Recombinant lecithin:cholesterol acyltransferase containing a Thr_{123}→Ile mutation esterifies cholesterol in low density lipoprotein but not in high density lipoprotein

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Abstract Fish-eye disease is a rare genetic disorder of high density lipoprotein (HDL) metabolism that is characterized biochemically by a partial deficiency of the enzyme lecithin:cholesterol acyltransferase (LCAT). One of the mutations that is causative for fish-eye disease occurs at codon 123 of the LCAT gene. This mutation results in the exchange of a threonine residue for an isoleucine in the LCAT protein (Thr_{123}→Ile). In order to understand the functional significance of this exchange, we have used site-directed mutagenesis to reconstruct this mutation in an LCAT cDNA followed by expression of the mutant LCAT in COS-1 cells. The fish-eye disease mutation resulted in a 50% decrease in LCAT mass in the culture medium compared to wild type enzyme. The secreted mutant protein was incapable of esterifying cholesterol in HDL and HDL analogues. However, this protein retained the ability to esterify cholesterol in plasma and low density lipoprotein. These results support the hypothesis that this mutation is responsible for biochemical abnormalities of LCAT observed in fish-eye disease and the mutant LCAT protein has lost the potential to esterify cholesterol in the HDL pool but retains the ability to esterify cholesterol from other lipoproteins.—**O, K., J. S. Hill, X. Wang, and P. H. Pritchard.** Recombinant lecithin:cholesterol acyltransferase containing a Thr_{123}→Ile mutation esterifies cholesterol in low density lipoprotein but not in high density lipoprotein. *J. Lipid Res.* 1993. **34:** 81–88.

Supplementary key words transient transfection • site-directed mutagenesis • recombinant LCAT • cholesterol esterification

Lecithin:cholesterol acyltransferase (LCAT, phosphatidylcholine:sterol O-acyltransferase, EC 2.3.1.43) is the enzyme responsible for the formation of cholesteryl ester in plasma via transfer of the *sn*-2 fatty acid from phosphatidylcholine to the 3-hydroxyl group of cholesterol (1). A great deal of information on the biochemistry and pathophysiology of this enzyme is now available and it has been the topic of numerous reviews (2, 3). Furthermore, studies on patients with familial LCAT deficiency have clearly illustrated the central role that this enzyme plays in plasma cholesterol homeostasis (4).

LCAT is a glycoprotein with an apparent molecular mass of 67,000 daltons and the human enzyme has been purified to homogeneity (2, 5). The substrates of LCAT are primarily high density lipoproteins (HDL) (2, 6, 7) but several investigators have provided evidence that LCAT may act directly on the lower density lipoproteins (8, 9). The major structural protein of HDL, apolipoprotein A-I, is believed to be the principle activator of LCAT (3). The gene for the human LCAT has been sequenced and is made up of 6 exons and 5 introns. LCAT mRNA contains 1550 nucleotides and is expressed primarily in the liver (10, 11). Mature LCAT protein contains 416 amino acids and 25% of total LCAT mass is carbohydrate that is covalently linked to four potential N-glycosylation sites (3, 5).

A number of mutations in the LCAT gene have been demonstrated to be causative for familial LCAT deficiency. These include mutations at codons 10, 141, 147, 228, and 293 (12–15). A variant of familial LCAT deficiency was recently reported by Funke et al. (16) in collaboration with our laboratory. It was found that a molecular defect at codon 123, which resulted in the exchange of a threonine (Thr) residue for an isoleucine (Ile) residue, was the defect underlying this familial partial LCAT deficiency (also known as fish-eye disease) in homozygotes from two unrelated families. Fish-eye disease is an unusual disorder because the apparent loss of LCAT activity does not result in a marked increase in the ratio of cholesterol to cholesteryl ester in plasma (16–19). It has been postulated that partial familial LCAT deficiency seen in fish-eye disease is caused by a defective

Abbreviations: HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein; LCAT, lecithin:cholesterol acyltransferase; PCR, polymerase chain reaction; TBS, Tris-buffered saline.

LCAT that has lost the ability to esterify cholesterol in the HDL pool but retains its activity on cholesterol in very low density lipoprotein (VLDL) and low density lipoprotein (LDL) (16, 18). Carlson and Holmquist (18) have suggested that there are two different LCAT activities present in normal plasma. One of these activities, denoted α-LCAT, is proposed to be specific for HDL and the other, β-LCAT, is specific for LDL and VLDL. Thus, they have suggested that fish-eye disease is caused by the lack of α-LCAT activity in plasma (18). In order to further investigate this hypothesis, we have recreated the C to T substitution in codon 123 of an LCAT cDNA and expressed this mutant LCAT (Thr123-Ile) in COS-1 monkey kidney cells. The secreted mutant LCAT protein exhibited a loss of the activity against HDL as substrate which mimics the fish-eye disease phenotype.

MATERIALS AND METHODS

Mutagenesis of LCAT cDNA

The natural mutation at codon 123 of LCAT is a substitution of thymidine for cytosine which causes an amino acid change from Thr to Ile. We introduced this mutation into the LCAT cDNA (a gift from Dr. J. McLean, Genentech, San Francisco, CA) using oligonucleotide-directed mutagenesis. The mutagenic oligonucleotide carried a mismatched base for substitution of Thr_{123} to Ile (5'-CCTGCACATACTGGTGC-3'). The full-length wild type LCAT cDNA in pUC19 was used as a template for the polymerase chain reaction (PCR). First round amplification with PCR was carried out by using 10 pg of template DNA, 100 pmol of a mutagenic oligonucleotide as a forward primer, and 100 pmol of the reverse pUC primer which hybridizes to the vector downstream from the LCAT cDNA sequence. PCR was carried out for 30 cycles, with step cycles of 95°C for 30 sec, 55°C for 30 sec, and 74°C for 60 sec. After the thirtieth cycle, the reaction was extended at 74°C for 5 min. The PCR product was purified by electrophoresis in a 0.7% agarose gel. A second round of PCR contained 20 pg of the cDNA LCAT template in pUC19, 10 pmol of universal primer, and the purified first round amplification product as the other primer. PCR conditions were the same as that for the first round, except that the extension time for each cycle was increased to 90 sec. The amplified DNA was purified and digested with restriction enzymes KpnI and PstI to generate a 527 bp fragment encompassing the 123 mutation. This fragment was inserted as a cassette in the wild type LCAT cDNA in pUC19 vector. DNA sequencing was performed to identify a clone that contained the desired mutation at codon 123 but no other mutations. The LCAT cDNA from this clone was cut out with restriction enzymes XhoI and BamHI and subcloned into the mammalian expression vector pNUT (20, 21). Prior to the transfection experiments, the final construct was sequenced to confirm that it contained the 123 mutation.

Transient transfection of COS cells

COS-1 monkey kidney cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) (GIBCO BRL Canada). Subconfluent COS-1 cell monolayers were washed twice with transfection buffer (25 mM Tris-HCl, pH 7.4, 140 mM NaCl, 1 mM $CaCl_2$, 3 mM KCl, 0.5 mM $MgCl_2$, 0.9 mM Na_2HPO_4). The expression vector pNUT containing either wild type or mutant LCAT cDNA was transfected into COS-1 cells by DEAE-dextran transfection. After incubation of the cells at 37°C for 30 min, the DNA solution was removed and replaced with DMEM containing 10% FBS and 80 μM chloroquine. After a 3-h incubation at 37°C, transfection was completed by a 3-min incubation with DMEM containing 10% DMSO at room temperature. After washing with transfection buffer, the cells were incubated in DMEM containing 10% FBS for 12 h and subsequently in serum-free medium (OptiMEM, GIBCO BRL Canada) for 48 h.

Radiolabeling and immunoprecipitation of recombinant LCAT

After 48 h of incubation in DMEM containing 10% FBS, transfected COS-1 cells were incubated in methionine-free DMEM (DMEM-Met) for 20 min at 37°C to deplete the methionine pool. The cells were then incubated for 30 min in DMEM-Met supplemented with 100–200 μCi/ml [^{35}S]methionine (700 Ci/mmol, New England Nuclear) followed by incubating in DMEM with 10% FBS. After 4 h incubation, the medium was collected and the cellular protein was harvested in lysis buffer (50 mM Tris-HCl, pH 8.0, 62.5 mM EDTA, 1% Nonidet P40, 0.4% sodium deoxycholate, 1 mM phenylmethylsulfonylfluoride).

Solid phase immunoprecipitation was performed to detect the presence of LCAT in the culture medium and the cellular lysate. Polyclonal goat anti-human LCAT antibodies (kindly provided by Dr. A. Lacko, Texas College of Osteopathic Medicine, Fort Worth) were pre-adsorbed onto agarose-immobilized protein G (GammaBind G Agarose, Genex Corporation, Gaithersburg, MD) for 30 min at 4°C. The LCAT antibodies were specific for LCAT protein in the culture medium as determined by Western blot analysis of SDS PAGE gels. An aliquot of medium or cell lysate was added to the antibody-protein G-agarose suspension and the mixture was rotated overnight at 4°C. Agarose beads were pelleted by centrifugation and then washed twice with 1 ml of Tris-buffered saline (TBS) containing 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% sodium azide. The pellet was resuspended in the buffer containing 0.1 M Tris-HCl, pH 6.8, 2% SDS, 40%

glycerol. The adsorbed materials were then eluted from the agarose beads by heating at 90°C for 10 min. The agarose beads were removed by centrifugation. The supernatant was subjected to electrophoresis on SDS-polyacrylamide gels (10%) with ^{14}C-labeled methylated protein standards (Amersham Canada Ltd., Oakville, Ontario) as molecular weight markers. After electrophoresis, the gels were agitated in 25% methanol–5% acetic acid for 30 min, then in Amplify (Amersham Canada Ltd.) for 15 min. The gels were dried and autoradiography was performed with Kodak X-Omat AR film (Eastman Kodak) at −70°C for 12–24 h.

Determination of LCAT mass

The mass of secreted LCAT protein was determined by solid-phase immunoassay. The aliquots of culture media and the purified human LCAT standard (kindly provided by Dr. A. Lacko) were applied to nitrocellulose membrane (Schleicher & Schuell, Keene, NH) in a Bio-Dot SF apparatus (Bio-Rad, Richmond, CA). Culture medium from cells transfected with plasmid DNA that did not contain the LCAT cDNA insert was used as a control. The membrane was blocked with 5% non-fat dry milk in TBS for 30 min at 37°C. The bound protein was allowed to react with polyclonal goat anti-human LCAT antibodies by incubating the membrane overnight in antibody solution. After washing three times with TBS containing 0.02% Tween 20, the membrane was incubated with protein G conjugated to horseradish peroxidase (GammaBind-G-HRP, Genex Corp.) for 60 min. The color was developed in a solution containing 25 mg diaminobenzidine (Sigma Chemical Co.), 15 mg $CoCl_2$, and 0.010 ml of 30% H_2O_2 in 50 ml TBS. The quantitation of LCAT protein was carried out by scanning the membrane with a Bio-Rad Model 620 Video Densitometer. The LCAT mass was proportional to absorbance up to 80 ng of protein. The interassay coefficient of variation was 7.3% for a single mass measurement in nine separate assays.

Preparation of plasma and lipoproteins

Blood was collected from normal volunteers after 12 h fasting and plasma was prepared by low speed centrifugation (1,200 *g*, 20 min). The different lipoproteins were isolated by preparative ultracentrifugation using lipoprotein fractions defined by their densities: $1.006 < d < 1.063$ g/ml for LDL and $1.063 < d < 1.21$ g/ml for HDL (22). Total lipoprotein-depleted plasma was prepared by ultracentrifugation at density 1.21 g/ml. In some experiments, the infranatant (inf. $d > 1.21$ g/ml) was recombined with the isolated LDL fraction. All lipoprotein preparations were dialyzed extensively at 4°C against 0.01 M Tris-HCl (pH 7.4) containing 0.15 M NaCl and 0.005 M EDTA. The plasma, HDL fraction, and LDL + inf. $d > 1.21$ g/ml were heat-inactivated at 56°C for 30 min to eliminate LCAT activity associated with those preparations. The heat-inactivation procedure has been carried out in a number of laboratories to elucidate the catalytic defects in LCAT protein (9, 23) and has been recently shown not to disturb the gradient gel pattern of HDL compared to a native preparation (23). There was no measurable LCAT activity associated with the isolated LDL fraction. The concentration of unesterified cholesterol in plasma and lipoproteins was determined enzymatically by a reagent kit (Boehringer-Mannheim GmbH).

Determination of LCAT activity and cholesterol esterification rates

The enzyme activities of wild-type and mutant LCAT gene products were determined using a proteoliposome as a substrate. The egg yolk phosphatidylcholine:cholesterol liposome was prepared by ethanol injection according to Batzri and Korn (24). The substrate mixture containing 4.66 nmol [^{3}H]cholesterol (0.03 μCi/nmol), 18.46 nmol phosphatidylcholine, and 7.5 μg purified human apoA-I in 10 mM Tris-HCl (pH 7.4)–150 mM NaCl–5 mM EDTA was preincubated at 37°C for 30 min. Subsequently, 5 mM β-mecaptoethanol and 1.5% bovine serum albumin (essentially fatty acid-free) were added to the substrate mixture. The reaction was initiated by the addition of 100–200 μl cell culture medium (containing 0.08–0.18 μg/ml LCAT protein) to a final volume of 0.3 ml. The reaction was carried out at 37°C for 2 h and was terminated by the addition of 4 ml of chloroform–methanol 2:1 (v/v) and water was added to achieve phase separation. Unesterified cholesterol and cholesteryl ester in the organic phase were separated by thin-layer chromatography (Sil G 60F-254, BDH Inc. B.C.) with the solvent system petroleum ether–diethyl ether–acetic acid 70:12:1 (v/v). The radioactivity associated with cholesterol and cholesteryl ester was determined by liquid scintillation counting.

Radiolabeled plasma or lipoprotein was prepared by equilibration with [^{3}H]cholesterol at 4°C as described by Dobiasova and Schutzova (25). An aliquot of heat-inactivated substrate (plasma, HDL fraction, LDL + inf. $d > 1.21$ g/ml) or isolated LDL was added to a pre-cooled test tube containing [^{3}H]cholesterol-labeled filter paper discs and the mixture was incubated at 4°C for 20 h. The labeled plasma or lipoprotein fractions were incubated with recombinant LCAT and the rates of [^{3}H]cholesterol esterification in plasma and lipoproteins were determined. Briefly, an aliquot (0.2–0.3 ml) of cell culture medium containing LCAT protein (0.025 μg) was added to a mixture containing labeled plasma or HDL or LDL or LDL + inf. $d > 1.21$ g/ml, 5 mM β-mercaptoethanol, and 1.5% bovine serum albumin (essentially fatty acid-free) to a final volume of 0.40 ml. The reaction mixture

was incubated at 37°C for 1–6 h. The reaction was terminated by adding 2 ml of ethanol. After the mixture was centrifuged at 2000 rpm for 10 min, the cholesterol and cholesteryl ester in the supernatant were separated by thin-layer chromatography (Sil G 60F-254, BDH Inc. B.C.). The results were expressed as nmoles of free cholesterol esterified per h per μg LCAT protein which refers to the rate of cholesteryl ester formation in plasma or isolated lipoprotein fraction catalyzed by the recombinant enzyme.

RESULTS

Secretion of wild type and mutant LCAT by transiently transfected COS-1 cells

In the present study, oligonucleotide-directed mutagenesis was used to introduce an amino acid substitution (Thr_{123}→Ile) into a human LCAT cDNA. This mutant LCAT cDNA and the wild type LCAT cDNA were independently inserted into the expression vector pNUT. After transfection of the constructs into COS-1 cells, synthesis and secretion of mutant and wild type LCAT were studied by a pulse-chase experiment with [^{35}S]methionine. As depicted in **Fig. 1,** COS cells transiently transfected with wild type or mutant LCAT cDNA secreted a major protein with an apparent molecular mass of about 67,000 daltons which was comparable to fully glycosylated plasma LCAT. This LCAT protein was not seen in the culture medium isolated from the cells transfected with vector DNA which did not contain the LCAT cDNA insert (control). In some experiments, a faint band of immunoprecipitable protein with a lower molecular mass (47,000 daltons) was observed in the media. However, this band also appeared in the medium from the cells transfected with the empty expression vector. Furthermore, an equivalent band was observed when irrelevant antibodies were used for the immunoprecipitation. Thus, it appears that the 47,000-dalton protein does not originate from the LCAT cDNA. Immunoprecipitation of the cell lysate revealed a specific protein band with a molecular mass lower than the mature LCAT (about 52,000 daltons) in all experiments except the cells transfected with the expression vector DNA alone (Fig. 1). This might represent a partially glycosylated form of LCAT that accumulates in the transfected cells prior to its final glycosylation and secretion. In addition, a specific band of immunoprecipitable protein with a molecular mass of about 32,000 daltons was observed in the cells transfected with the vector containing the mutant LCAT cDNA. We speculate that this may be a product of degraded mutant LCAT protein.

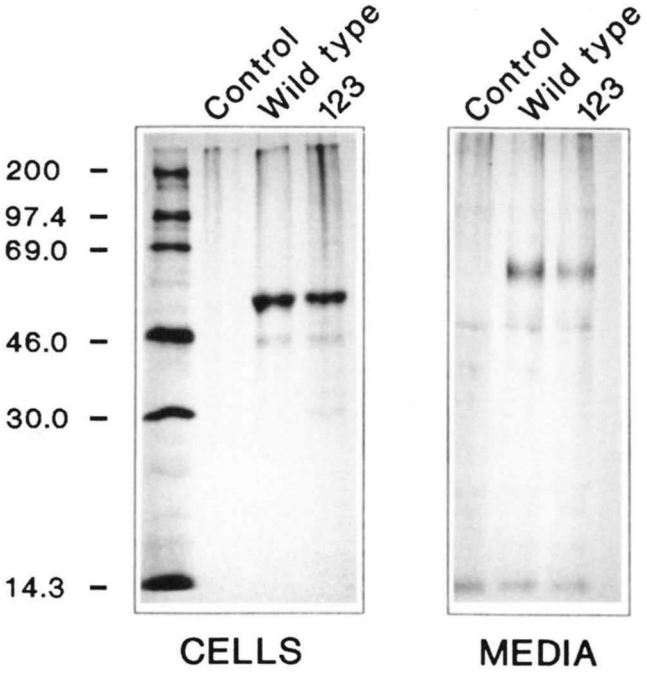

Fig. 1. Immunoprecipitation of wild type and mutant LCAT. The transfected COS-1 cells were pulse-labeled with [^{35}S]methionine (200 μCi/ml) for 30 min followed by a chase incubation with nonradiolabeled methionine for 4 h. Subsequently, proteins in the culture medium and cells were immunoprecipitated with polyclonal anti-human LCAT antibodies and electrophoresed in 10% SDS polyacrylamide gels. The radiolabeled proteins of wild type and mutant LCAT were visualized by autoradiography. Migration position and size (in kilodalton) of protein standards are indicated.

TABLE 1. Protein mass and enzyme activity of LCAT secreted by COS cells

	Wild Type	Thr_{123}-Ile
Protein mass (μg/ml)	0.19 ± 0.02 (6)	0.08 ± 0.01 (6)
LCAT activity (nmol/h/ml)	2.49 ± 0.26 (6)	0.017 ± 0.006 (6)
Specific activity (nmol/h/μg)	12.49 ± 0.88 (6)	0.03 ± 0.008 (6)

After a 48-h incubation of transfected COS-1 cells in serum free medium, the culture medium was analyzed for protein mass and activity of wild type and mutant LCAT (Thr^{123}-Ile) as described in Materials and Methods. The results are depicted as mean ± standard deviation (number of experiments). Control experiments in which the cells were transfected with vector containing no insert were also carried out. In all cases, no LCAT activity or LCAT mass could be detected.

To quantify the amount of LCAT in the culture medium, we determined the mass of the secreted LCAT protein using polyclonal antibodies specific for human LCAT. As shown in **Table 1,** after a 48-h incubation in serum-free medium, the transfected COS cells secreted an average mass of LCAT of 0.19 μg/ml for wild type and 0.08 μg/ml for mutant LCAT.

The enzyme activity of the secreted LCAT protein was determined with a proteoliposome containing [^{3}H] cholesterol–egg phosphatidylcholine–apolipoprotein A-I. As shown in Table 1, the specific activity of the mutant LCAT was less than 1% of that observed for the wild type enzyme. There was no detectable LCAT activity or mass in the culture medium collected from the cells transfected

with the vector DNA alone. These results indicate that the transient transfection of COS-1 cells with an LCAT cDNA is an appropriate model system for the study of secretion and enzyme function of recombinant human LCAT.

Lipoprotein substrate specificity of wild type and mutant LCAT

Our next objective was to examine the ability of recombinant LCAT to esterify cholesterol in human plasma. When radiolabeled heat-inactivated plasma (40 nmol cholesterol/ml) was incubated with culture medium containing either wild type or mutant LCAT, cholesterol esterification rate was linear during 1–6 h of incubation (**Fig. 2A**). After 6 h incubation, the cholesterol esterification rate catalyzed by mutant LCAT was 35% of that obtained with wild type enzyme (Fig. 2A). The dependence of LCAT activity on the amount of unesterified cholesterol in plasma was also investigated. When the rate of cholesterol esterification was determined with various amounts of plasma cholesterol (15–80 nmol/ml plasma), the activities of both wild type and mutant LCAT increased in a substrate-dependent manner (Fig. 2B).

The major purpose of this study was to examine the differences between wild type and mutant LCAT with respect to the utilization of different lipoprotein classes. This was achieved by incubating the secreted recombinant LCAT protein with isolated lipoprotein fractions. [^{3}H]cholesterol was pre-equilibrated with lipoproteins and the esterification of cholesterol catalyzed by recombinant LCAT was determined. When radiolabeled heat-inactivated HDL fraction was used as substrate, the reaction catalyzed by wild type LCAT was linear up to 6 h of incubation (**Fig. 3A**). The highest activity was obtained when 22 nmol/ml HDL cholesterol was present in the assay mixture (Fig. 3B). However, there was no detectible [^{3}H]cholesterol esterification in HDL when mutant LCAT was used as an enzyme source (Fig. 3A, B).

Although the HDL fraction is believed to serve as the major substrate for LCAT, a small proportion of cholesterol esterification catalyzed by LCAT has been found in association with LDL (9, 26, 27). The data described above demonstrated that the mutant LCAT was able to esterify free cholesterol in plasma but not in HDL or HDL analogues. This suggests that LDL, and not HDL, is utilized as a substrate by the mutant enzyme. Hence, we determined the activities of both wild type and mutant LCAT against isolated LDL as substrate. As depicted in **Fig. 4A,** [^{3}H]cholesterol esterification rates in LDL catalyzed by both enzymes were low but measurable after a 3-h incubation. When increased amounts of LDL cholesterol were added to the assay mixture, the activities of both enzymes increased to a similar extent (Fig. 4B). When the LCAT activity was determined using heat-inactivated LDL + inf. d > 1.21 g/ml as a substrate, the

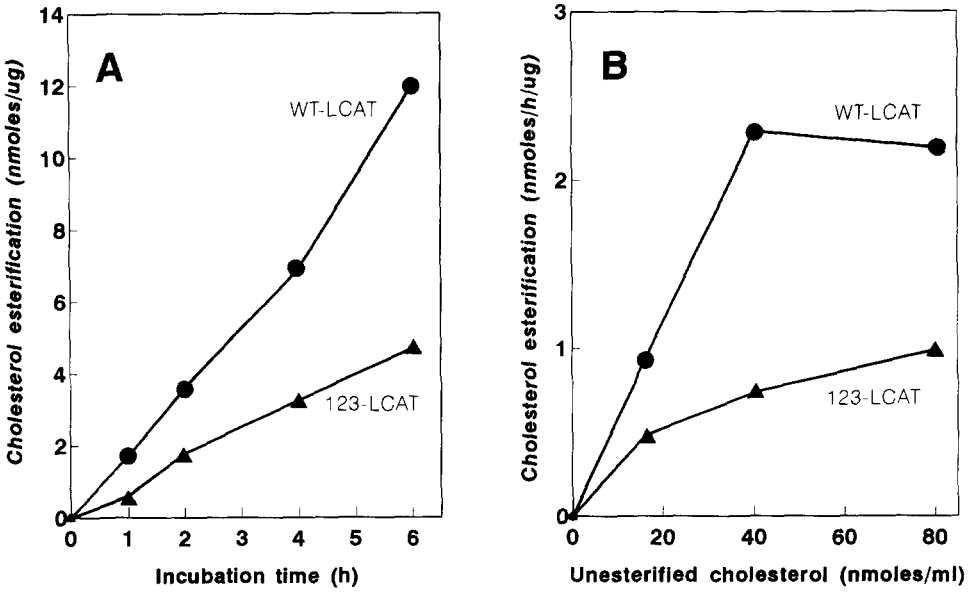

Fig. 2. Cholesterol esterification in plasma catalyzed by wild type and mutant LCAT. A: An aliquot of cell culture medium containing 0.025 μg wild type LCAT (WT-LCAT●) or mutant LCAT (123-LCAT, ▲) was incubated with heat-inactivated plasma pre-equilibrated with [^{3}H]cholesterol (each reaction contained 40 nmol unesterified cholesterol per ml). The incubation was carried out at 37°C for various time periods. Cholesterol esterification rates were determined as described in Materials and Methods and were expressed as nmoles of cholesteryl ester formed per μg LCAT protein. B: An aliquot of cell culture medium containing WT-LCAT or 123-LCAT was incubated with various amounts of heat-inactivated [^{3}H]cholesterol plasma for 5 h at 37°C. The cholesterol esterification rate was determined and expressed as nmoles of cholesteryl ester formed per h per μg LCAT protein.

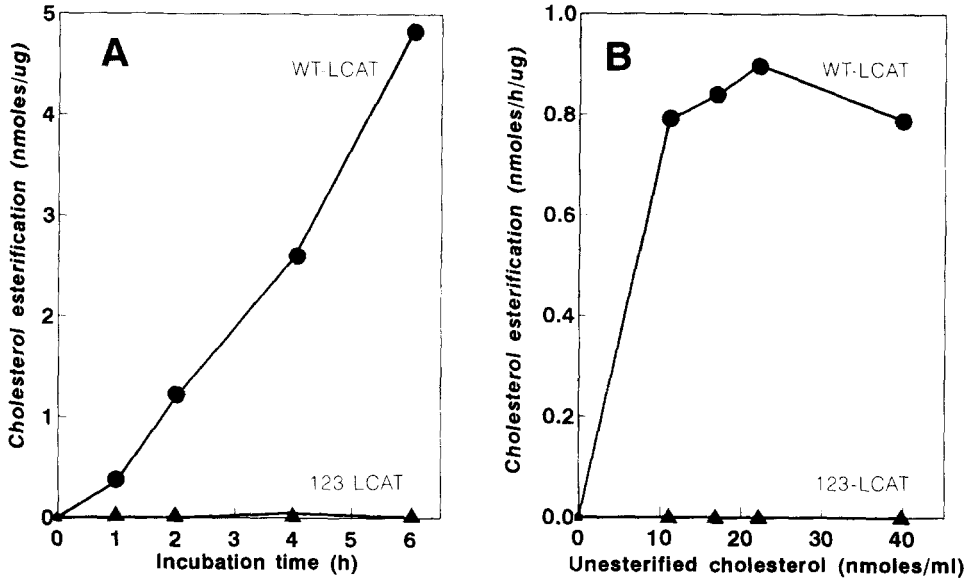

Fig. 3. Cholesterol esterification in the HDL fraction catalyzed by wild type and mutant LCAT. A: An aliquot of cell culture medium containing 0.025 μg wild type (WT-LCAT; ●) or mutant LCAT (123-LCAT; ▲) was incubated with heat-inactivated HDL fraction that was pre-equilibrated with [^{3}H]cholesterol (the reaction mixtures contained 17 nmol unesterified cholesterol per ml) and the incubations were carried out at 37°C for the indicated time periods. Cholesterol esterification rates were determined and expressed as nmoles of cholesteryl ester formed per μg LCAT protein. B: An aliquot of cell culture medium containing WT-LCAT (●) or 123-LCAT (▲) was incubated with various amounts of a heat-inactivated [^{3}H]cholesterol HDL at 37°C for 5 h. Cholesterol esterification rates were determined and expressed as nmoles of cholesteryl ester formed per h per μg LCAT protein.

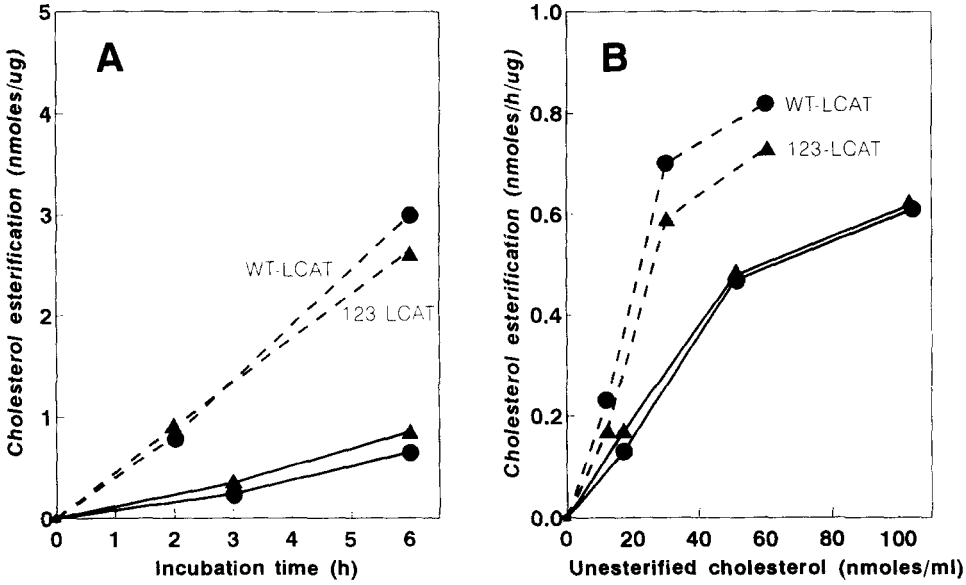

Fig. 4. Cholesterol esterification in LDL and LDL + inf. d > 1.21 g/ml fractions catalyzed by wild type and mutant LCAT. A: An aliquot of cell culture medium containing 0.025 μg wild type (WT-LCAT; ●) or mutant LCAT (123-LCAT; ▲) was incubated with [^{3}H]cholesterol LDL (18 nmol unesterified cholesterol per ml) (solid lines) or heat-inactivated LDL + inf. d > 1.21 g/ml (21 nmol unesterified cholesterol per ml) (dotted lines) at 37°C for various time periods. Cholesterol esterification rates were determined and expressed as nmoles of cholesteryl ester formed per μg LCAT protein. B: An aliquot of cell culture medium containing WT-LCAT or 123-LCAT was incubated with various amounts of labeled LDL or heat-inactivated LDL + inf. d > 1.21 g/ml at 37°C for 5 h. Cholesterol esterification rates were expressed as nmoles of cholesteryl ester formed per h per μg LCAT protein.

rate of cholesterol esterification was increased approximately threefold (Fig. 4A, B). Using either LDL or LDL + inf. d > 1.21 g/ml as substrate, the rates of cholesterol esterification were comparable in the reactions catalyzed by wild type and mutant LCAT. These results are consistent with the notion that the mutant LCAT retains the ability to esterify cholesterol in LDL in vivo.

DISCUSSION

Fish-eye disease is a rare genetic disorder characterized by severe corneal opacities and plasma lipoprotein abnormalities (3, 17, 21). It has been well documented that the cholesteryl ester content in fish-eye disease plasma is low in HDL but normal in LDL and VLDL (17, 18). Recently, a defect in codon 123 of the LCAT gene was identified in four individuals from two unrelated fish-eye disease families (16). Although the LCAT in fish-eye disease plasma was unable to esterify free cholesterol in the exogenous proteoliposome substrate, the endogenous cholesterol esterification rate appeared to be unaffected. In order to resolve this paradox and to determine the functional significance of this mutation, we have expressed and characterized the recombinant LCAT which carries the fish-eye disease mutation (Thr_{123}→Ile). The mutant LCAT secreted from transfected COS-1 cells displayed the same molecular weight as the wild type protein, but its mass in culture medium was about 50% of the wild type enzyme. There was no accumulation of the fully glycosylated LCAT protein detected within the cells from either the mutant or wild type cDNA product. However, the occurrence of a unique immunoprecipitable protein band of approximately 32,000 daltons associated with the fish-eye disease mutation suggests that increased cellular degradation of this mutant protein may occur.

Based on their extensive work on normal and fish-eye disease plasma, Carlson, Holmquist, and colleagues (9, 18) have postulated that there are two different LCAT activities in human plasma: α-LCAT (acting on HDL) and β-LCAT (acting on LDL). Although HDL is the preferred substrate for LCAT, a small proportion of cholesterol esterification takes place on LDL (9, 18, 26, 27). In the present study, we have observed that recombinant LCAT is capable of esterifying HDL and LDL cholesterol. Our results with recombinant LCAT derived from a single human LCAT cDNA clearly indicate that both LCAT activities (α- and β-LCAT) reside on the same protein.

We have also investigated the ability of the recombinant mutant LCAT to esterify cholesterol in native lipoprotein substrates. We found that the substitution of single amino acid residue (Thr_{123}→Ile) abolished the activity of the enzyme to esterify free cholesterol in the HDL fraction. However, this mutant protein retained the ability to esterify cholesterol in whole plasma as well as in isolated LDL. These data agree with the hypothesis of Carlson and Holmquist (18) which suggests that LCAT in fish-eye disease plasma esterifies cholesterol only in lower density lipoproteins (β-LCAT activity) and that its ability to esterify cholesterol in HDL (α-LCAT activity) is lost. In addition, we have observed that adding the inf. d > 1.21 g/ml to LDL results in an increase in the rate of cholesterol esterification catalyzed by recombinant LCAT. The possibility of experimental artifact was excluded by the fact that there was no measurable LCAT activity associated with LDL + inf. d > 1.21 g/ml after heat-inactivation. A similar effect of inf. d > 1.21 g/ml on cholesterol esterification in LDL has been previously reported with human plasma LCAT (26). The exact mechanism, however, by which this stimulation occurs is not known.

The similarity between the properties of the recombinant mutant LCAT and the enzyme in fish-eye disease plasma provide supporting evidence that the mutation at codon 123 (The_{123}→Ile) in the LCAT gene is causative for the biochemical changes seen in this disorder. In addition, these data provide direct experimental evidence that this mutant LCAT is incapable of esterifying cholesterol in HDL. The near normal rates of cholesterol esterification in fish-eye disease plasma (16) are a result of esterification of cholesterol from lower density lipoproteins by the mutant LCAT. The exact mechanism by which this is brought about cannot be determined from the present study.

In summary, this study represents the first report of the recreation and expression of a natural mutation of LCAT that is responsible for the altered specificity of the enzyme in fish-eye disease. The results clearly demonstrate that a mutation at codon 123 of the LCAT gene (Thr_{123}→Ile) is responsible for the loss of activity against HDL as substrate. A complete elucidation of the molecular basis of these observations is a prerequisite if disorders such as fish-eye disease are to yield a major increase in our understanding of the factors that regulate cholesterol metabolism.

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REFERENCES

1. Glomset, J. A., and J. L. Wright. 1964. Some properties of a cholesterol esterifying enzyme in human plasma. *Biochim. Biophys. Acta.* **89:** 266–276.

2. Fielding, C. J. 1990. Lecithin:cholesterol acyltransferase. *In* Advances in Cholesterol Research. M. Esfahani and J. Swaney, editors. Telford Press, Caldwell, NJ. 270–314.
3. Jonas, A. 1991. Lecithin-cholesterol acyltransferase in the metabolism of high density lipoproteins. *Biochim. Biophys. Acta.* **1084:** 205–220.
4. Frohlich, J., R. McLeod, P. H. Pritchard, J. Fesmire, and W. McConathy. 1988. Plasma lipoprotein abnormalities in heterozygotes for familial lecithin:cholesterol acyltransferase deficiency. *Metabolism.* **37:** 3–8.
5. Yang, C. Y., D. Manoogian, Q. Pao, F. S. Lee, R. D. Knapp, A. M. Gotto, Jr., and H. J. Pownall. 1987. Lecithin:cholesterol acyltransferase. *J. Biol. Chem.* **262:** 3086–3091.
6. Jahani, M., and A. G. Lacko. 1982. Study of the lecithin:cholesterol acyltransferase reaction with liposome and high density lipoproteins. *Biochim. Biophys. Acta.* **713:** 504–511.
7. Barter, P. J., G. J. Hopkins, and L. Gorjatschko. 1985 Lipoprotein substrates for plasma cholesterol esterification. Influence of particle size and composition of high density lipoproteins. *Atherosclerosis.* **58:** 97–107.
8. Rajaram, O. V., and P. J. Barter. 1985. Increases in the particle size of high density lipoproteins induced by purified lecithin:cholesterol acyltransferase: effect of low density lipoproteins. *Biochim. Biophys. Acta.* **835:** 41–49.
9. Carlson, L. A., and L. Holmquist. 1985. Evidence for the presence in human plasma of lecithin:cholesterol acyltransferase activity (β-LCAT) specifically esterifying free cholesterol of combined pre-β and β-lipoproteins. Studies of fish eye disease patients and control subjects. *Acta Med. Scand.* **218:** 197–205.
10. McLean, J., C. Fielding, D. Drayna, H. Dieplinger, B. Baer, W. Kohr, W. Henzel, and R. Lawn. 1986. Cloning and expression of human lecithin-cholesterol acyltransferase cDNA. *Proc. Natl. Acad. Sci. USA.* **83:** 2335–2339.
11. McLean, J., K. Wion, D. Drayna, C. Fielding, and R. Lawn. 1986. Human lecithin-cholesterol acyltransferase gene: complete gene sequence and sites of expression. *Nucleic Acids Res.* **14:** 9397–9406.
12. Bujo, H., J. Kusunoki, M. Ogasawara, T. Yamamoto, Y. Ohta, T. Shimada, Y. Saito, and S. Yoshida. 1991. Molecular defect in familial lecithin:cholesterol acyltransferase (LCAT) deficiency: a single nucleotide insertion in LCAT gene causes a complete deficient type of the disease. *Biochem. Biophys. Res. Commun.* **181:** 933–940.
13. Taramelli, R., M. Pontoglio, G. Candiani, S. Ottolenghi, H. Dieplinger, A. Catapano, J. Albers, C. Vergani, and J. McLean. 1990. Lecithin:cholesterol acyl transferase deficiency: molecular analysis of a mutated allele. *Hum. Genet.* **85:** 195–199.
14. Maeda, E., Y. Naka, T. Matozaki, M. Sakuma, Y. Akanuma, G. Yoshino, and M. Kasuga. 1991. Lecithin-cholesterol acyltransferase (LCAT) deficiency with a missense mutation in exon 6 of the LCAT gene. *Biochem. Biophys. Res. Commun.* **178:** 460–466.
15. Gotoda, T., N. Yamada, T. Murase, M. Sakuma, N. Murayama, H. Shimano, K. Kozaki, J. Albers, Y. Yazaki, and Y. Akanuma. 1991. Differential phenotypic expression by three mutant alleles in familial lecithin:cholesterol acyltransferase deficiency. *Lancet.* **338:** 778–781.
16. Funke, H., A. von Eckardstein, P. H. Pritchard, J. J. Albers, J. J. P. Kastelein, C. Droste, and G. Assmann. 1991. A molecular defect causing fish eye disease: an amino acid exchange in lecithin-cholesterol acyltransferase (LCAT) leads to the selective loss of α-LCAT activity. *Proc. Natl. Acad. Sci. USA.* **88:** 4855–4859.
17. Carlson, L. A. 1982. Fish eye disease: a new familial condition with massive corneal opacities and dislipoproteinemia. *Eur. J. Clin. Invest.* **12:** 41–53.
18. Carlson, L. A., and L. Holmquist. 1985. Evidence for deficiency of high density lipoprotein lecithin:cholesterol acyltransferase activity (α-LCAT) in fish-eye disease. *Acta Med. Scand.* **218:** 189–196.
19. Frohlich, J., G. Hoag, R. McLeod, M. Hayden, D. V. Godin, L. D. Wadsworth, J. D. Critchley, and P. H. Pritchard. 1987. Hypoalphalipoproteinemia resembling Fish Eye Disease. *Acta Med. Scand.* **221:** 291–298.
20. Palmiter, R. D., R. R. Behringer, C. J. Quaife, F. Maxwell, I. H. Maxwell, and R. L. Brinster. 1987. Cell lineage ablation in transgenic mice by cell-specific expression of a toxin gene. *Cell.* **50:** 435–443.
21. Funke, W. D., R. T. A. MacGillivray, A. B. Mason, S. A. Brown, and R. C. Woodworth. 1990. Expression of the amino-terminal half-molecule of human serum transferrin in cultured cells and characterization of the recombinant protein. *Biochemistry.* **29:** 1654–1660.
22. Sparks, D. L., and P. H. Pritchard. 1989. Transfer of cholesteryl ester into high density lipoprotein by cholesteryl ester transfer protein: effect of HDL lipid and apoprotein content. *J. Lipid Res.* **30:** 1491–1498.
23. Karpe, F., J. Johansson, and L. A. Carlson. 1990. Studies on lecithin:cholesterol acyltransferase substrate properties of HDL as determined by its subclass distribution analyzed by gradient gel electrophoresis. *Biochim. Biophys. Acta.* **1042:** 310–314.
24. Batzi, S., and E. D. Korn. 1973. Single layer liposomes prepared without sonication. *Biochim. Biophys. Acta.* **298:** 1015–1019.
25. Dobiasova, M., and M. Schutzova. 1986. Cold labelled substrate and estimation of cholesterol esterification rate in lecithin:cholesterol acyltransferase radioassay. *Physiol. Bohemoslov.* **35:** 319–327.
26. Barter, P. 1983. Evidence that lecithin:cholesterol acyltransferase acts on both high density and low density lipoproteins. *Biochim. Biophys. Acta.* **751:** 261–270.
27. Knipping, G., A. Birchbauer, E. Steyrer, J. Groener, R. Zechner, and G. M. Kostner. 1986. Studies on the substrate specificity of human and pig lecithin:cholesterol acyltransferase: role of low-density lipoproteins. *Biochemistry.* **25:** 5242–5249.