Human Acyl-CoA:Cholesterol Acyltransferase-1 in the Endoplasmic Reticulum Contains Seven Transmembrane Domains*

(Received for publication, May 4, 1999, and in revised form, June 2, 1999)

Song Lin‡, Dong Cheng‡, Ming-Sun Liu§, Jun Chen‡, and Ta-Yuan Chang‡

From the ‡Department of Biochemistry, Dartmouth Medical School, Hanover, New Hampshire 03755 and §Beckman Coulter Inc., Fullerton, California 92634

Acyl-CoA:cholesterol acyltransferase (ACAT) plays important roles in cellular cholesterol homeostasis and is involved in atherosclerosis. ACAT-1 protein is located mainly in the ER. The hydropathy plot suggests that ACAT-1 protein contains multiple transmembrane segments. We inserted either the hemagglutinin tag or the HisT7 tag at various hydrophilic regions within the human ACAT-1 protein and used immunofluorescence microscopy to determine the topography of the tagged proteins expressed in mutant Chinese hamster ovary cells lacking endogenous ACAT. All of the tagged proteins are located mainly in the ER and retain full or partial enzyme activities. None of the tagged proteins produces detectable intracellular degradation intermediates. Treating cells with digitonin at 5 μg/ml permeabilizes the plasma membranes while leaving the ER membranes sealed; in contrast, treating cells with 0.25% Triton X-100 or with cold methanol permeabilizes both the plasma membranes and the ER membranes. After appropriate permeabilization, double immunostaining using antibodies against the N-terminal region and against the inserted tag were used to visualize various regions of the tagged protein. The results show that human ACAT-1 in the ER contains seven transmembrane domains.

In mammals, liver and intestine synthesize and secrete lipoproteins (very low density lipoproteins and chylomicrons) that serve as lipid carriers in the blood. The cholesteryl esters present as part of the neutral lipid core within these lipoproteins are mainly produced through the action of acyl-coenzyme A:cholesterol acyltransferase (ACAT)1 in hepatocytes and intestinal mucosal cells (for reviews, see Refs. 1 and 2). In addition, the accumulation of cholesteryl esters as cytotoxic lipid droplets within macrophages constitutes the hallmark of foam cells during atherogenesis (for a review, see Ref. 3). Most of the cholesteryl esters during early stages of foam cell formation are believed to be derived from ACAT. For these reasons, ACAT has been a potential target for pharmaceutical interventions of hypercholesterolemia and atherosclerosis (for recent reviews, see Refs. 4 and 5). ACAT uses long chain fatty acyl coenzyme A and cholesteryl as its substrates to catalyze the formation of cholesteryl esters and coenzyme A. The early work of Mukherjee et al. (6) and Goodman et al. (7) established essential biochemical characteristics of ACAT as an enzyme. The ACAT activities in various cell types were shown to be enriched in the microsomal fraction (reviewed in Refs. 5, 8, and 9); it could not be solubilized without the use of detergents. Due to its minute quantity and its susceptibility to inactivation by a variety of detergents, attempts to purify this enzyme from natural sources had not been successful (reviewed in Ref. 10). The first ACAT gene (designated as ACAT-1), identified from a human macrophage cDNA library, was cloned in 1993 (11), by its ability to restore the ACAT activity of a Chinese hamster ovary cell mutant lacking endogenous ACAT activity (Refs. 12 and 13; reviewed in Ref. 14). Expression of this cDNA in insect cells devoid of endogenous ACAT activity produced ample ACAT activity in vitro (15). The recombinant human ACAT-1 (hACAT-1) protein expressed in CHO cells has recently been purified to homogeneity with retention in catalytic activity (16). Human ACAT-1 gene localization and organization have been reported (17–18).

Protein sequence analysis predicts that hACAT-1 is a hydrophobic protein with 550 amino acid residues and contains multiple potential transmembrane (TM) domains; however, the first 130 amino acids at the N-terminal of hACAT-1 contain a long stretch of hydrophilic region. Through genetic engineering, this region was fused in frame with bacterial glutathione S-transferase to produce a glutathione S-transferase-hACAT-1 gene fusion protein; the purified fusion protein was used as antigen to produce high titer, specific anti-hACAT-1 rabbit polyclonal antibodies (designated as DM-10) (19). Western blotting with DM 10 (15, 16, 19) could detect the hACAT-1 protein in various human cells and tissues as a single 50-kDa protein. Immunodepletion experiments using this anti-ACAT-1 IgG suggest that the hACAT-1 protein plays a major role in ACAT catalysis in human fibroblasts, HepG2 cells, hepatocytes, macrophages, adrenal glands, and kidneys (19–21). In contrast, in human intestines, approximately 80% of the total measurable ACAT activity in vitro is resistant to immunodepletion, suggesting that ACAT activity in this particular tissue may be largely due to the presence of a different ACAT protein (20). Orthologs of ACAT-1 in various species have been identified (reviewed in Ref. 5). In mice, ACAT-1 gene knockout mice have been generated (22). The homozygous knockout mice show markedly reduced ACAT activity and reduced amounts of cholesteryl esters in adrenal glands and in macrophages but not in the liver, suggesting the presence of at least one additional ACAT gene. Two different ACAT genes are present in yeast (23, 24). This and other evidence lead to the recent cloning of a second ACAT gene, designated as ACAT-2 (25–27). The predicted ACAT-2 protein sequence shares significant homology with that of the...
ACAT-1 protein, particularly near the C-terminal halves of the polypeptides. Whether ACAT-2 protein is responsible for most of the observed ACAT activity in human intestines is not clear at present. Unlike the ACAT-1 mRNAs, the expression levels of ACAT-2 mRNAs in various species examined thus far are tissue-restricted (25–27).

We have begun structure-function studies of hACAT-1. Previously, immunofluorescence studies showed that the hACAT-1 protein in human melanoma cells or expressed in CHO cells mainly resides in the endoplasmic reticulum (ER) (19). The topographical arrangement of ACAT-1 in membranes is unknown. Based on various protein structure prediction programs available, ACAT-1 protein may have up to eight potential transmembrane domains (TMDs). Our current goal is to provide experimental data to define the exact numbers and locations of the TMDs in ACAT-1. In this report, we used a well-established procedure for studying membrane protein topography in the ER of intact cells (for two examples, see Refs. 28 and 29). This procedure consists of fixing cells with formaldehyde, exposing cells with low concentrations of digitonin, which only permeabilizes the plasma membranes, or fixing cells with agents such as Triton X-100 or cold methanol, which permeabilizes both the plasma membranes and internal membranes. Using immunofluorescence microscopy, one then monitors the locations of various specific antigenic sites within the protein. We created various antigenic sites within the ACAT-1 protein by epitope tagging. To express the tagged hACAT-1 proteins, we performed transient transfections in CHO cell mutant AC29 (12). AC29 cells lack endogenous ACAT-1 protein (19) and have previously been shown to serve as an excellent host for ACAT protein expression studies (11, 16). Our results show that hACAT-1 expressed in CHO cells contains seven TMDs.

**EXPERIMENTAL PROCEDURES**

**Reagents**

Anti-HA mouse monoclonal antibody (HA11) was from Babco; anti-GRF75 (BIF) Igs were from Affinity Bioreagents, Inc.; anti-T7 monoclonal antibody was from Novagen; and goat anti-rabbit IgG conjugated with Texas Red and goat anti-mouse IgG conjugated with FITC were from Vector Laboratories, Inc. Anti-tubulin monoclonal antibody was from Sigma. The impure digitonin (80%) and Triton X-100 were from Sigma. The highly purified digitonin, also called saponin, was from Calbiochem-Novabiochem. Restriction enzymes were from New England Biolabs and from Life Technologies, Inc. The DNA primers were synthesized by the molecular biology core facility at Dartmouth or purchased from Life Technologies, Inc. [9,10-3H]oleic acid was from Amersham Pharmacia Biotech.

**Methods**

The **Construct ACAT1-HisT7**—This construct (indicated in Fig. 1C) contains a six-histidine tag immediately followed by the T7 tag, designated as the HisT7 tag, at the C terminus of ACAT-1. It was generated by a two-step procedure. First, a DNA fragment containing the sequence encoding the HisT7 tag (34 amino acids, Met-Arg-Gly-Ser-His-His-His-His-His-Gly-Met-Ala-Ser-Thr-Gly-Gln-Gln-Met-Gly-Arg-Asp-Leu-Tyr-Asp-Asp-Asp-Lys-Arg-Try-Gly) flanked with the EcoRI and XbaI sites, was polymerase chain reaction-amplified from the pBlue BaciHis A vector sequence (Invitrogen). The polymerase chain reaction product was confirmed by DNA sequencing. This fragment was ligated into the pCDNA3 vector (Invitrogen) at the multiple linker region between the EcoRI and the XbaI sites. The new expression vector designated as pCDNA3HisT7c, provides a HisT7 tag at the C terminus of any protein if the protein coding sequence is inserted in frame with the HisT7 tag at the EcoRI site. Next, the stop codon present in the pCDNA3HisT7c DNA was deleted by polymerase chain reaction (PCR) reaction between the ACAT1 cDNA and the oligonucleotide 

\[
\text{GGGCTGATCCGTCGATGTTGGTTGAAGAAGATGTCG (designated as Nontop ACAT1-3')}
\]

as the 3'-primer; the resultant ACAT-1 without the stop codon was ligated into the vector pCDNA3HisT7c between the BamHI and EcoRI sites. The identity of the HisT7-tagged ACAT1 construct in pCDNA3 was confirmed by DNA sequencing. This new construct, designated as pCDNA3-ACAT1-HisT7, was used as the template for various site-specific mutagenesis experiments described below.

**ACAT1 Constructs containing the HA Tag—**The hemagglutinin epitope tag (HA tag, 9 amino acids: Tyr-Pro-Tyr-Asp-Val-Pro-Tyr-Ala) (30, 31) was inserted into the ACAT-1 protein at each of 12 specific sites as indicated in Fig. 1, A and C; the specific amino acid after which the HA tag was inserted has been indicated in parentheses in Fig. 1C. For constructs ACAT1-HA1a, -HA2a, -HA3, -HA4, -HA6a, -HA7, -HA8, -HA6m, and -HA7m, the tag was inserted by site-specific mutagenesis using the Quick Change Site-Directed Mutagenesis Kit (Stratagene). Each of the mutagenic primers used was 67 bp in length, containing 27 bp encoding the HA tag (TACCATGATGCTCCGGACTACGC), flanked by two 20-bp ACAT-1 coding sequences, located at each site of a given insertion site. The constructs ACAT1-HA1a and ACAT1-HA5 (Fig. 1) do not contain the HisT7 tag at their 3'-ends of the coding region. These two constructs were produced by the same site-specific mutagenesis procedure described above, using ACAT1 cDNA in pUC19 as the template; afterward, the resultant mutant cDNA constructs were transferred directly from the pUC 19 vector to pCDNA3 between the unique HindIII and BamHI sites. The sequences of the mutant cDNAs covering the insertion sites were all confirmed by DNA sequencing.

The construct ACAT1-HA2a was generated by polymerase chain reaction by the following method. Using hACAT-1 in pCDNA3 as the template, the N-ACAT1 primer described above and the primer with the sequence CCGAATTCCCGGTAGCCGGAGTCATATGGTTATCGTC- TAAACCAACATATG (designated as ACAT1-HA2aU) were employed to produce an ACAT-1 N-terminal fragment, containing the first 735 bp of the ACAT-1 coding sequence (11), flanked with BamHI at the 5'-end and the HA tag sequence and the EcoRI sequence at the 3'-end; the other two primers with the sequences ATGAGTTCTTACTATCCCTG- CACCAGC (designated as ACAT1-HA2aD) and TTTCTATGGCGCGCC- AACGTCTAAAACGCTAAGCAGATGC (designated as C-ACAT1) were employed to produce a ACAT-1 C-terminal fragment, containing the last 915 bp of the ACAT-1 coding sequence flanked with EcoRI sequence at the 5'-end and the NotI sequence at the 3'-end. The product containing the N-terminal fragment was cut by BamHI and EcoRI; the product containing the C-terminal fragment was cut by EcoRI and NotI; and the expression vector pCDNA3 (from Invitrogen) was cut by BamHI and NotI. Afterward, the two fragments and the vector underwent three-piece ligation reaction. The resultant ACAT1-HA2a inserted in pCDNA3 was confirmed by DNA sequencing.

**Cells and Transient Transfection Experiments**—The ACAT-deficient mutant CHO cell line AC29 (12, 19) was grown in Medium F-12 supplemented with 10% fetal bovine serum (FBS) in 5% CO2 incubator at 37 °C. Human melanoma 2058 cells were grown in Dulbecco's modified Eagle's medium with 10% FBS in 10% CO2 incubator at 37 °C. The vector pCDNA3 was used for expressing various ACAT-1 constructs in AC29 cells. For Western blotting analysis and ACAT activity in intact cell assay, the cells were grown to around 70% confluency in 25-cm2 flasks and then transfected with 3 μg of the tagged ACAT-1 constructs per flask. For cytimmunofluorescence assay, the cells were plated on glass coverslips in six-well plates (Corning Glass), grown to 50% of confluency, and then transfected with 2 μg of the tagged ACAT-1 constructs/well. For transfection, Lipofectamine and OPTI-MEM medium (Life Technologies, Inc.) were used according to the manufacturer's instructions. The transfection time was 8 h.

**Immunoblot Analysis**—After transfection, AC 29 cells were grown in F-12 with 10% FBS for 48 h and then lysed with 0.5 ml of 2% SDS per flask. Protein concentration was determined by a method previously described (32). One-fifth volume of 5 × loading buffer (5% SDS, 1 × b-mercaptoethanol, 25% glycerol, 0.02% bromphenol blue, 20 mM Tris-HCl at pH 6.8) was then added to each sample containing 150 μg of protein. The samples were incubated at 37 °C for 15 min and then subjected to SDS-PAGE and immunoblot analysis as described previously (15, 19).

**ACAT Activity in Intact Cells**—This was assayed by measuring the rate of [3H]cholesteryl oleate synthesis in intact cells as described previously (33). To briefly describe the procedure, after transfection, AC 29 cells were grown in F-12 with 10% FBS for 24 h at 37 °C in the CO2 incubator. Then 20 μl of 10 mM [3H]oleate (1.34 × 106 cpm/10 μl) in 10% bovine serum albumin was added per flask for 2 h at 37 °C. The lipid analysis was as described previously. The cells were given a fresh medium change of 2 ml of F-12 with 10% FBS per flask, incubated for 1 h (33).
Stable Transfectants Expressing Various ACAT-1 Constructs—Using AC29 cells as the recipients, clones stably expressing each of the constructs hACAT1-HA1, HA5, HisT7, and hACAT1-ORF have been isolated. The general procedure for isolating and maintaining stable transfectants has been previously described (16). Transient transfectants were used in experiments described in Figs. 2, 3, and 5. stable transfectants were used in experiments described in Fig. 4.

Cytomembrane Assay—After transfection, AC 29 cells on glass coverslips in six-well plates were grown in F-12 with 10% FBS for 48 h and then processed for immunofluorescence studies. For fixation and staining, the procedure described by Harlow and Lane (34) was followed with minor modification. The cells were washed three times with PBS and then fixed with 4% of paraformaldehyde in PBS for 10 min at room temperature. After three more washes with PBS, the plasma membrane was selectively permeabilized with impure digitonin at 5 μg/ml in buffer D (0.3 M sucrrose, 2.5 mM MgCl2, 0.1 M KCl, 1 mM EDTA, and 10 mM Pipes at pH 6.8) for 5 min on ice. Alternatively, both the plasma membranes and the ER membranes were permeabilized with 0.25% of Triton X-100 in PBS for 10 min on ice or with cold methanol ( precooled at −20 °C) for 2 min at room temperature or with 0.25% (2.5 mg/ml) aponin (highly purified digitonin) in PBS for 5 min at room temperature. After the permeabilization, the cells were incubated with 1% bovine serum albumin in PBS for 30 min at room temperature to block nonspecific binding. Cells were then incubated for 1 h at room temperature with one of the following three primary antibodies: the rabbit polyclonal IgGs against the N-terminal 131 amino acids of hACAT-1 (DM10) (19) at 3 μg/ml and the mouse monoclonal IgG against the HA epitope at a 1:1000 dilution; 2) DM10 at 3 μg/ml and the mouse monoclonal IgG against the T7 epitope at 2 μg/ml; or 3) the rabbit polyclonal IgG against the resident ER protein BIP (anti-GRP 78) at 2 μg/ml and the mouse monoclonal IgG against the cysteolic protein tubulin at a 1:1000 dilution. Cells were then washed three times with PBS; the mixtures of goat anti-rabbit IgG conjugated with Texas Red at a 1:100 dilution and goat anti-mouse IgG conjugated with FITC at a 1:500 dilution were then added as secondary antibodies. The secondary antibodies were incubated with the cells for 1 h at room temperature. After three 15-min incubations with PBS at room temperature, the coverslips were mounted onto the glass plates, with a drop of FITC guard (from Testog, Inc.) added between the plates at room temperature, the coverslips were mounted onto the glass plates, for viewing. Each picture shown represents what was typically observed under a Zeiss universal microscope with a 67 achromat oil immersion plot (Fig. 1A), hACAT-1 is a hydrophobic protein with multiple TMDs. We prepared plasmid DNAs containing various ACAT-1 constructs described above and performed transient transfections using CHO cell mutant AC29 as the host. To monitor expressions of tagged or untagged ACAT-1 proteins, we analyzed homogenates of transient transfectant cells by Western blot analyses using the specific anti-ACAT-1 antibodies DM10. We also inserted the HisT7 tag at the C terminus of ACAT-1 and designated that construct as ACAT1-HisT7 (Fig. 1C). Pilot experiments have indicated that insertion of the HisT7 tag at the C terminus does not alter intracellular localization, nor does it inactivate the enzyme activity of ACAT-1 (results not shown). Therefore, we also included the HisT7 tag at the C termini of constructs ACAT1-HA1a, -HA2, -HA3, -HA4, -HA5, -HA6, -HA7, and -HA8 (Fig. 1, A and C). In addition, two other constructs, ACAT1-HA6m and ACAT1-HA7m, which will be described below, were also made. We used the HisT7 tag as the negative controls. For positive controls, we used untagged ACAT-1 protein migrated as a 50-kDa band, while the HisT7-tagged ACAT-1 protein migrated as a 54-kDa band in SDS-PAGE (16, 19). We now find that all of the tagged constructs have been expressed, and none of these tagged proteins produces detectable intracellular degradation intermediates as analyzed by SDS-PAGE. A typical result, representing several independent experiments, is shown in Fig. 2.

We next examined the ability of each of the tagged ACAT-1 proteins to produce cholesteryl esters in intact cells. We monitored rates of cholesteryl ester synthesis by performing [3H]oleate pulse in intact cells. In parallel experiments, untransfected AC29 cells, which express a very low but still measurable rate in cholesteryl ester synthesis (11–12), or AC29 cells transfected with the expression vector (pcDNA3) only were used as the negative controls. For positive controls, we used untagged wild-type ACAT-1 (designated as the ACAT1-ORF) construct. The results, which represent the means of three separate experiments, show that all of the constructs are able to augment cholesteryl ester synthesis rate beyond what is present endogenously in the AC29 cells (Fig. 3). We not predicted by the TMpred program; 2) the TMpred program predicts a TMD (T7, between aa 501 and 518) not predicted by the PhD program.

Expression of Epitope-tagged ACAT-1 Constructs in AC29 Cells—We noted that short, hydrophilic regions exist between various potential TMDs (Fig. 1A). To test the validity of the multiple TMD model, we had made numerous attempts to produce anti-peptide antibodies against various hydrophilic regions but were not able to produce any antibodies other than the antibodies DM-10, which were against the antigenic site(s) within the first 130 amino acids (19). We decided to create various antigenic sites within the ACAT-1 protein by epitope tagging. We made 10 different hACAT-1 cDNA constructs by inserting the HA tag at the predicted hydrophilic loops flanking all of the potential TMDs as predicted by both programs. These constructs are designated as ACAT1-HA1, -HA1a, -HA2, -HA3, -HA4, -HA5, -HA6, -HA7, and -HA8 (Fig. 1A and C). In addition, two other constructs, ACAT1-HA6m and ACAT1-HA7m, which will be described below, were also made. We also inserted the HisT7 tag at the C terminus of ACAT-1 and designated that construct as ACAT1-HisT7 (Fig. 1C). Pilot experiments have indicated that insertion of the HisT7 tag at the C terminus does not alter intracellular localization, nor does it inactivate the enzyme activity of ACAT-1 (results not shown). Therefore, we also included the HisT7 tag at the C termini of constructs ACAT1-HA1a, -HA2, -HA3, -HA4, -HA5, -HA7, and -HA8 (Fig. 1C). The additional HisT7 insertions enable us to monitor the location of the C termini of various HA-tagged ACAT-1 proteins.

RESULTS
The Potential Transmembrane Domains as Predicted by Two Different Algorithms—Based on the Kyte-Doolittle hydrophathy plot (Fig. 1A), hACAT-1 is a hydrophobic protein with multiple TMDs. We employed two widely used programs to predict the TMDs in ACAT-1: the PhD program by Rost (35) (available on the Internet), using an empirical filter to restrict the length of a given predicted TM helix, predicts eight TMDs, located between aa 141 and 158, 183 and 200, 225 and 242, 247 and 264, 326 and 343, 368 and 387, 443 and 460, and 465 and 484 (Fig. 1B, first row); TMDs depicted as dark boxes T1–T7; the TMpred program by Hofmann and Stoffel (36) (available on the Internet) predicts 7 TMDs, located between aa 144 and 162, 181 and 203, 225 and 248, 322 and 341, 361 and 385, 470 and 486, and 501 and 518 (Fig. 1B, second row); TMDs depicted as dark boxes T1–T7. The latter algorithm is based on the statistical analysis of data available on all naturally occurring transmembrane proteins, assuming a minimum of 17 aa and a maximum of 33 aa to form a TM helix. Three key differences exist between the two predictions: 1) the PhD program predicts two TMDs (T4 and T7; between aa 247 and 264 and between 443 and 460) not predicted by the TMpred program; 2) the TMpred program predicts a TMD (T7, between aa 501 and 518) not predicted by the PhD program.
define normalized ACAT activities as the relative cholesteryl ester synthesis rate shown in Fig. 3 minus 1.0, divided by the relative ACAT protein expression level shown in Fig. 2. The normalized ACAT activities are as follows: ACAT1-HA1 (4.5); ACAT1-HA1a (5); ACAT1-HA2 (2.5); ACAT1-HA2a (7.5); ACAT1-HA3 (3); ACAT1-HA4 (5); ACAT1-HA5 (5); ACAT1-HA6 (1.6); ACAT1-HA7 (3); ACAT1-HA8 (8.3); ORF (7). This analysis reveals that the proteins tagged at sites HA1, HA1a, HA2a, HA4, HA5, HA8, or HisT7 express activities at more than 50% of the untagged protein, while the proteins tagged at sites HA2, HA3, HA6, or HA7 express activities at less than 50% of the untagged protein. This analysis can only be considered qualitative in nature for the following reasons: 1) estimation of protein levels based on Western blotting can only be considered as a semiquantitative method and 2) Fig. 2 represents the result of a single experiment, while Fig. 3 shows the averages of results from three separate experiments. Nevertheless, we can conclude that all of the tagged ACAT-1 proteins described above are at least partially active, with activities ranging from 20 to 100% of the untagged ACAT-1 enzyme activity.

Validation of the Cytoimmunofluorescence Assay—Using
confocal fluorescent microscopy, we had earlier shown that hACAT-1 stably expressed in AC29 cells was mainly localized in the ER and the nuclear membranes (19). We now show that the HA-tagged protein ACAT1-HA1 (Fig. 4, row I; frame B; red) stably expressed in AC29 cells significantly colocalizes with BiP, an ER protein marker (29) (Fig. 4, row I, frame A; green). Frame C in Fig. 4, row I, demonstrates the very high degree of overlap (80–90% by visual examination) between the hACAT-1 signal and the BiP signal. We also found that the untagged hACAT-1, the ACAT1-HA5, or the ACAT1-HisT7 proteins stably expressed in AC29 cells gave essentially identical results (i.e. approximately 80–90% of the total ACAT-1 immunoreactive signals overlap with the BiP signals; data not shown). The result in frame C of Fig. 4, row I, also indicates that in addition to the ER localization, a small amount of the hACAT-1 signal (red) can be found at the juxtanuclear region. This observation is similar to the earlier observations made in mouse macrophages (37). The functional significance of the non-ER localization of ACAT-1 is not clear at present.

In various mammalian cells, the unpurified detergent digitonin at low concentration preferentially permeabilizes the plasma membranes, while leaving the ER membranes sealed (28, 29). To find the optimal condition in our system, we exposed formaldehyde-fixed cells with varying concentrations of digitonin. We then used antibodies against tubulin, a structural protein in the cytosol, as a marker for cytoplasmic accessibility and used the antibodies against BiP as a marker for ER luminal accessibility. Our results show that treating cells with digitonin at 5 mg/ml completely permeabilizes the plasma membranes (Fig. 4, row II; frames A–C; signal with anti-tubulin becomes clearer with increasing concentrations of digitonin), while leaving the ER membranes sealed (Fig. 4, row II; frame D; no signal with anti-BiP staining). In contrast, treating these cells with 0.25% Triton X-100 completely permeabilizes the ER membranes (Fig. 4, row II; frame E; clear signal with anti-BiP staining). Additional experiments showed that treating these cells directly with cold methanol for 2 min or with 0.25% saponin in PBS for 5 min at room temperature could also completely permeabilize the ER membranes (results not shown). Using optimized conditions for selectively permeabiliz-
Fig. 4. Row I, colocalization of ACAT-1 (red) and the ER marker BIP (green) as probed by confocal microscopy. A transf ectant of AC29 cells stably expressing the ACAT1-HA1 construct was isolated and grown on glass coverslips. Cells were fixed with paraformaldehyde, permeabilized with 0.25% Triton X-100, probed with a mixture of mouse monoclonal antibody against BiP and rabbit polyclonal antibodies against ACAT-1 (DM10), stained with a mixture of FITC-conjugated goat anti-mouse and Texas Red-conjugated goat anti-rabbit secondary antibodies, and then viewed with FITC optics (frame A), or with Texas Red optics (frame B). Overlap between the green and the red produces the yellow. Frame C demonstrates a high degree of overlap between the localization patterns of BiP and ACAT-1 in these cells. To indicate the cell periphery, AC29 cells provided the same result as shown in Row II.

Additional experiments showed that treating these cells directly with cold methanol for 2 min or with 0.25% saponin in PBS for 5 min at room temperature could also completely permeabilize the ER membranes (results not shown). The protocol for cell fixation and permeabilization is described under “Experimental Procedures.” Row II, degree of permeabilization of plasma membranes and the ER membranes by different agents. AC29 cells plated on glass coverslips and grown in F-12 with 10% FBS were fixed with paraformaldehyde, permeabilized with various agents, and then probed with anti-tubulin or with anti-BiP antibodies. Frames A–C show the degrees of immunostaining with the mouse monoclonal antibody against the cytoplasmic marker tubulin after cells were treated with 1, 3, or 5 µg/ml of digitonin. Frame D shows that the cells treated with 5 µg/ml digitonin were not stained with the antibodies against BiP, a protein marker for the ER lumen. Frame E shows that cells treated with 0.25% Triton X-100 were stained with antibodies against BiP. Additional experiments showed that treating these cells directly with cold methanol for 2 min or with 0.25% saponin in PBS for 5 min at room temperature could also completely permeabilize the ER membranes (results not shown). The protocol for cell fixation and permeabilization is described under “Experimental Procedures.” Row III, ACAT-1 localization patterns in various cell types treated with 5 µg/ml digitonin. Cells were probed with the ACAT-1 N-terminal specific antibodies (DM10), stained with FITC secondary antibodies, and then viewed with FITC optics. Frame A, AC29 cells stably transfected with ACAT1-ORF, frame B, AC29 cells stably transfected with the construct ACAT1-HA1, frame C, human melanoma cells; frame D, AC29 cells. Additional control experiments showed that the degrees of immunostaining were essentially unaltered if these cells were permeabilized with Triton X-100, or with cold methanol (results not shown).

ing cells, we used the DM10 antibodies to determine the orientation of the N-terminal region of the ACAT-1 protein in various cell types. The DM10-positive signals are clearly present in cells stably expressing the hACAT1-HA1 protein permeabilized with digitonin only (Fig. 4, row III; frame A). Under the same conditions, staining with the DM10 antibodies using AC29 cells provided no signal (Fig. 4, row III, frame D), demonstrating the specificity of the DM10-positive signal. Additional results show that staining with the HA antibody in cells expressing the ACAT1-HA1 protein provided the same result as demonstrated in frame A of Fig. 4, row III; staining with the HA antibody in AC29 cells provided the same result as shown in frame D of Fig. 4, row III (results not shown). We also found that staining with the DM10 antibodies in AC29 cells stably expressing the ACAT1-ORF protein or in human melanoma cells expressing endogenous hACAT-1 protein produced the same staining patterns (frames B and C, Fig. 4, row III). Additional experiments showed that the pattern and the degree of staining by DM10 antibodies or by HA antibody remained unaltered if any of these cells described above were permeabilized directly with agents known to permeabilize the ER membranes, i.e. with 0.25% Triton X-100 or with cold methanol, or with 0.25% saponin (results not shown). These results indicate that the N-terminal region of hACAT-1 in various cell types is located at the cytoplasmic side of the ER membranes.

Determination of the Orientation of the Antigenic Tags in ACAT-1—We next performed a series of transient transfection experiments in AC29 cells, using one of the ACAT-1 constructs described in Fig. 1 as the transfecting plasmid. The transfected cells were permeabilized with 5 µg/ml of digitonin only (Fig. 5, A and B; left halves) or with Triton X-100 (Fig. 5, A and B; right halves). The appropriately permeabilized cells were simultaneously stained using antibodies against the N-terminal region of ACAT-1 (DM10) and the HA tag (or the T7 tag) and then examined under the fluorescence microscope. For visualization, appropriate secondary antibodies were chosen such that the DM10-positive signals are in red, while the HA- or the H7-positive signals are in green. The results (Fig. 5, A and B) show that each of these tagged proteins exhibits uniformly distributed staining pattern throughout the reticular network in the transfected cells, consistent with the interpretation that the tagged ACAT-1 proteins are located mainly in the ER. In addition, the results show that the HA1 site, the HA2 site, the HA3 site, the HA4 site, and the HA7 site can all be visualized in digitonin-permeabilized cells; the degrees and patterns of stainings are essentially unaltered in cells permeabilized with digitonin or with Triton X-100, indicating that these sites are all in the cytoplasmic side of the ER. In contrast, the HA1a site, the HA2a site, the HA5 site, the HA6 site, the HA8 site, as well as the HisT7 site extending the C terminus are not visible in digitonin-permeabilized cells; they only become visible when cells are permeabilized with Triton X-100 or with cold methanol, indicating that these sites are all within the luminal side of the ER. The results shown in Fig. 5, A and B, are representative of approximately 20–30 different microscopic areas in a single microscopic slide, covering 30 or more transfected cells. Also, for each ACAT-1 construct employed, the results described here have been consistently seen in at least three independent transfection experiments. The results using Triton X-100 for permeabilizing the ER membranes have been confirmed by using cold methanol. Since the constructs ACAT1-HA1a, -HA2, -HA3, -HA4, -HA6, -HA7, and -HA8 all contain the
HisT7 tag as an extension at the C terminus (Fig. 1C), we performed additional double immunostaining experiments, using the DM10 antibodies (in red) and the T7 antibody (in green) to monitor the sidedness of the C termini of the HA-tagged ACAT-1 proteins. Our results show that for all of the proteins derived from the tagged constructs listed in Fig. 1C, the T7 site was visible in Triton X-100-permeabilized cells, but not in digitonin-permeabilized cells (results not shown), supporting the assumption that the presence of the HA tags at sites indicated in Fig. 1 does not alter the ACAT-1 topography at the C terminus.

Our results are consistent with the interpretation that one TMD is present between each of the following sites: the HA1 and the HA1a (between amino acids 25 and 166), the HA1a and the HA2 (between amino acids 166 and 202), the HA2 and HA2a (between amino acids 202 and 245), HA2a and HA3 (between amino acids 245 and 277), the HA4 and the HA5 (between amino acids 308 and 349), the HA6 and the HA7 (between amino acids 404 and 449), and the HA7 and the HA8 (between amino acids 494 and 523). These results are not entirely consistent with the computer-based predictions made by either the PhD program or the TMpred program. Specifically, we find that no TMD exists between the HA5 and the HA6 sites, in contrast to the predictions made by both programs (one TMD). Also, our results show that two TMDs exist between the HA2 and HA3 sites, which is consistent with the PhD program but not with the TMpred program; one TMD exists between the HA6 and HA7 sites and one TMD exists between the HA7 and HA8 sites, which is consistent with the TMpred program but not with the PhD program. Based on these data, we propose a seven-TMD topographical model for ACAT-1 in the ER, as shown in Fig. 6, with TMDs located between aa 144 and 162, 181 and 201, 225 and 242, 247 and 264, 326 and 343, 470 and 486, and 501 and 518. In drawing the model, we use a combination of the PhD program and the TMpred program to assign the numbers of amino acids constituting various TMDs. The exact locations for each of the TMDs in fact cannot be determined by the method employed in this manuscript.

We note that between the HA6 and the HA7 sites, many hydrophobic amino acids are present (Fig. 1. A and C; amino acids 404–494). In an effort to further delineate the exact location of the sixth TMD, we made one additional ACAT-1 construct, by inserting the HA tag at the 459th amino acid (ACAT1-HA7m; see Fig. 1A and C, for the location of the HA

---

**FIG. 5.** Determining the sidedness along the ER membranes of the inserted tags in ACAT-1 proteins by indirect cytoimmunofluorescence. Cells transiently transfected with various epitope-tagged ACAT-1 constructs (indicated at the left) were doubly immunostained with the ACAT-1 N-terminal specific antibodies DM10, providing the red color, and the HA antibody or the T7 antibody, providing the green color. For A and B, the left two columns show immunostainings after cells were permeabilized with digitonin; the right two columns show immunostainings after cells were permeabilized with Triton X-100. For each horizontal set of images shown, the same basal instrument settings, including light intensity and light exposure time, were kept constant. This point was verified by the results demonstrating that for each set of images the intensities of the red color in digitonin-treated cells did not differ significantly from those in Triton X-100-treated cells. A, immunostainings of the ACAT1-HA1, -HA1a, -HA2, -HA2a, -HA3, or -HA4 proteins. B, immunostainings of the ACAT1-HA5, -HA6, -HA7, -HA8, or HisT7ACAT1 proteins. Additional control experiments showed that the T7 tags located at the C termini of ACAT-1 proteins tagged with HA1a, HA2, HA3, HA4, HA6, HA7, or HA8 were immunostained only after the cells were treated with methanol but not with digitonin (results not shown).
We found that as expected, the ACAT1-HA7m construct expresses the tagged ACAT-1 protein with the predicted molecular weight without detectable intracellular degradation intermediates; the N terminus of the expressed protein is in the cytoplasmic side, while its C terminus is in the luminal side of the ER. However, we were unable to detect the HA signal in the ACAT1-HA7m protein, either by Western blot analysis or by cytoimmunofluorescence analysis (results not shown); the expressed ACAT1-HA7m protein is enzymatically inactive. Thus, the exact location of the TMD between amino acids 404 and 494 remains uncertain. We also noted that no TMD could be detected between the HA5 and HA6 sites (amino acids 349–404; Fig. 6). Within this area, many hydrophobic amino acids are present. Hydrophobic peptides not part of a TM segment may be closely associated with lipids in membranes (for a review on this subject, see Ref. 38). To test this possibility, we made one additional ACAT-1 construct by inserting the HA tag at the 369th amino acid (ACAT1-HA6m; see Fig. 1, A and C, for location of the HA tag). The ACAT1-HA6m construct expresses the expected tagged ACAT-1 protein, with its N terminus in the cytoplasmic side and its C terminus in the luminal side of the ER, and is enzymatically active. However, its HA signal cannot be detected by Western blot analysis or by cytoimmunofluorescence analysis (results not shown). These results are consistent with the idea that the region located at or near the HA6m site (369th amino acid) may be in close contact with the lipid bilayer; alternatively, this region may be interacting with other protein(s) in the intact cells.

The data described above were collected by using transfec-

tant cells grown in lipoprotein-containing medium. It is possible that when cellular cholesterol content of the host cells is significantly depleted, the membrane topography of ACAT-1 in intact cells may be altered. To test this possibility, we grew stable transfectant cells that express the ACAT1-HA1, -HA5, or -His T7 proteins in cholesterol starvation condition (39, 40) for 2 days and then performed cytoimmunofluorescence assays, using the DM10 antibodies, the HA antibody, and the T7 antibody as probes. The results obtained are the same as those obtained by growing cells in lipoprotein-containing medium, indicating that the ACAT-1 membrane topography is not altered by cellular cholesterol content (results not shown).

DISCUSSION

We used the method of epitope tagging coupled with cytoimmunofluorescence assay to study the orientation of hACAT-1 in the ER of intact cells. This method has previously been used by other investigators to study the topographical arrangements of a variety of membrane proteins (for three examples, see Refs. 28, 29, and 41). In using the epitope tag strategy, one assumes that the insertion of the tag only causes minor alteration in the functional domain(s) within the ACAT-1 protein, without causing gross structural change(s) that may lead to altering the intracellular localization and/or membrane topography. In our case, this assumption has been supported by the findings demonstrating that the N termini of these tagged proteins exhibit the same orientations as the untagged ACAT-1 protein in the ER. We also found that other than the ACAT1-HA7m protein, all of the tagged ACAT-1 proteins described in this manuscript
express full or partial enzyme activity in intact cells. Prior to using this method, we had attempted to isolate microsomal vesicles and use the method of sensitivity to protease digestion for assigning the sidedness of certain segments of ACAT-1 protein. However, we found that results using the latter method were not always reproducible. Otto and Smith (29) had earlier shown that microsomal vesicles isolated in various cell types might not always be 100% intact; the luminal portion of the ER protein may not always be resistant to protease digestion.

Our results led us to propose a seven-TM topographical model for hACAT-1. It should be pointed out that the current study is restricted to examining the topography of ACAT-1 expressed in transfected cells only. Although unlikely, it is possible that the ACAT-1 protein in different cell types may assume a different topographical arrangement. Also, the design of experiments described here is based on the two programs that predict the numbers and locations of transmembrane helices within ACAT-1. It is possible that other than the seven TM helices proposed in this study, additional nonhelical transmembrane peptide segment(s), such as what has been found in the subunits of nicotinic acetylcholine receptor (42), may exist. Despite these reservations, this is the first working model outlining two-dimensional membrane topography of ACAT-1. The validity of this model can now be further tested by various biochemical and biophysical experiments.

Our model proposes that, in addition to the seven TMDs, the hACAT-1 protein is composed of one large cytoplasmic domain (located at aa 1–143), one large luminal domain (located at aa 344–470), three small cytoplasmic loops (located at aa 202–224, 265–325, and 487–500), and three small, luminal domains (located at aa 163–181, 243–246, and 519–550). ACAT utilizes long chain fatty acyl-coenzyme A and cholesterol as the two substrates, producing cholesteryl esters and coenzyme A as the two products. At present, very little is known about the ACAT active site(s) as well as its catalytic mechanism. Long chain acyl-CoA is not permeable to the ER membranes (reviewed in Ref. 43). Therefore, the acyl-CoA binding motif within ACAT has to be located in the cytoplasmic side. By comparing available ACAT-1 and ACAT-2 sequences from various species including mammals, yeast and *C. elegans* (Refs. 5 and 25–27), a highly homologous 6-aa peptide sequence has been found; in hACAT-1, this sequence is MKASHF (amino acids 265–270). In hamsters, a Ser to Leu mutation within this sequence results in loss of ACAT-1 enzyme activity, suggesting that this peptide motif may be involved in ACAT catalysis (44). According to our model, this peptide is located within the second small cytoplasmic domain, immediately outside the fourth TMD (Fig. 6). It is possible that this peptide may serve as part of the fatty acyl-CoA binding motif. ACAT is believed to utilize cholesterol in the ER (5). Within the seven TM domains of ACAT-1, several highly homologous peptide sequences among various ACAT sequences exist; in hACAT-1, they are located at amino acids 144–151 (FIALLILF, within the first TMD); 188–195 (WWIMFLST; within the second TMD), 248–260 (LPPASRFIIIFEQ; within the fourth TMD), 327–336 (FAQVFGCFFY; within the fifth TMD), and 471–486 (FYPVLFVLFMPFGMAF; within the sixth TMD). It is possible that some of these sequences may constitute part of the cholesterol binding site(s) in ACAT. Consistent with this proposal, we note that none of these four sequences is conserved in the enzyme diacylglycerol acyltransferase (45), which utilizes diacylglycerol, not cholesterol, as its substrate. Three other conserved sequences have been identified; the 298–313 peptide is located in the second small cytoplasmic domain, while the 391–425 and the 447–465 peptides are located within the large luminal domain. These three sequences are also highly homologous in the diacylglycerol acyltransferase protein sequence. The specific functions of these conserved peptide sequences are unknown at present; they may be involved in forming part of the enzyme catalytic center and/or involved in facilitating the release of cholesteryl ester or triacylglycerol to the luminal side of the ER, where VLDL

![Fig. 6. The proposed membrane topography of hACAT-1 protein expressed in the ER of CHO cells. Amino acids are shown in single letter code. The amino acids after which the tags were inserted are shown in black. This model shows that the N terminus of ACAT-1 is in the cytoplasmic side of ER; the C terminus is in the luminal side of ER. The protein traverses the ER lipid bilayer seven times.](image-url)
synthesis and assembly takes place (for reviews, see Refs. 46–48). Other functions are also possible. Currently, we are designing various experiments to test the proposed functions of these conserved peptide sequences. It has been proposed that the topographical arrangements of ACAT-1 and ACAT-2 may be different (25), and this difference, if it exists, may form the molecular basis for the two isoenzymes to perform different physiological functions. We are in the process of testing this hypothesis in our laboratory.

Acknowledgments—We thank Dr. Duane Compton for helpful advice and Dr. Gus Lienhard for careful reading of the manuscript; we thank Ann Cole, Akira Miyazaki, Kristin Cobb, and Andy Menshol for participation in various stages of this work; and we thank Cathy Chang, Chunjiang Yu, and Albert F. Chen for helpful discussions. We thank Helina Morgan for careful editing of the manuscript.

REFERENCES

1. Dietchy, J. M., Turley, S. D., and Spady, D. K. (1983) J. Lipid Res. 24, 1637–1660
2. Wilson, M. D., and Rudel, L. L. (1994) J. Lipid Res. 35, 943–955
3. Tabas, I. (1995) Curr. Opin. Lipidol. 6, 260–268
4. Krause, B. R., and Bocan, T. M. A. (1995) in Inflammation: Mediators and Pathways (Ruffolo, R. R., Jr., and Hollinger, M. A., eds) pp. 173–196, CRC Press, Inc., Boca Raton, FL
5. Chang, T. Y., Chang, C. C. Y., and Cheng, D. (1997) Annu. Rev. Biochem. 66, 613–638
6. Mukherjee, S., Kunitake, G., and Alfin-Slater, R. B. (1985) J. Biol. Chem. 260, 91–96
7. Goodman, D. S., Deykin, D., and Shiratori, T. (1964) J. Biol. Chem. 239, 1353–1354
8. Suckling, K. E., and Stange, E. F. (1985) J. Lipid Res. 26, 647–671
9. Suckling, K. E., and Stange, E. F. (1985) J. Biol. Chem. 260, 20747–20755
10. Chang, C. C. Y., Huh, H. Y., Cadigan, K. M., and Chang, T. Y. (1993) J. Biol. Chem. 268, 20747–20755
11. Cadigan, K. M., Heider, J. G., and Chang, T. Y. (1988) J. Biol. Chem. 263, 24274–24283
12. Cadigan, K. M., Chang, C. C. Y., and Chang, T. Y. (1989) J. Cell Biol. 108, 2201–2210
13. Chang, C. C. Y., Chang, C. C. Y., and Cadigan, K. M. (1994) Trends Cardiovasc. Med. 4, 223–230
14. Chang, C. C. Y., Chang, C. C. Y., Qu, X., and Chang, T. Y. (1995) J. Biol. Chem. 270, 685–695
15. Chang, C. C. Y., Lee, C. G. Y., Chang, E. T., Cruz, J. C., Levesque, M., and Chang, T. Y. (1998) J. Biol. Chem. 273, 35132–35141
16. Chang, C. C. Y., and Noll, W., Nuttle-McMenemy, N., Lindsey, E. A., Baldini, A., Chang, W., and Chang, T. Y. (1994) Somatic Cell Mol. Genet. 20, 71–74
17. Li, B. L., Li, X. L., Duan, Z. J., Lee, O., Lin, S., Ma, Z. M., Chang, C. C. Y., Yang, X. Y., Park, J. P., Mohandas, T. K., Noll, W., Chan, L., and Chang, T. Y. (1999) J. Biol. Chem. 274, 11060–11071
18. Lee, O., Chang, C. C. Y., Lee, W., and Chang, T. Y. (1998) J. Lipid Res. 39, 1722–1727
19. Miyazaki, A., Sakahashi, N., Lee, O., Takahashi, K., Horiuichi, S., Hakamata, H., Morangel, P. M., Chang, C. C. Y., and Chang, T. Y. (1998) Arterioscler. Thromb. Vasc. Biol. 18, 1568–1574
20. Meiner, V. L., Cases, S., Myers, H. M., Sande, E. R., Belliotta, S., Schambelan, M., Pitas, R. E., McGuire, J., Heuz, J., and Farese, R. V. J. (1996a) Proc. Natl. Acad. Sci. U. S. A. 93, 14041–14046
21. Yang, H., Bard, M., Bruner, D. A., Gleeson, A., Deckelbaum, R. J., Aijinovic, G., Pohl, T. M., Rothstein, R., and Sturley, S. L. (1996) Science 272, 1353–1356
22. Yu, C., Kennedy, N. J., Chang, C. C. Y., and Rothblatt, J. A. (1996) J. Biol. Chem. 271, 24157–24163
23. Anderson, R. A., Joyce, C., Davis, M., Reagan, J. W., Clark, M., Shelnaz, G. S., and Rudel, L. L. (1998) J. Biol. Chem. 273, 26747–26754
24. Cases, S., Novak, S., Zheng, Y. W., Myers, H., Lear, S. R., Sande, E., Welch, C. B., Luise, A. J., Spencer, T. A., Krause, B. R., Erickson, S. K., and Farese, R. V., Jr. (1998) J. Biol. Chem. 273, 26755–26764
25. Oelkers, P., Behari, A., Cromley, D., Bilheimer, J. T., and Sturley, S. L. (1998) J. Biol. Chem. 273, 26765–26771
26. Boitelman, J., Olenker, E. H., Bar-Nun, S., Dunn, W. A., Jr., and Simon, R. D. (1992) J. Cell Biol. 117, 593–573
27. Otto, J. C., and Smith, W. L. (1994) J. Biol. Chem. 269, 18668–18675
28. Wilson, I. A., Nimah, H. L., Houghten, R. A., Chenerson, A. R., Connolly, M. L., and Lerner, R. A. (1984) Cell 37, 767–778
29. Koboldej, P. A., and Young, R. A. (1993) Methods Enzymol. 194, 599–519
30. Peterson, G. L. (1977) Anal. Biochem. 83, 346–356
31. Chang, C. C. Y., Doolittle, G. M., and Chang, T. Y. (1986) Biochemistry 25, 1693–1699
32. Khelef, N., Buton, X., Beutin, N., Wang, H., Meiner, V., Chang, T. Y., Farese, Jr., R. V., Maxfield, F. R., and Tabas, I. (1998) J. Biol. Chem. 273, 11218–11224
33. Brazeur, R., Piolet, T., Lins, L., Vandekeerkhove, J., and Rosseneu, M. (1997) Trends Biochem. Sci. 22, 167–171
34. Cadigan, K. M., Spillane, D. M., and Chang, T. Y. (1990) J. Cell Biol. 116, 295–308
35. Spillane, D. M., Reagan, J. W., Kennedy, N. J., Schneider, D. L., and Chang, T. Y. (1995) Biochim. Biophys. Acta 1254, 283–294
36. Kas, C., Canfield, V., Levenson, R., and Gres, P. (1996) J. Biol. Chem. 271, 9240–9248
37. Unwin, N. (1993) J. Mol. Biol. 229, 1101–1124
38. Coleman, R. A., and Bell, R. M. (1983) Enzymes 16, 605–625
39. Cao, G., Goldstein, J. L., and Brown, M. S. (1996) J. Biol. Chem. 271, 14672–14684
40. Cases, S., Smith, S. J., Zheng, Y. W., Myers, H. M., Lear, S. R., Sande, E., Novak, S., Collins, C., Welch, C. B., Luise, A. J., Erickson, S. K., and Farese, R. V., Jr. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 13018–13023
41. Chang, T. Y., Chang, C. C. Y., and Lee, O. (1998) in Intracellular Cholesterol Trafficking (Chang, T. Y., and Freeman, D., eds) pp. 1–14, Kluwer Academic Press, Boston, MA
42. Nyte, J., and Doolittle, R. F. (1982) J. Mol. Biol. 157, 105–132