293T cells were used in this experiment. In addition to non-transfected HEK-293T cells, cells transfected with empty plasmid and cells transfected with LRAT cDNA were collected 24 h after transfection and included in the analysis. 10 μg of microsomal protein from each sample in buffer containing 1% 2-mercaptoethanol was boiled for 2 min and loaded onto a 5% SDS-polyacrylamide gel electrophoresis. Blot analysis was performed on nitrocellulose filters according to Towbin et al. (27) using antiserum diluted to 1:1000 for the identification of LRAT. Protein bands were detected by the ECL system.

LRAT Activity Assay—Unless otherwise mentioned, all procedures were performed under dim red light with samples kept on ice. 28 pmol of 11,12-3H]all-trans-retinol (solvent dried under N₂ stream and retinol dissolved in 5 μl of 10% BSA) were added to 100 μl of an HEK 293 membrane suspension (0.38 mg of protein, 100 μM Tris-HCl, pH 8.5, 0.28 μM final retinol concentration, 0.5% final BSA concentration). The membranes were incubated at room temperature, and 50-μl aliquots of the reaction mixture were taken out for analysis after 15 and 60 min, respectively. The reactions were quenched with methanol (500 μl/sample), 100 μl of H₂O was added, and 500 μl of hexane (containing butylated hydroxytoluene at 1 mg/ml) was used for extraction of retinoids. The isomeric retinols were analyzed on a 5-μm PVA-Sil column (250 × 4.00 mm, YMC) and the eluant was 7% dioxane in hexane at a flow rate of 1.5 ml/min. Retinyl esters were separated on a 5-μm Maxsil column (250 × 4.00 mm, Phenomenex) with 0.4% ether (preservative free) in hexane, 0.8 ml/min; detection of added standards was at 313 nm.

RESULTS

Inhibition of LRAT by BACMK—Previous studies have demonstrated that RBA is an active-site directed affinity labeling agent of LRAT (20). Other hydrophobic halomethyl ketones (Scheme 1) such as dodecylchloromethyl ketone and dodecylbromomethyl ketone, are also potent affinity labeling agents of this enzyme. In order to identify the labeled protein without resorting to radioactive synthesis, a biotinyl-containing dodecylchloromethyl ketone analog, BACMK (Scheme 1), was prepared and tested as an inactivator of LRAT.

As shown in Fig. 1A, the rate of conversion of retinol into retinyl esters by 0.52 mg of partially purified enzyme was gradually reduced when incubated for a fixed time period of 10 min with increasing concentrations of BACMK. The same amounts of enzyme were used to determine the remaining LRAT activity by a fixed concentration of 2 μM BACMK at several time intervals (Fig. 1B). This graph clearly demonstrates a time-dependent mode of LRAT inactivation by BACMK. After a 10-min incubation, 2 μM BACMK was able to inhibit around 45% of the LRAT activity as opposed to the activity of cells in the absence of BACMK. These data suggested that the inhibitory effect of BACMK for LRAT is as potent as the previously described RBA.

Labeling of LRAT with BACMK—Since LRAT has been recalcitrant to complete purification, it was of interest to identify the enzyme, or a component thereof, using BACMK as a probe. Since the partially purified enzyme preparation still contains undesirable proteins, it was important to first eliminate the possibility that proteins other than LRAT would react with the affinity labeling agent BACMK. To this end, partially purified LRAT was first incubated with cholesterol chloroacetate to block extraneous nucleophilic proteins (probably thiol dependent) other than LRAT. Cholesterol chloroacetate does not inhibit LRAT, and has been used to block any non-LRAT related nucleophiles prior to treatment with RBA (20). This pretreatment dramatically simplifies the labeling pattern when using affinity labeling reagents. Fig. 2 shows the ECL labeling patterns of partially purified LRAT when using a concentration of 25 μM BACMK. Lane 1, shows a major labeled product of approximately 25 kDa molecular mass. A 35-kDa protein also appeared to be labeled in this procedure. Lane 2 shows the labeling of partially purified LRAT with 10 μM RBA, followed by treatment with BACMK. Interestingly, when the partially purified LRAT was first passed through a streptavidin column to remove endogenously biotinylated proteins, and afterward

2 Y.-H. Lim, I. Hubacek, and R. R. Rando, unpublished data.
dependent inhibition of the LRAT by BACMK. Data points are mean values from duplicate assays. Units on the ordinate are percent retinyl ester formation from total retinoids after 10 min of incubation. A, time course for the inhibition of LRAT by BACMK. Triplicate assays were performed in all cases. The means of these values are shown in the figure (standard deviation ± 5%). Control experiments were also performed by incubating LRAT for 0, 10, 20, 30, 40, and 60 min in the absence of BACMK.

The specificity of a polyclonal antiserum raised in rabbits against a mixture of two LRAT peptides was tested by Western blot analysis (Fig. 5). As shown in lane 1, this antiserum reacted specifically with a 25-kDa band treated with BACMK, a clear sharp band of 25 kDa was identified after ECL detection, lane 3.

The band in Fig. 2, lane 3, was subsequently extracted from the gel and subjected to protein sequencing. The N terminus was not blocked, and proved to possess the following sequence: MKNPMLAEAVSLVLEKLFISHFYKFK representing the first 24 amino acids. After enzymatic degradation of the 25-kDa product with trypsin, an internal peptide sequence was determined to be HLTHYGIYLGDNR. Neither peptide sequences were found homologous to any known protein previously deposited into the GenBank sequence database.

Molecular Cloning of LRAT—A 189-bp DNA fragment encoding a portion of the putative LRAT was generated by RT-PCR of poly(A) RNA from human RPE cells and used as a probe to screen a human RPE cDNA library. Five cDNA clones with sizes of 1942 bp (clone-A), 2521 bp (clone-B), 855 bp (clone-C), 481 bp (clone D), and 824 bp (clone E) were isolated. Further characterization was performed on these clones by sequence analysis. The overlapping nucleotide sequence of clones A and B spanned 2718 bp (Fig. 3, panel A). This sequence contained 92 nucleotides of 5'-untranslated region (UTR) and an open reading frame of 690 bp encoding for a deduced 230-amino acid polypeptide (Fig. 3, panel B) with a calculated mass of 25.3 kDa. The 3'-UTR contained 1936 nucleotides which included a poly(A) tail of 20 residues (GenBank accession number AF071510). The methionine initiation codon ATG at nucleotides (+1 to +3) is presumed to initiate the transcription process of the LRAT protein and is in reasonable agreement with consensus sequences for the translation initiation site of eukaryotic mRNAs (30). The amino acid sequence predicts a potential glycosylation site, N-X-T at position 21–23. Whether this glycosylation process occurs in vivo has not been experimentally determined. Collectively, clones C, D, and E spanned part of the coding sequence starting at position 392, with respect to the first nucleotide of the ATG initiation codon through the 1335 position on the 3'-UTR. An atypical polyadenylation signal ATTAAA was localized 20 nucleotides upstream from the poly(A) tail. Other polyadenylation signals were also found at positions 1487–1492 and 1839–1844. The entire nucleotide sequence and amino acid sequence encoding for LRAT reported in this study was compared with sequences deposited in the GenBank data base. The only high homology observed was with partial nucleotide sequences of two unidentified EST clones with accession numbers AA243120 and W90617 deposited in GenBank by the IMAGE Consortium which, respectively, mapped at positions 1–177 and 1446–1850 in the LRAT nucleotide sequence.

Northern Blot Analysis—The cDNA clone B was labeled with [α-32P]dCTP by nick translation and used as a probe to analyze the tissue distribution of RNA species encoding for LRAT in different human tissues (Fig. 4). A major RNA transcript of 5.0 kb was observed in several tissues, particularly in tissues known for their high vitamin A processing activity. In fetal tissues, the specific message was expressed in RPE, liver, and barely in brain. In adult tissues, the highest level of expression was observed in testis and liver, followed by the RPE, small intestine, prostate, pancreas, colon, and low expression in brain. When using clone B as a probe, additional smaller messages which could represent polyadenylation variants, were also detected in these tissues and others such as adult skeletal muscle, spleen, thymus, and uterus. A 470-bp DNA fragment corresponding to position 1996–2465 near the end of the LRAT 3'-UTR nucleotide sequence hybridized exclusively with the 5.0-kb message (data not shown), eliminating the lower molecular weight bands and suggesting that they represent polyadenylation variants.

Western Blot Analysis of the LRAT Protein—The specificity of a polyclonal antiserum raised in rabbits against a mixture of two LRAT peptides was tested by Western blot analysis (Fig. 5). As shown in lane 1, this antiserum reacted specifically with the 25-kDa LRAT protein.
a single protein band of about 25–26 kDa in human RPE cells. The same band was also observed in HEK-293T cells transfected with LRAT cDNA (lane 4, arrowhead). In contrast, this product was clearly absent in cells that were nontransfected or transfected with the empty plasmid (lanes 2 and 3, respectively). This result was in agreement with the molecular mass for LRAT obtained by affinity labeling experiments and the calculated mass of the amino acid polypeptide deduced from the cDNA sequence. The antiserum also cross-reacted nonspecifically with a HEK-293T kidney protein which is not present in RPE.

Identification of the Reaction Product Formed by HEK-293 Cells Transfected with LRAT cDNA following Addition of \( ^{3} \text{H} \)-All-trans-Retinol—Upon addition of \( ^{3} \text{H} \)-all-trans-retinol, membranes from HEK-293 cells transfected with LRAT cDNA formed a product that eluted between 2 and 3 min after loading (Fig. 6, peak 1). This elution time is characteristic for all-trans-retinyl palmitate (Sigma) under the conditions described under “Experimental Procedures.” The radioactive peak also coeluted with authentic, nonradioactive all-trans-retinyl palmitate. The only other product found in the same analysis was \( ^{3} \text{H} \)-all-trans-retinol (Fig. 6, peak 2) that had not yet been converted by the membranes into retinyl palmitate after 1 h incubation. The substance suspected to be all-trans-retinyl palmitate (peak 1) was collected in subsequent runs with higher amounts of material, spiked with cold all-trans-retinyl palmitate (Sigma) and

![Image](50x242 to 554x729)

**Fig. 3. Analysis of human LRAT cDNAs, nucleotide sequence, and predicted primary structure of LRAT.** A, schematic representation of the human RPE cDNA clones A and B spanning 2718 nucleotides encoding for LRAT protein. The hatched box represents the open reading frame of LRAT. The position of each cDNA clone with respect to the open reading frame is indicated. B, 5′-UTR nucleotide sequence is indicated as 5′ position. The 11 position represents the first nucleotide of the start codon ATG. The deduced amino acid sequence shown under the nucleotide sequence is indicated by the one-letter code. A potential N-linked glycosylation site is indicated by an asterisk. The 3′-UTR contains a consensus polyadenylation signal at positions 1457–1492 and 1839–1844 and an atypical signal shown in bold is found 21 nucleotides upstream from the poly(A) tail. Peptide sequences obtained by microsequencing are underlined.
subjected to I<sub>2</sub>-catalyzed isomerization.

Spectrophotometric analysis at 313 nm of the I<sub>2</sub>-catalyzed isomerization reaction in hexane lead to an isomeric mixture containing predominately trans and 13-cis, as well as a small amount of 9-cis and traces of 9,13-cis-retinyl palmitate (Fig. 7A). This finding was in good accordance with the results described earlier for the I<sub>2</sub>-catalyzed isomerization of all-trans-retinyl palmitate in heptane (29). The reaction product formed by LRAT-transfected cell membranes following addition of \[^{[3H]}\text{all-trans-retinol}\] coeluted with all-trans-retinyl palmitate (Fig. 7B). In the same manner, the I<sub>2</sub>-catalyzed isomerization products of this radioactive entity yielded an isomeric mixture identical to that of the added cold all-trans-retinyl palmitate.
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The two additional peaks eluting between 13-cis and 9,13-cis-retinyl palmitate and after all-trans-retinyl palmitate are probably 13-cis-retinyl stearate and all-trans-retinyl stearate, respectively (28). Therefore, added radioactive all-trans-retinol can be transformed into all-trans-retinyl ester in an apparently LRAT-dependent process.

Analysis of All-trans-retinyl Ester Formation by Membranes from HEK-293 Cells Transfected with LRAT cDNA—The previously described experiments showed that transfected HEK-293 cell membranes exhibit LRAT activity. The relative amounts of retinyl ester formed (as percent of total retinoids) was analyzed for membranes from nontransfected HEK-293 cells and cells transfected with empty plasmid, and cells transfected with LRAT plasmid. The precise amount of specific LRAT protein contained in each sample analyzed is unknown, therefore only relative enzymatic activity is reported. Each group of transfected cells collected at 24, 48, and 72 h after transfection showed similar levels of activity, therefore average data are presented. Nontransfected cells showed a considerably lower apparent LRAT activity. After 15 min and 1 h incubation time, 22.5 and 38.1% of retinyl ester was generated, respectively, by these cells. Cells transfected with empty plasmid showed no LRAT activity within the first 15 min, probably due to a competitive expression of endogenous cell proteins and exogenous proteins such as neomycin induced by the vector itself. However, after 1-h reaction time, these cells generated 21.4% of retinyl ester. Interestingly, LRAT-transfected cells formed 87.1% of retinyl ester within the first 15 min, and 91.6% within 1 h. Apparent LRAT activity was about 4 times higher in cells transfected with LRAT plasmid than in cells transfected with empty plasmid (at 1-h reaction time). Compared with nontransfected cells, the LRAT-transfected cells showed an increase in apparent LRAT activity of 4-fold after 15 min, and 2-fold after 1 h. The relatively high ester synthetase activity in nontransfected cells was surprising given the fact that HEK-293 cells did not have detectable transcripts for LRAT by RT-PCR and Northern blot analysis and expression of LRAT message was only induced in cells transfected with LRAT cDNA (data not shown). One possible explanation for this finding is that the retinyl ester forming activity was not due to LRAT, but rather to the activity of other retinyl ester-forming enzymes. To test this hypothesis, HEK-293 membranes were sedimented in 1 ml of buffer (100 mM Tris-Cl, pH 8.0, 100,000 × g, 30 min, 4 °C) to remove soluble, cytosolic retinyl ester synthetase activity. Washed membranes were tested for LRAT activity as described above. No retinyl ester formation was observed after a 15-min reaction time in either nontransfected cells or cells transfected with empty plasmid. After 1 h incubation, washed membranes of nontransfected cells formed 7% retinyl esters, whereas cells transfected with empty plasmid generated 3% retinyl esters. In contrast, LRAT-transfected cells generated 53% retinyl esters during the same incubation time. Combined, these data suggest that specific LRAT activity is present only in the LRAT transfected cells and absent in nontransfected cells and cells transfected with empty plasmid.

**DISCUSSION**

In the present study, we describe for the first time the deduced amino acid sequence of LRAT, an essential enzyme in the processing of vitamin A (all-trans-retinol) into 11-cis-retinoids in the RPE and for transport and processing of retinoids in other tissues. LRAT has been exceedingly difficult to purify to homogeneity and consequently its primary structure and molecular identification has been lacking. As is typical of many membrane-bound enzymes, enzyme denaturation occurs as purification proceeds. Therefore, indirect biochemical means were sought to clearly identify this important enzyme as an initial step toward its further molecular characterization.

Affinity labeling experiments were performed by using the LRAT specific affinity labeling agent doxycycloromethyl ketone coupled to biotinylated BACMK. Previously, radioactive RBA has been shown to label a protein with an apparent mass of 25 kDa (20). In agreement with this report, BACMK staining of the BACMK affinity labeled product also showed a 25-kDa protein as the labeled entity. The fact that the same polypeptide band was shown to be labeled by both BACMK and RBA affinity labeling agents provided stronger evidence that the LRAT protein was identified and has a mass of 25 kDa.

Additional evidence for the identification of LRAT was supported by the sequence analysis of cDNA clones obtained from a human RPE cDNA library. Encompassing nucleotide sequences of the two major clones indicated an open reading frame of 690 bp encoding for a 230-amino acid polypeptide with a calculated mass of 25.3 kDa, in close agreement with the protein size obtained by the affinity labeling procedures. In addition, the sequences of both bovine N-terminal and internal peptide data from the biochemical characterization were present within the human LRAT polypeptide, confirming the correlation of both peptides with the deduced amino acid sequence from the cDNAs. Amino acid variations were seen particularly in the N-terminal peptide residues which are probably due to species differences since the peptide sequences were derived from bovine tissue. A hydroxyl analysis (31) of the LRAT amino acid sequence suggests 2 possible transmembrane regions within the protein at positions 9–31 and 195–222 (http://ulrec3.unil.ch/software/TMPRED). Thus, within the endoplasmic reticulum where the protein is presumed to be located, the N- and C-terminal would be found in the same luminal (extracellular) orientation. It is therefore hypothesized that the active site for LRAT would be localized somewhere in the putative cytotoxic loop formed by residues 31–195 which includes cysteine residues at position 161, 168, and 182.

A nucleotide and amino acid comparison with sequences deposited in GenBank showed no homology with any previously described protein. Surprisingly, when LRAT was compared with sequences encoding for other closely related acyltransferases such as the 60-kDa soluble protein lecithin:cholesterol acyltransferase (32, 33) low homology was found in the N-terminal peptide residues which are probably due to species differences since the peptide sequences were derived from bovine tissue. A hydroxyl analysis (31) of the LRAT amino acid sequence suggests 2 possible transmembrane regions within the protein at positions 9–31 and 195–222 (http://ulrec3.unil.ch/software/TMPRED). Thus, within the endoplasmic reticulum where the protein is presumed to be located, the N- and C-terminal would be found in the same luminal (extracellular) orientation. It is therefore hypothesized that the active site for LRAT would be localized somewhere in the putative cytotoxic loop formed by residues 31–195 which includes cysteine residues at position 161, 168, and 182.

A nucleotide and amino acid comparison with sequences deposited in GenBank showed no homology with any previously described protein. Surprisingly, when LRAT was compared with sequences encoding for other closely related acyltransferases such as the 60-kDa soluble protein lecithin:cholesterol acyltransferase (32, 33) low homology was found and no obvious sequence to account for a possible common active site was identified. Although it is known that both acyltransferases remove acyl groups from lecithin to esterify their respective substrates, retinol for LRAT and cholesterol for lecithin:cholesterol acyltransferase, it is possible that each process occurs in a mechanistically different manner. In fact, LRAT has regiospecificity for acyl groups at position sn-1, whereas lecithin:cholesterol acyltransferase possesses regiospecificity for acyl groups at position sn-2 of membrane phospholipids (11, 22, 34, 35).

Northern blot analysis suggested a major 5.0-kb mRNA for LRAT. The overlapping sequences of the two major cDNA clones contain 2718 bp of this message. The nucleotide sequence required to encompass the remainder of the 5.0-kb message remains to be resolved. The existence of alternative
polyadenylation sites in the 3′-UTR or in the initiation of transcription sites at the 5′-UTR could account for the variation in the size of the observed species in the Northern blots. Our current analysis of genomic clones for the gene characterization of LRAT will eventually help to elucidate these issues. Interestingly, Fig. 4 also shows an apparent higher level of expression of LRAT RNA transcripts in adult tissues compared with fetal stages. If the levels of mRNA in these tissues have a direct correlation with higher protein synthesis and therefore higher enzymatic activity, it is possible that an increased demand for esterification is required at the adult stage of development.

In summary, evidence that the deduced amino acid sequence corresponds to LRAT is provided by several techniques. Western blot detection of a 25-kDa protein in native RPE cells and LRAT-transfected cells is in agreement with data obtained by affinity labeling of the protein. The ability of HEK-293 cells transfected with LRAT cDNA to convert retinol into retinyl palmitate (Figs. 6 and 7) provides additional compelling evidence. Although it is known that, physiologically, LRAT uses as a substrate retinol bound to BSA, a method whose efficacy has been well established in earlier studies (12, 13, 16). Chemical proof on the identification of trans-retinyl esters by the LRAT-transfected cells comes from chemical transformation. The expected retinyl ester isomerization of LRAT will eventually help to elucidate these issues.

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