Characterization of C-terminal histidine-tagged human recombinant lecithin:cholesterol acyltransferase

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Abstract   Lecithin:cholesterol acyltransferase (LCAT) is the plasma enzyme that catalyzes esterification of the *sn*-2 fatty acid of phospholipid to cholesterol. To facilitate the isolation of large quantities of LCAT and to assist in future structure–function studies, LCAT containing a carboxy-terminal histidine-tag (H6) was expressed in Chinese hamster ovary cells (CHO). A high level of CHO-hLCATH6 expression ($\approx$15 mg L^{-1}) was achieved over a 72-h period using 10 mM sodium butyrate to enhance transcription and PFX-CHO protein-free medium. The pure enzyme ($\approx$96%) was isolated by cobalt metal affinity chromatography with an activity yield of 82 $\pm$ 26%. CHO-hLCATH6 and CHO-hLCAT species had identical specific activities (26 $\pm$ 6 and 26 $\pm$ 3 nmol CE formed μg^{-1} h^{-1}, respectively). The enzymatic activity of CHO-hLCATH6 was stable at 4°C in excess of 60 days. Substrate saturation studies, using rHDL composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), cholesterol, and apolipoprotein A-I (80:5:1) indicated that the appK_m for CHO-hLCATH6, CHO-hLCAT, and purified plasma LCAT were nearly identical at $\approx$2 μM substrate cholesterol.■ We conclude that carboxy-terminal histidine-tagged LCAT is a suitable replacement for both plasma LCAT and CHO-hLCAT.—Chisholm, J. W., A. K. Gebre, and J. S. Parks. **Characterization of C-terminal histidine-tagged human recombinant lecithin:cholesterol acyltransferase.** *J. Lipid Res.* 1999. **40:** 1512–1519.

Supplementary key words phosphatidylcholine-O-cholesterol acyltransferase • LCAT • CHO cells • cobalt metal affinity chromatography • butyric acid • his-tag • glycosylation analysis • PFX-CHO

LCAT (lecithin:cholesterol acyltransferase) is the plasma enzyme that catalyzes the transacylation of an *sn*-2 fatty acid from phospholipid to the 3β-hydroxyl of cholesterol (1). LCAT is responsible for nearly all HDL cholesteryl esters in human plasma and, in species having cholesteryl ester transfer protein (CETP), a significant proportion of VLDL and LDL cholesteryl esters. LCAT through the esterification of cholesterol is thought to play a major role in the reverse cholesterol transport pathway (2–4) by facilitating the net movement of cholesterol from peripheral tissues back to the liver for excretion. Through this action, LCAT appears to aid in the maintenance and determination of plasma HDL levels, as transgenic overexpression of LCAT increases plasma HDL concentrations (5, 6), whereas LCAT deficiency results in a very low concentration of plasma HDL (7, 8).

Three different recombinant cell systems have been previously described for the production of LCAT. LCAT from both stably transfected CHO (9, 10) and BHK cells (11) has been shown to be very similar to plasma LCAT with the exception that they are more heavily and heterogeneously glycosylated. LCAT has also been expressed in insect cells using the baculovirus system (9, 12) and although enzymatically active, contains much less carbohydrate than LCAT from mammalian sources. Most LCAT purifications from plasma and recombinant cell lines (9, 13–17) are based primarily on a combination of phenyl-Sepharose and hydroxylapatite chromatography. However, a number of investigators (10, 11) have had success in purifying recombinant wild-type LCAT using a single phenyl-Sepharose step. The reliability and reproducibility of this method for LCAT mutants and chimeras remains to be determined. An alternate and theoretically more reliable method would be to add an epitope tag to recombinant LCAT and LCAT mutants.

While numerous epitope-tagged proteins have previously been described in the literature, the application of this technology towards LCAT has been lacking. Recently, Vinogradov, Honggun, and Owen (18) have reported that carboxy-terminal histidine 6 LCAT is catalytically active and immunodetectable using an anti-His$_4$ antibody. However, the secretion efficiency of the CHO-hLCATH6 cell line described was very low (3 mg L^{-1}) and the recombinant enzyme was not characterized. The investigators rationale for using a carboxy-terminal epitope tag, like ours, was based on LCAT carboxy-terminal deletion mutant studies that indicated the carboxy-terminus was not obligatory for enzymatic activity and secretion (19, 20). We re-

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port in this paper the development of a CHO cell line that produces large amounts of CHO-hLCATH6 ($\approx$15 mg L^{-1}) using sodium butyrate to enhance expression.

METHODS

Engineering and cloning of His6-tagged human LCAT

The human LCAT cDNA (21) (a gift from John MacLean, Genentech) in the pCMV5 vector was used as a template for PCR using a 5′ complementary forward primer (5′-AGC TTC AAC TAC ACA GGC CG-3′) and a 3′ reverse primer (5′-CG GGA TCC TTA ATG ATG ATG ATG ATG ATG TTC AGG AGG CGG GGG CTC-3′) designed to append the sequence for 6 C-terminal histidine residues, a stop codon, and a 3′ BamH I restriction site. The resulting $\approx$500 bp human LCAT-H6 fragment was restriction digested with Pst I and BamH I and ligated into Pst I and BamH I digested pCMV5 human LCAT cDNA. The ligation reactions were transformed into chemically competent DH5α cells (Gibco-Brl) and transformed cells were selected with ampicillin (100 μg/ml) on LB (Lennox L Broth)-agar plates. The entire LCAT cDNA sequence was sequenced in both directions for three individual clones, using an ABI autosequencer (Perkin-Elmer). All three clones were found to be identical and one was selected for further use. hLCATH6 plasmid for transfection was amplified in LB cultures (containing 100 μg/ml ampicillin) and purified using a Maxiprep purification kit (Genomed). Isolated plasmid stocks were stored at −20°C.

Generation of hLCATH6 stable cell line

Chinese hamster ovary (CHO-K1) cells were co-transfected with hLCATH6 plasmid and pSV3-neo by the calcium phosphate transfection method (22) using a 19:1 pCMV5-hLCATH6: PSV3-neo ratio. Transfected cells were selected using Geneticin (G418, Gibco-Brl, 700 μg ml^{-1}) and 100 clones were selected and screened for LCAT activity. The expression of hLCATH6 was assessed by LCAT assay and normalized to cell protein. One clone, 16-2, was found to yield the highest level of LCAT expression and was subjected to one round of dilution cloning. Cells were plated at 0.5 cells per well in 24-well plates (Falcon) until colonies were visible. Individual colonies were screened by activity and normalized to cell protein. One clone, 16-2-6, was identified as the highest hLCATH6 expressing clone. This cell line was maintained under continuous selection using 700 μg ml^{-1} of G418 in DMEM/F12 (Mediatech), 10% heat-inactivated fetal bovine serum, 0.01% penicillin–streptomycin, 2 mm l-glutamine, and vitamins.

Expression and purification of CHO-hLCATH6

CHO-hLCATH6 cells (95–100% confluent) were washed 2× with Hanks's Balanced Salt Solution (BSS, Mediatech) and incubated for 72 h in a minimal volume (50 ml for a 500 cm^2 triple flask, NUNC) of PFX-CHO (Hyclone) serum-free complete growth medium, containing 0.01% penicillin–streptomycin, 2 mm l-glutamine, and vitamins, in either the presence or absence of 0.025 mCi/ml ^{35}S cys/met labeling mix (New England Nuclear). The specific activity of ^{35}S-labeled LCAT was approximately 16000 CPM μg^{-1}. To boost expression, sodium butyrate (10 mm final concentration) was added to the medium after 24 h. Collected medium was immediately cooled on ice, centrifuged (1500 g for 15 min) to pellet debris, and incubated with Talon™ cobalt metal affinity resin (Clontech) for 1–2 h at 4°C. The medium:resin ratio was routinely maintained less than 20:1. The resin was pelleted by centrifugation 1500 g for 15 min and batch-washed 4 times with one medium volume of 10 mm Tris HCl, 140 mm NaCl, 0.02% NaN$_3$, pH 7.4, and then transferred to

a small column (Bio-Rad). Recombinant hLCATH6 was eluted from the column with the wash buffer containing 50 mm imidazole (Fisher) and 10% glycerol (Fisher). Fractions containing recombinant hLCATH6 were identified by either LCAT activity or O.D.$_{280}$.

Expression and purification of CHO-hLCAT

Plasma LCAT was purified from human plasma obtained from the Red Cross as previously described (9). CHO-hLCAT was isolated from the medium of stably transfected CHO cells as previously described (9). Nearly confluent cell monolayers grown in DMEM/F12 (containing 10% heat-inactivated fetal bovine serum, 0.01% penicillin–streptomycin, 2 mm l-glutamine, and vitamins) were washed 2× with BSS and incubated with RPMI 1640 (phenol red-free) medium (Mediatech), containing 0.01% penicillin–streptomycin, 2 mm l-glutamine, and vitamins. PFX-CHO could not be used as a production medium, as it contains phenol-red which co-purifies with CHO-hLCAT. Cells were incubated for 72 h and at 24 h sodium butyrate was added to a final concentration of 10 mm. Medium was centrifuged at low speed 1500 g for 15 min to pellet debris and adjusted to 0.5 m NaCl with solid NaCl (added 23.4 g L^{-1}). Medium was batch bound to phenyl-Sepharose (Pharmacia) that had been pre-washed with 5 mm NaPO$_4$, 0.5 m NaCl, by inversion for 2 h at 4°C using a medium:resin ratio of 50:1 (v/v). The resin was recovered by low speed centrifugation 1500 g and washed 3× with 1 medium volume of wash buffer (5 mm NaPO$_4$, 0.5 m NaCl, pH 7.4). The resin was transferred to a disposable mini-column and washed until the O.D.$_{280}$ was <0.010. Recombinant CHO-hLCAT was eluted in 1–2-ml fractions with deionized H$_2$O, pH 7.4, and LCAT-containing fractions were identified by activity assay. LCAT-containing fractions were pooled and adjusted to 4 mm NaPO$_4$, 0.15 m NaCl, pH 6.9, by the addition of a 40 mm NaPO$_4$, 1.5 m NaCl, pH 6.9, stock solution. The sample was applied to a hydroxylapatite column (Bio-Gel HT, Bio-Rad) (same resin volume as used for phenyl-Sepharose step) that had been pre-equilibrated with 4 mm NaPO$_4$, 0.15 m NaCl, pH 6.9. The column was washed with 4 mm NaPO$_4$ until the O.D.$_{280}$ was <0.010. LCAT was eluted using a linear gradient from 4 to 60 mm NaPO$_4$, 0.15 m NaCl, pH 6.9. LCAT-containing fractions that were identified by O.D.$_{280}$ and LCAT activity were pooled, adjusted to 10% glycerol, and stored at −70°C.

LCAT characterization

Exogenous LCAT activity measurements were performed as previously described (23) using recombinant HDL (rHDL) composed of 1-palmitoyl-2-oleoyl-sn-glycerol-3-phospocholine (POPC)–cholesterol–apolipoprotein A-I 80:5:1. Human apolipoprotein A-I was purified from human plasma (Red Cross) as previously described (24).

LCAT esterase activity was measured using a water-soluble substrate p-nitrophenylvalerate (Sigma), as previously described (25).

Purified LCAT was analyzed by SDS-PAGE on 7.5% gels using a buffer system as previously described (26). Gels were loaded with 0.5 μg of protein, focused for 1 h at 25 mA, and run for approximately 3 h at 40 mA on 16-cm gels using the Bio-Rad Protein II system. Gels were silver stained as previously described (9).

Protein in purified LCAT preparations was either measured by a modified Lowry method (27) or by OD$_{280}$ using a molar extinction coefficient of 2.0 mg^{-1} cm^2 at 280 nm (28). Both 50 mm imidazole and 10% glycerol were found to interfere with the standard Lowry method. To compensate, samples containing both reagents were measured by adding an equivalent amount of glycerol and imidazole to the Lowry standards.

Deglycosylation

Endo and exoglycosidases were purchased from either Glyko or New England Biolabs (NEB) and used according to the in-

cluded methods or under non-denaturing conditions using NEB's recommended buffer for N-glycanase F.

RESULTS

After colony selection and subsequent dilution cloning, as described in Methods, the CHO-hLCATH6 cell line 16-2-6 was used for LCAT expression and purification. Initial time-course experiments indicated that the expression of LCAT under serum-free conditions (DMEM/F12) was linear in excess of 72 h. Furthermore, the addition of sodium butyrate (10 mm final concentration), 24 h after switching to serum-free medium (DMEM/F12), resulted in a doubling of LCAT volume activity (data not shown). Sodium butyrate, an inhibitor of histone deacetylase, induces the transcription of some genes (29–31). To further optimize the expression conditions for CHO-hLCATH6, serum-free DMEM/F12 was compared with an essentially protein-free complete CHO specific medium (PFX-CHO). In **Fig. 1**, the two media were compared over a 72 h time-course both with and without the addition of sodium butyrate. LCAT expression, measured by volume activity, was similar and linear for both media over a time course of 72 h when sodium butyrate was not added. LCAT expression using PFX-CHO and sodium butyrate was enhanced approximately 3-fold at the 72 h time point, compared to PFX-CHO alone. Adding sodium butyrate at 48 h did not appear to increase expression by the 72 h time point (data not shown). These combined results indicated that significantly increased LCAT expression may be obtained when PFX-CHO medium is used over a 72 h time-course in conjunction with addition
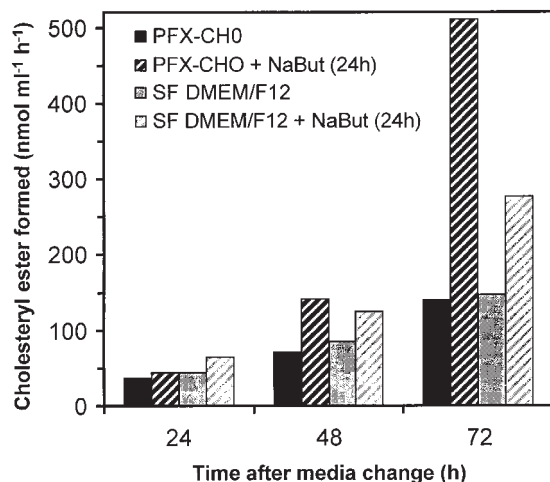

Fig. 1. Optimization of CHO-hLCATH6 production. Equal numbers of stable expressed CHO cells were seeded into 6-well plates. The next day, the cells were washed 2× with BSS and incubated with either serum-free DMEM/F12 (Mediatech) or PFX-CHO (Hyclone) containing 0.01% penicillin–streptomycin, 2 mm l-glutamine, and vitamins. Sodium butyrate (Sigma) was added to a final concentration of 10 mm at 24 h, to one set of wells, as described in the figure legend. A small aliquot of medium was taken at each time point for LCAT activity determinations and immediately frozen at −20°C. The sampled volume was replaced with fresh medium.

of sodium butyrate at 24 h. The only other low or protein-free medium that we have evaluated was CCM5 (HyClone). This medium yielded results similar to PFX-CHO in terms of medium LCAT volume activity; however, the purification of CHO-hLCATH6 was complicated by the co-elution of a heme-containing compound from the cobalt affinity column.

Binding of CHO-hLCATH6 from culture medium using a cobalt affinity resin followed by elution in 50 mm imidazole yielded a total activity recovery of 82 ± 26% (see **Table 1**). This was very efficient when compared to the 17 ± 11% total activity recovery achieved by isolating CHO-hLCAT using a combination of phenyl-Sepharose and hydroxylapatite chromatography. The purity of each preparation, as assessed by SDS-PAGE followed by silver staining, was estimated to be ≈96%. The specific activities of both CHO-hLCATH6 and CHO-hLCAT were identical 26 ± 6 and 26 ± 3 nmol cholesteryl ester formed h^{-1} μg^{-1}, respectively (see Table 1). Based on the specific activity and volume activity, the CHO-hLCATH6 cell line was estimated to produce >5-fold (15 ± 5 mg L^{-1}) more LCAT than the CHO-LCAT (2.6 ± 0.1 mg L^{-1}) cell line. The esterase activity of CHO-hLCATH6 using a water-soluble substrate p-nitrophenylvalerate was similar to CHO-hLCAT and purified plasma LCAT (data not shown).

Results from the sequential deglycosylation of CHO-hLCATH6 (**Fig. 2**) suggested that the mature protein had an estimated molecular mass of 65 ± 1 kDa with the deglycosylated core protein having an apparent molecular mass of 48 ± 1 kDa. The protein was composed of 26% carbohydrate of which 8% was sialic acid. The majority of the chains were N-linked with a small amount (1%) of O-linked sugars. The presence of O-linked sugars was variable and detection was limited by the resolution of SDS-PAGE.

The native molecular mass, deglycosylated molecular mass, and carbohydrate content of CHO-hLCATH6, CHO-hLCAT, and pure plasma hLCAT were compared by SDS-

TABLE 1. Summary of CHO-hLCATH6 and CHO-hLCAT purifications

	n	Medium LCAT	LCAT POPC specific activity	% Yield (Total volume activity)	LCAT Purity
		mg L^{-1}	nmol formed $h^{-1}\mu g^{-1}$	%	%
CHO-hLCATH6	7	15 ± 5	26 ± 6	82% ± 26%	96 ± 4%[a]
CHO-hLCAT	2	2.6 ± 0.1	26 ± 3	17% ± 11%	95 ± 1%[b]

CHO-hLCATH6 was produced from confluent monolayer cultures in 500 cm² triple flasks (NUNC). PFX-CHO was used as the production medium with 10 mm sodium butyrate added at 24 h. Medium was collected at 72 h and CHO-hLCATH6 was purified by cobalt metal affinity chromatography. CHO-hLCAT was produced as described in Methods. LCAT protein in purified preparations was measured by a modified Lowry procedure as described in Methods. The medium LCAT concentration was estimated from the specific activities of the purified LCAT preparations and medium volume LCAT activities using rHDL composed of POPC:cholesterol:apolipoprotein A-I (80:5:1). Purity was assessed by densiotometric scanning of silver-stained SDS-PAGE gels using NIH Image.

[a] Mean ± SD of 4 analyses using two separate CHO-hLCATH6 preps.

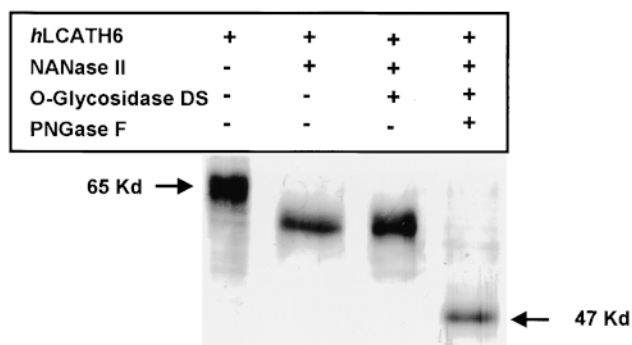[b] Mean ± SD of 2 separate measurements.

Fig. 2. Sequential deglycosylation of purified CHO-hLCATH6. CHO-hLCATH6 was purified by affinity chromatography as described in Methods and sequentially deglycosylated using enzymes supplied in a kit (Glyko). NANase II, neuraminidase ($\alpha2-3,6$); PNGase F, n-glycosidase F. Samples were analyzed on 7.5% SDS-PAGE gel and silver-stained as described in Methods. Molecular mass determinations using silver stain standards (Bio-Rad) estimated the CHO-hLCATH6 molecular mass (n = 5) at 65 ± 1 kDa with 8 ± 2% ($\alpha2-3$, 6) neuraminic acid, 25 ± 1% N-linked sugars, and 1 ± 1% O-linked sugars (n = 3). The core protein had a molecular mass of 48 ± 1 kDa.

PAGE (**Fig. 3**). Both LCAT species from CHO cells exhibited similar broad bands less than 66 kDa (average 63.6 kDa), whereas, pure plasma LCAT had a somewhat tighter band and an estimated molecular mass of 62.5 kDa. Treatment of all three enzymes with NANase resulted in a similar degree of desialation. Surprisingly, both CHO-LCAT species maintained broad bands, which is indicative of carbohydrate heterogeneity. Removal of all the N-linked carbohydrate with PNGase F resolved the protein into two distinct bands. We speculate that the higher molecular mass
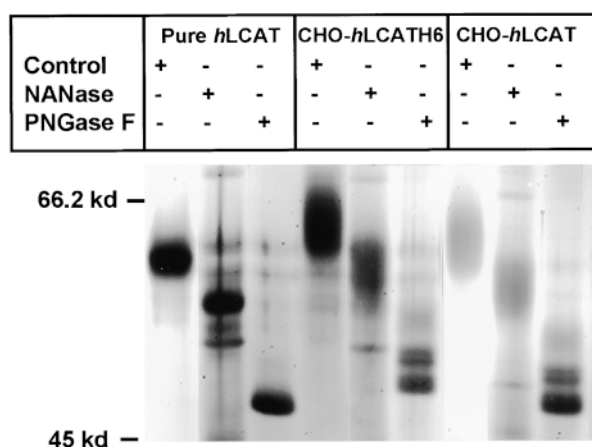

Fig. 3. Deglycosylation of purified hLCAT. His-tagged CHO-hLCATH6 and CHO-hLCAT were purified as described in Methods. Pure plasma hLCAT was prepared as previously described (9). Deglycosylation enzymes (NEB) were NANase, neuraminidase ($\alpha2-3,6,8$); PNGase F, N-glycosidase F. Deglycosylation reactions were performed under non-denaturation conditions using the buffer supplied with PNGase. Samples were incubated overnight at 25°C, analyzed on 7.5% SDS-PAGE gels, and silver-stained. Molecular masses were estimated using silver stain standards (Bio-Rad).

band may contain 1–2% of O-linked sugars as this doublet was not observed during sequential glycosylation studies (Fig. 2) in which O-glycosidase was used. The difference in apparent molecular mass between the deglycosylated form of the CHO-hLCATH6 and the other two LCAT proteins (CHO-hLCAT and purified plasma LCAT) was consistent with the addition of 6 histidine residues to CHO-hLCATH6.

Previously, purified CHO-hLCAT has been reported to self-associate into SDS-resistant (3% SDS in sample buffer) aggregates upon storage at greater than 450 μg/ml (10). Concentrated samples of CHO-hLCATH6 (up to 1500 μg/ml) were analyzed by SDS-PAGE (0.5% SDS in sample buffer) along with purified plasma LCAT (**Fig. 4A**). SDS-resistant LCAT multimers could not be identified in any of the samples after silver staining. Western blotting of a duplicate gel (not shown) using chicken anti-LCAT IgY (9) suggested a small amount of aggregation in the purified plasma LCAT preparation but none in any of the CHO-hLCATH6 samples. Non-denaturing size-exclusion FPLC of ^{35}S-radiolabeled LCAT (Fig. 4B) indicated that only 10 ± 3% (n = 10) of the total radiolabeled LCAT eluted in the void volume consistent with the presence of only a very small amount of multimers.

The stability of purified CHO-hLCATH6 at 4°C (**Fig. 5A**) and 25°C (Fig. 5B) was assessed by periodic activity sampling of CHO-hLCATH6 stored at both temperatures. CHO-hLCATH6 was very stable at 4°C with the 60-day activity measurement similar to the initial activity. Between 10 and 40 days there was an apparent increase in LCAT activity that could not be explained by assay variation. We speculate that this may be due to spontaneous desialation of the protein, which has been reported to increase the activity of the enzyme (32). The slow decline in LCAT activity after 20 days may indicate slow degradation of the enzyme. The enzyme preparation was also remarkably stable at 25°C, only exhibiting a steady decline in activity after 48 h. The half-life of the enzyme at 25°C appears to be approximately 10 days. There was no apparent advantage to storing the enzyme in liquid form in 10% glycerol at either 4°C or 25°C.

A number of CHO-LCATH6 preparations, CHO-hLCAT and purified plasma LCAT underwent kinetic analysis to compare the appK_m and appV_{max} of each LCAT species. The results are tabulated in **Table 2** and representative substrate saturation profiles are shown in **Fig. 6**. The results (Table 2) suggest that the appK_m of both CHO-hLCATH6 and CHO-hLCAT are very similar at approximately 2 μm rHDL cholesterol. The appV_{max} for the three CHO-hLCATH6 preparations was variable and, with the exception of preparation B, was higher than both CHO-hLCAT and purified plasma LCAT. The appV_{max} for CHO-hLCATH6 preparation B and CHO-hLCAT were comparable and similar to the specific activities reported in Table 1.

DISCUSSION

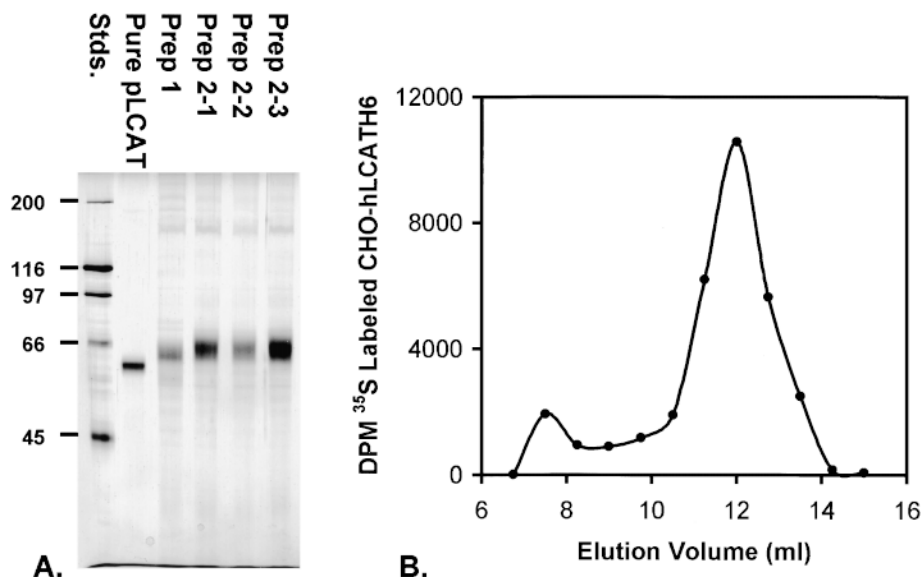The CHO-hLCATH6 cell line, described in this paper, using a protein-free complete medium (PFX-CHO) and

Fig. 4. Effect of CHO-hLCATH6 concentration on the aggregation status of LCAT. A: Purified CHO-hLCATH6–Prep 1 (145 μg ml^{-1}) and CHO-hLCATH6–Prep 2 at 3 different concentrations (330 μg ml^{-1} [2–1], 660 μg ml^{-1} [2–2], and 1500 μg ml^{-1} [2–3]) were analyzed by electrophoresis of 0.5-μg aliquots on a 7.5% SDS-PAGE gel. Proteins were visualized by silver staining as described in Methods. Protein samples were concentrated in a Centricon-30 (5000 g, Amicon). ^{35}S-labeled CHO-hLCATH6 (5 μg in 100 μl of 10 mm Tris, 140 mm NaCl, 0.01% Na$_2$EDTA, 0.01% NaN$_3$, and 0.01% BSA, pH 7.4) was chromatographed using a Superose 12 10/30 column (Pharmacia) at a flow rate of 1 ml min (10 mm Tris, 140 mm NaCl, 0.01% Na$_2$EDTA, 0.01% NaN$_3$, and 0.01% BSA, pH 7.4), and eluted into 0.75-ml fractions. ^{35}S-labeled CHO-hLCATH6 elution was monitored by liquid scintillation counting.

induction by sodium butyrate yielded medium LCAT concentrations of $\approx$15 mg L^{-1}. This level of expression was significantly higher than any other previously described LCAT secreting mammalian cell line (9–12, 18). Basal secretion of LCAT from the CHO-hLCAT6 cells was comparable to other CHO-hLCAT cell lines in the absence of sodium butyrate. The induction of LCAT secretion by sodium butyrate was enhanced in cells incubated with PFX-CHO medium compared to serum-free DMEM/F12 me-

dium. Under optimized conditions, PFX-CHO medium appeared superior in providing the necessary nutrients required for ongoing protein synthesis and secretion. For most of the previously described recombinant LCAT cell lines (10, 11), Opti-Mem (Gibco) has been used as a complete serum-free medium. While we have no experience with this medium, PFX-CHO may be advantageous as it is specifically formulated for use with CHO cells.

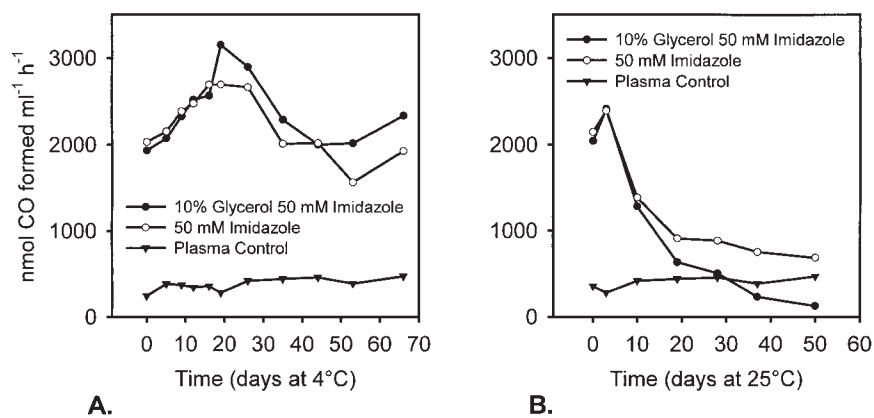CHO-hLCAT6, isolated by cobalt metal affinity chroma-

Fig. 5. LCAT stability at 4°C and 25°C. CHO-hLCATH6 was purified by affinity chromatography and eluted in 50 mm imidazole (Fisher) with and without 10% glycerol (Fisher). An aliquot of each purified enzyme preparation was maintained at either 4°C (A) or 25°C (B) and LCAT activity was measured periodically using a POPC rHDL substrate. Assay and substrate variability were monitored by analyzing a freshly thawed aliquot of frozen human plasma at the time of each LCAT activity determination.

TABLE 2. Summary of POPC rHDL substrate saturation studies

	appK_m	appV_{max}	R^2
	μM rHDL cholesterol	nmol CE formed h^{-1} $μg^{-1}$	
CHO-hLCATH6 Prep A	3.7 ± 0.6	37.3 ± 2.4	0.990
CHO-hLCATH6 Prep B	2.0 ± 0.3	23.0 ± 1.1	0.988
CHO-hLCATH6 Prep C	2.3 ± 0.7	41.1 ± 4.1	0.957
CHO-hLCAT	1.9 ± 0.4	25.7 ± 1.5	0.982
Plasma LCAT	1.7 ± 0.3	20.5 ± 0.9	0.989

The substrate saturation kinetics for three CHO-hLCATH6 preparations were measured and compared to a CHO-hLCAT preparation and purified plasma LCAT. Kinetic parameters were determined by non-linear regression analysis using the single rectangular hyperbole function in Sigma Plot (Jandel Scientific). Results are appV_{max} and appK_m ± the standard error of the curve fit ($P < 0.02$).

tography, was nearly homogeneous (≈96% purity) and was obtained in high yield (82 ± 26%). Nickel metal affinity resins are also commonly used for the purification of histidine-tagged proteins. Vinogradov et al. (18) previously used a nickel column to isolate histidine-tagged LCAT. While we have not directly compared the two resins, nickel columns have the possible disadvantage of requiring a 4-fold higher concentration of imidazole for elution. Typically, we do not remove the imidazole from our LCAT preparations, however, imidazole does interfere with both the Lowry (described in Methods) and BCA protein assays and is not compatible with the measurement of esterase activity using water-soluble substrates. However, imidazole and glycerol can be removed without adversely affecting the enzyme by using a Centricon™ (Amicon) 10 or 30 concentrator and exchanging the buffer 3–4 times.

Isolation of CHO-hLCATH6 by cobalt metal affinity chromatography was highly efficient. The yield and purity, by SDS-PAGE, were comparable to those reported for the single-step purification of recombinant LCAT using phenyl-Sepharose (10, 11). In our hands, phenyl-Sepharose chromatography yields purified LCAT, that by SDS-PAGE appears to be free of major contaminants. However, the specific activity of preparations tends to be lower than achievable by performing an additional hydroxylapatite chromatography step (data not shown), suggesting that phenyl-Sepharose-isolated LCAT preparations may contain an inhibitor or contaminating proteins not visible by silver staining of SDS polyacrylamide gels. Indeed, Nair et al. (17) have reported a low molecular weight contaminant in their phenyl-Sepharose-purified LCAT preparations. The contaminant was removed by a gel filtration step and resulted in a yield only 2-fold higher than typically attainable using phenyl-Sepharose followed by hydroxylapatite chromatography.

CHO-hLCATH6 was stable in excess of 60 days when stored at 4°C and several days at 25°C. Albers, Lin, and Roberts (22) and Chen and Albers (34) have reported LCAT to have a half-life at 4°C of 22–30 days, whereas others have reported the half-life to be measured in hours (35). Our enzyme preparation appears to be at least as stable as that described by Albers for purified plasma LCAT. The reason for the stability of our CHO-hLCATH6 preparation remains unclear and may be due to the methods used for purification, handling, and storage; however, we cannot rule out a stabilizing effect imparted by either the histidine tag or storage in 50 mm imidazole.

Sequential deglycosylation analysis of CHO-hLCATH6 and comparison of CHO-hLCATH6 to purified plasma LCAT and CHO-hLCAT confirmed that all three LCAT species were sialated and nearly all of the carbohydrate was N-linked. Furthermore, CHO-hLCATH6 and CHO-hLCAT appeared to yield identical products after deglycosylation with NANase or PNGase, the only difference being that CHO-hLCAT6 ran slower on the gel due to the presence of the additional six histidine residues. The molecular weights and carbohydrate composition of all three LCAT species were consistent with that previously reported (9, 11, 16, 36). Both CHO-LCAT species had characteristically broader bands by SDS-PAGE when compared to plasma LCAT and this was likely due to greater heterogeneity in the carbohydrate chains. This has previously been described for recombinant CHO-LCAT (9) and BHK-LCAT (11).

The appK_m of the three LCAT species appeared to be very similar at approximately 2 μm rHDL cholesterol
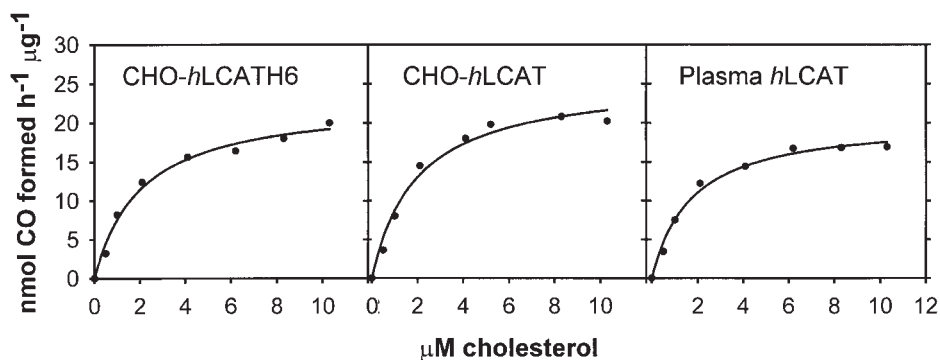

Fig. 6. POPC rHDL LCAT substrate saturation curves. The activities of purified CHO-hLCATH6 (prep B), CHO-hLCAT, and plasma LCAT were determined as a function of cholesterol concentrations and the appV_{max} and appK_m for each LCAT species was determined by non-linear regression analysis using the single rectangular hyperbole function in Sigma Plot (Jandel Scientific).

under our assay conditions. The V_{max} for CHO-hLCATH6 ranged from 23 to 41 nmol cholesteryl ester formed h^{-1} μg^{-1} and the app V_{max} for CHO-hLCAT (26 nmol cholesteryl ester formed h^{-1} μg^{-1}) was within this range. Given that freshly isolated CHO-hLCATH6 and CHO-hLCAT had identical specific activities (Table 1), the V_{max} for the two CHO-LCAT appears to be very similar. The app V_{max} for the purified plasma LCAT preparation (21 nmol cholesteryl ester formed h^{-1} μg^{-1}) was somewhat lower than LCAT produced in CHO cells, as well as measurements we have previously reported for purified plasma LCAT (53 (9) and 61 (37) nmol cholesteryl ester formed h^{-1} μg^{-1}). We attribute this difference to degradation of the preparation after 3 years stored at $-70°$C and speculate that the app V_{max} of freshly purified plasma LCAT is approximately 2-fold higher than that produced by CHO cells in agreement with Jin, Lee, and Jonas (10).

In conclusion, our optimized CHO-hLCATH6 cell line and production conditions allow for the isolation of mass quantities of recombinant LCAT. Large amounts of highly purified LCAT are required for structure and function studies such as chemical modification, structural analysis, and catalytic studies. Use of the histidine-tag is widely applicable to studies utilizing both LCAT and LCAT mutants and the availability of anti-histidine tag monoclonal antibodies will further facilitate both structure and function studies and intracellular trafficking studies. ∎

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