The Disulfide Linkage and the Free Sulfhydryl Accessibility of Acyl-Coenzyme A:Cholesterol Acyltransferase 1 As Studied by Using $mPEG_{5000}$-Maleimide†

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ABSTRACT: Acyl-coenzyme A:cholesterol acyltransferase (ACAT) is a membrane protein located in the endoplasmic reticulum (ER). It plays important roles in cellular cholesterol homeostasis. Human ACAT1 (hACAT1) contains nine cysteines (C). To quantify and map its disulfide linkage, we performed thiol-specific modifications by $mPEG_{5000}$-maleimide (PEG-mal) and iodoacetamide (IA) under denatured condition, using extracts that contain wild-type or various single C to A mutant hACAT1s. With the wild-type enzyme, seven Cs could be modified before dithiothreitol (DTT) treatment; nine Cs could be modified after DTT treatment. With the C528A or the C546A enzyme, all eight Cs could be modified before or after DTT treatment. With all other remaining single C to A mutant enzymes, six Cs could be modified before DTT treatment, and eight Cs could be modified after DTT treatment. We next performed Lys-C protease digestion on hACAT1 with a hemagglutinin (HA) tag at the C-terminus. The digests were treated with or without DTT and analyzed by SDS−PAGE and Western blotting. The two predicted C-terminal fragments (K496−K531 and N532−F550−HA tag) were trapped as a single peptide band, but only when the digests were treated without DTT. Thus, C528 and C546 near the enzyme's C-terminus form a disulfide. PEG-mal is impermeable to ER membranes. We used PEG-mal to map the localizations of the seven free sulfhydryls and the disulfide bond of hACAT1 present in microsomal vesicles. The results show that C92 is located on the cytoplasmic side of the ER membrane and the disulfide is located in the ER lumen, while all other free Cs are located within the hydrophobic region(s) of the enzyme.

Acyl-coenzyme A:cholesterol acyltransferase (ACAT)[1] catalyzes the formation of cholesterol esters using long-chain fatty acyl-coenzyme A and cholesterol as substrates. It plays important roles in cellular cholesterol homeostasis and is involved in various pathophysiological events that are associated with cholesterol metabolism (*1*). In mammals, two ACAT genes (*acat1* and *acat2*) have been identified (*2*−*5*). In adult humans, ACAT1 is ubiquitously expressed in various tissues, including hepatocytes, Kupffer cells, macrophages, adrenals, intestines, and neurons (*6*−*8*), while ACAT2 is mainly located at the apical region of the small intestine (*8*). Under various pathological conditions, macrophages and livers also express ACAT2 in addition to ACAT1 (*9*, *10*). Because of their biomedical importance, both enzymes are drug targets for therapeutic intervention for various human diseases (*11*−*13*). Our laboratory had previously purified the recombinant human ACAT1 (hACAT1) overexpressed in CHO cells to homogeneity (*14*). However, the yield of the purified ACAT1 protein is low. Thus, current studies on ACAT1 are feasible only at the enzymological and cell biological level, but not at the structural biology level. ACAT1 is a resident endoplasmic reticulum (ER) membrane protein with multiple transmembrane domains (*15*, *16*). It is a homotetramer in vitro and in vivo (*17*), and its N-terminal region contains a dimer-forming motif (*18*). When dispersed in vesicles and in mixed micelles, the cholesterol saturation curves of the enzyme assayed both in mixed micelles and in reconstituted vesicles are highly sigmoidal. The oleoyl-coenzyme A substrate saturation curves of the enzyme assayed under the same conditions are both hyperbolic (*14*). This and other evidence suggests that ACAT1 is an allosteric enzyme that can be activated by its own substrate cholesterol (*19*, *20*). These studies provide the first example that a membrane-bound enzyme can be allosterically regulated by cholesterol.

In the 1980s, before the ACAT1 genes were identified, chemical modification studies suggested that certain cysteine (C) and histidine (H) residue(s) might be near or in the active site of the enzyme (*21*, *22*). Recent site-directed mutagenesis results show that H460 in ACAT1 (or its equivalent H432 in ACAT2) is essential for enzyme activity (*23*−*25*).

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[1] Abbreviations: ACAT, acyl-coenzyme A:cholesterol acyltransferase; CEs, cholesteryl esters; CHO, Chinese hamster ovary; ER, endoplasmic reticulum; IA, iodoacetamide; ME, 2-mercaptoethanol; PEG-mal, $mPEG_{5000}$-maleimide; PBS, phosphate-buffered saline; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; DTT, dithiothreitol; FBS, fetal bovine serum; GSH, reduced glutathione; TCA, trichloroacetic acid; PMA, phorbol 12-myristate 13-acetate; HA, hemagglutinin; TBS, Tris-buffered saline; WT, wild type.

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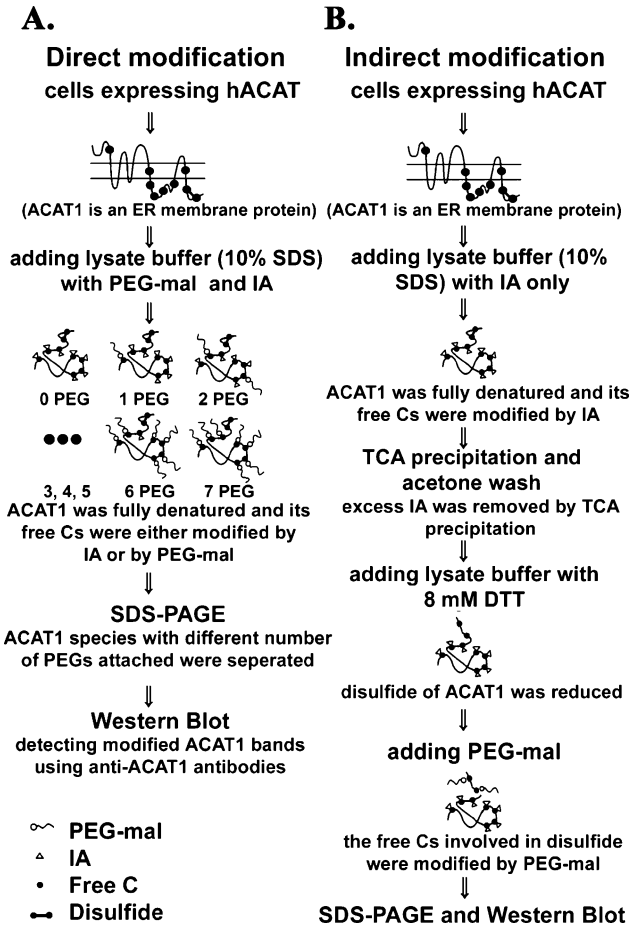

FIGURE 1: Strategies employed to deduce the number of disulfide linkages in hACAT1. (A) Direct modification by a mixture of IA and PEG-mal in SDS solution. (B) Indirect modification by sequential treatment of IA, DTT, and PEG-mal in SDS solution.

However, none of the nine Cs (C92, C333, C345, C365, C387, C467, C516, C528, and C546) is necessary for ACAT1 activity (*26*). C residues in proteins can exist either as free Cs or as a disulfide bond formed between two Cs. Since the purified ACAT1 protein is only available in limited quantity, it has been difficult to perform biochemical experiments that are only feasible with proteins in abundant quantities; as a result the disulfide pairing within ACAT1 remains unknown. $mPEG_{5000}$-maleimide (PEG-mal) is a membrane-impermeant C-specific reagent and has been used to study the topology of membrane proteins (*33*). It has a large molecular mass (MW 5000 Da), while iodoacetamide (IA) is a small reagent with a much smaller MW (185 Da). Both reagents mainly target free sulfhydryls in proteins. In the current study, we prepared extracts from cells that express wild-type or mutant hACAT1s containing various single C to alanine (A) mutations and performed direct modification by PEG-mal, with and without increasing concentrations of IA under denatured condition. In addition, we also performed indirect modification by sequential treatment of IA, DTT, and PEG-mal under denatured condition. The two strategies used to deduce the number of disulfides in hACAT1 are schematically presented in Figure 1. Depending upon the number of free cysteines available, direct modifications by a mixture of PEG-mal and IA of hACAT1 produce ladders of ACAT1 bands, with discontinuous increases in molecular mass on SDS−PAGE, while the modifications by IA do not. The modified and unmodified hACAT1 bands are detected by performing Western blotting using the ACAT1-specific antibodies. This new strategy allowed us to quantify and map the disulfide linkage of hACAT1. The methods described should also be applicable to other proteins with sparse quantities. To gain information on the environment of various cysteines of hACAT1 in the ER membrane, we also used PEG-mal to probe the environment of the free cysteines and the disulfide bond of ACAT1 present in microsomal vesicles.

MATERIALS AND METHODS

Materials. PEG-mal was purchased from Watershears. PEG-mal solution was freshly prepared in lysate buffer (10% SDS, 50 mM Tris-HCl, 1 mM EDTA, pH 8.7) for modification under denatured condition and in 50 mM Tris-HCl and 1 mM EDTA, pH 7.8, for modification under native condition. IA was from Sigma and was prepared as fresh stock solution in lysate buffer. DTT was from Invitrogen. 2-Mercaptoethanol (ME) was from Sigma. FuGENE 6 transfection reagent was from Roche Molecular Biology. [9,10-^{3}H]Oleic acid was from Amersham Pharmacia Biotech. Endoproteinase Lys-C was from Calbiochem. PVDF membranes (Immobilon P and Immobilon PSQ) were from Millipore. The monoclonal anti-HA11 antibody was from Covance. The rabbit polyclonal antibodies DM10 against the N-terminal fragment (1−131) of human ACAT1 were described previously (*14*). Goat anti-rabbit and goat anti-mouse IgG (L + H)−HRP conjugate was from Bio-Rad. The SuperSignal West Pico chemiluminescent substrate was from Pierce.

Cell Culture. The CHO cells were cultured in F-12/DMEM (50:50) medium supplemented with 10% FBS in a 5% CO_2 incubator at 37 °C. The human monocytic cell line THP-1 cells were cultured in medium RPMI 1640 plus 10% FBS in a 5% CO_2 incubator at 37 °C and transformed to macrophage-like cells by treatment with PMA (final concentration is 0.1 μM) for 5 days. The ACAT1-deficient CHO cell line AC29 (*27*) was used to express hACAT1 and its mutants. For transfections, the AC29 cells were cultured in six-well plates to 70−80% confluency and then transfected with 2 μg of pcDNA3 vectors encoding N-terminal 6× histidine-tagged hACAT1 (His-hACAT1) or its mutants, using FuGENE 6 transfection reagent according to the manufacturer's protocols. On the second day, the cells were trypsinized, divided equally into three wells, and continuously cultured for 2 days at 37 °C with G418 (0.3 mg/mL) present in the growth medium.

Recombinant DNA Technology. The His-ACAT1 mutants with various single C to A mutations were constructed previously (*26*) and subcloned from the pGEM-7Z(−) vector to the pcDNA3 vector according to standard protocols. Briefly, the 1.7 kb DNA fragment encoding His-ACAT1 mutants was released from the pGEM-7Z(−) vector by *Hin*dIII cleavage and filled-in by Klenow enzyme and then cleaved with *Eco*RI. Then, the DNA fragment encoding ACAT1 was ligated into the pcDNA3 vector pretreated with *Eco*RI and *Eco*RV. The C-terminal HA-tagged hACAT1 (ACAT1-HA) expression plasmid was constructed and described previously (*28*).

ACAT Activity Assay in Intact Cells. This method measures the rate of [^{3}H]cholesteryl oleate synthesis in intact cells (*29*). The transiently transfected AC29 cells were cultured in

6-well plates at 37 °C for 3 days. The cells were given a fresh media change (1 mL/well) 2 h before assay. Then 20 μL of 10 mM [^{3}H]oleate in 10% bovine serum albumin was added to the media, and the assay was carried out at 37 °C for 30 min.

hACAT1 Protein Content Analysis after Transfection. The cells were washed with 2 mL of PBS and lysed by 240 μL of lysate buffer containing 10 mM IA. The cell lysates were transferred to Eppendorf tubes, 60 μL per tube of SDS−PAGE loading buffer (10% SDS, 20% glycerol, 0.05% bromphenol blue, 50 mM Tris-HCl, pH 6.8) was added, and the solution was well mixed by vigorous vortexing. Then, 60 μL of sample was loaded onto a 9% SDS gel. After electrophoresis, the proteins were transferred to a PVDF (Immobilon P) membrane, and the bands of ACAT1 mutants were visualized by Western blot using DM10 as the primary antibodies. The relative amount of the hACAT1 mutants compared with that of WT hACAT1 was analyzed by densitometry.

Direct Modification under Denatured Condition. The cells expressing hACAT1 were cultured in six-well plates and lysed by 100 μL of lysate buffer containing different concentrations of IA and PEG-mal as indicated. The modification reaction was carried out at 37 °C for 30 min. During modification, 10% SDS was used to lyse the CHO cells, and the modification agents were dissolved in the lysate buffer. Therefore, it can be reasonably presumed that the ACAT1 is fully denatured and all of the free cysteines can be modified quickly and efficiently. Then one-fourth volume of SDS−PAGE loading buffer was added, and the solution was mixed well by vigorous vortexing. Appropriate amounts of modified cell lysates were loaded onto a 9% SDS gel. After electrophoresis, the proteins were transferred to a PVDF (Immobilon P) membrane, and the ACAT1 bands were visualized by Western blot using DM10 as the primary antibodies.

Indirect Modifications under Denatured Condition. The cells expressing hACAT1 were cultured in six-well plates and lysed by adding 400 μL of lysate buffer containing 10 mM IA. The modification reaction was carried out at 37 °C for 30 min. Subsequently, the proteins in the lysates were precipitated by 15% TCA. The pellets were washed with 1 mL of 15% TCA and then with 2 × 1 mL of cold acetone per tube, respectively. The pellets were then dissolved in the Lysate Buffer containing 8 mM DTT and incubated at 37 °C for 30 min. A negative control sample was prepared by dissolving the pellet in the Lysate Buffer without DTT. After incubation, equal volumes of 20 mM PEG-mal dissolved in the lysate buffer were added to each tube to modify the free cysteines exposed by DTT reduction of the disulfide linkage. The modified lysates were analyzed by SDS−PAGE, and proteins were transferred to a PVDF membrane (Immobilon P). The ACAT1 bands were visualized by Western blot using DM10 as the primary antibodies.

Direct PEG-mal Modification under Native Condition. The cells expressing hACAT1 were cultured in 90 mm dishes, lysed by hypotonic shock, and scraped from the bottom of the dish according to the previously published procedure (*30*). The intact cells and nucleus were removed by centrifugation (800*g*, 5 min). PEG-mal solution (final concentration indicated in each figure legend) dissolved in buffer A (50 mM Tris-HCl, 1 mM EDTA, pH 7.8) was then added to the cell lysate supernatants in the absence or presence of saponin, which permeabilizes the microsomal membranes (*15*, *25*). The modification reactions were carried out at 4 °C for 1 h. After incubation, 2-mercaptoethanol (ME) was added to the final concentration of 10 mM (to react with the excess PEG-mal). The reactions were carried out at 4 °C for an additional 30 min. Subsequently, equal volumes of 60 mM IA in lysate buffer were added to modify the remaining thiol groups of the denatured hACAT1. Finally, the modification mixtures were analyzed by SDS−PAGE, and the proteins were transferred to a PVDF (Immobilon P) membrane after electrophoresis. The ACAT1 bands were visualized by Western blot using DM10 as the primary antibodies.

Indirect PEG-mal Modification under Native Condition. The CHO cells stably expressing WT hACAT1 were cultured in 90 mm dishes to about 90% confluency. The cells were trypsinized and then washed with 2 × 1 mL of PBS. The cells were then resuspended in 200 μL of cold PBS containing 100 mM DTT and incubated on ice for 1 h. Afterward, the cells were washed with 4 × 1 mL of cold PBS. Finally, the cells were resuspended in 500 μL of cold buffer A and homogenized using a cold steel homogenizer. The intact cells and nucleus were removed by centrifugation (800*g*, 5 min), and the supernatants were used to carry out PEG-mal modifications under native condition as described above.

Lys-C Cleavage of C-Terminal HA-Tagged hACAT1 and Analysis of the HA-Tagged Peptide Fragment. The C-terminal HA-tagged hACAT1 (*28*) was expressed in AC29 cells by transient transfection. Cells were grown for 3 days, lysed by lysate buffer containing 10 mM IA, and incubated at 37 °C for 30 min. The IA treatment blocks all of the free cysteines and prevents possible disulfide-exchange reactions during later analysis. The cell lysate was loaded onto a 9% SDS gel. After electrophoresis, the proteins were transferred to a PVDF membrane (Immobilon P). The membrane was washed with TBS to remove SDS, and the ACAT1 band was cut and sliced into small pieces (1 × 1 mm). Then, appropriate amounts (50−70 μL) of digestion buffer (100 mM Tris-HCl, 1% Triton X-100, pH 8.5) and 0.5 μg of Lys-C endoproteinase were added (*31*). The digestion was carried out at 37 °C for 24 h. After digestion, the peptide fragments were eluted from the membrane by adding a 20% SDS solution to produce a final concentration of 3% and incubated at 80 °C for 5 min. The eluted solution was equally divided into two vials; DTT was added into one vial to the final concentration of 10 mM to reduce disulfides. After incubation at 37 °C for 30 min, IA solution was added to the final concentration of 40 mM to block the thiol groups. Then the samples were loaded onto a tricine SDS gel (16.5% T, 6% C) (*32*), which is capable of resolving peptides with molecular masses ranging from 5 to 100 kDa. After electrophoresis, the peptides were transferred to a PVDF membrane (Immobilon PSQ), and the C-terminal peptide fragment of hACAT1 was visualized by Western blot using monoclonal anti-HA11 (1:250 dilution) as the primary antibody.

RESULTS

Thiol-Specific Modification of Recombinant hACAT1 Expressed in CHO Cells under Denatured Condition. PEG-

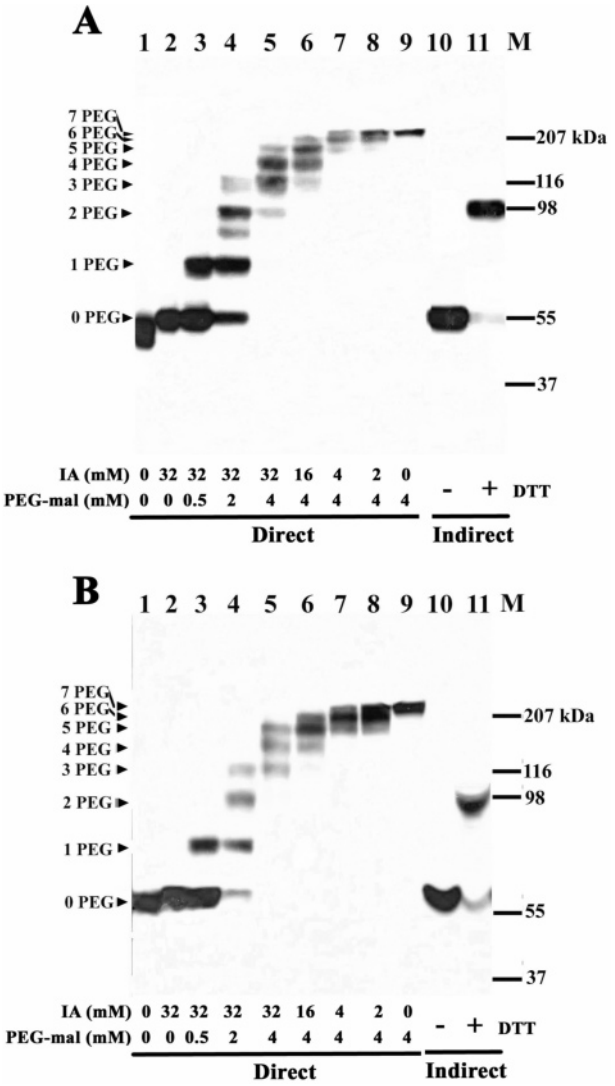

FIGURE 2: The direct and indirect PEG-mal modification of (A) hACAT1 stably expressed in CHO cells or (B) His-hACAT1 expressed in CHO cells after transient transfection. Lanes 1−9 are direct modifications of (A) hACAT1 (50 kDa) or (B) His-ACAT1 (55 kDa), with a mixture of IA and PEG-mal. In lanes 1−9, the ratio of IA to PEG-mal (mM/mM) is 0/0, 32/0, 32/0.5, 32/2, 32/4, 16/4, 4/4, 2/4, and 0/4, respectively. Lane 11 shows the results of indirect PEG-mal modification of (A) hACAT1 or (B) His-ACAT1. Lane 10, without DTT treatment, is the negative control of lane 11. The strategies are depicted in Figure 1, and the detailed procedures are described in Materials and Methods. Results represent one of two independent experiments.

mal is a thiol-specific alkylation agent that attaches one PEG_{5000} moiety to each free C of the target protein (*33*). The PEG-mal-modified proteins migrate more slowly than those of the unmodified protein on SDS−PAGE. We used this property and developed a new strategy to elucidate the disulfide linkage of hACAT1. The strategy is outlined in Figure 1. As shown in Figure 2 (lane 9), after modification by PEG-mal, the apparent molecular mass of the PEG-mal-modified ACAT1, or that of the His-tagged ACAT1, is approximately 200 kDa, which is much larger than that of the unmodified protein (50 kDa for hACAT1 or 55 kDa for His-tagged hACAT1). This result suggests that, under denatured condition, ACAT1 contains several free cysteine residues that can be alkylated by PEG-mal. However, it is difficult to determine how many PEGs are attached to the modified hACAT1 by estimating the mobility rate of the PEG-modified protein vs various protein markers on SDS−PAGE because, unlike the polypeptide backbone, the PEGs attached to the protein would not be expected to bind SDS. The apparent molecular mass of the modified hACAT1 on SDS−PAGE calculated on the basis of protein markers is expected to be much larger than its actual molecular mass. Therefore, it is necessary to find a method to determine how many PEGs are attached to the fully denatured hACAT1. IA is also a thiol-specific alkylation agent, but it is a small molecule. After modification by IA, the mobility rate of the modified protein on SDS−PAGE is not significantly altered. As shown in Figure 2 (lane 2), after modification by IA, the mobility rate of the modified hACAT1 remained the same as that of the unmodified hACAT1 on SDS−PAGE. We next used a mixture of IA and PEG-mal to modify hACAT1 under denatured condition. Under these conditions, the free Cs of hACAT1 would be modified by either PEG-mal or by IA, and a series of modified hACAT1 species with different numbers of PEG attached would be produced. Assuming the number of free Cs in ACAT1 is N, one predicts that there would be a total of $N + 1$ modified hACAT1 species with 0, 1, 2, and up to N molecule(s) of PEGs attached. IA would modify the free Cs of ACAT1 left unmodified by PEG-mal. By changing the ratio of IA to PEG-mal, a ladder of hACAT1 species would be observed on SDS−PAGE, with the highest total number of modified hACAT1 species equaling $N + 1$. Experimentally, as shown in Figure 2A (lanes 3−8), a total of eight discrete hACAT1 bands on SDS−PAGE appeared after modification by the mixtures of IA and PEG-mal; the same results were obtained when extracts of cells transiently expressing His-ACAT1 were used (Figure 2B). This result suggests that there are seven free C residues in hACAT1 expressed in CHO cells.

Since hACAT1 contains nine C residues, the above results suggest that two C residues either form a disulfide bond with each other or are blocked by small molecules, such as glutathione (GSH) or cysteine, and cannot be modified even under denatured condition. To test this interpretation, we carried out the indirect modification procedure (depicted in Figure 1B). As shown in Figure 2 (lane 11), after blocking all of the free Cs with IA under denatured condition and following with DTT treatment, two PEGs can be attached to hACAT1 or His-hACAT1. The control experiment showed that under the same conditions, but without DTT treatment, no Cs could be modified by PEG-mal (Figure 2, lane 10). Therefore, hACAT1 contains two bonded Cs that either form a disulfide bond with each other or are blocked by GSH, C, or other thiol-containing small molecules.

Thiol-Specific Modification of hACAT1 Expressed in Human Macrophage-like Cells under Denatured Condition. The results described above suggest that recombinant hACAT1 expressed in CHO cells has seven free and two bonded cysteines. To test this interpretation in a human cell line that expresses native hACAT1, we employed the human monocytic cell line THP-1 after PMA treatment as the hACAT1 source to carry out thiol-specific modification. It is known that PMA-treated THP-1 cells behave similarly to human macrophages and express relatively abundant amounts of the hACAT1 protein (*28*). As shown in Figure 3 (lane 2), the 50 kDa hACAT1 expressed in human THP-1 cells and the same protein expressed in CHO cells have identical

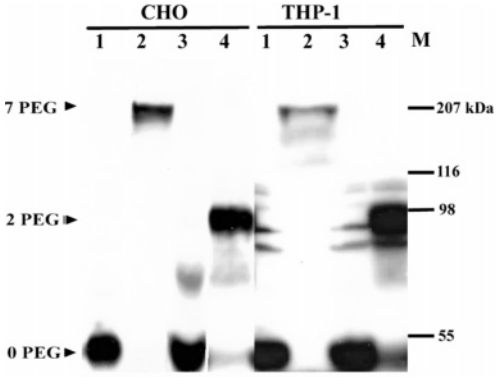

FIGURE 3: Direct and indirect PEG-mal modification of ACAT1 expressed in human macrophage-like THP-1 cells (marked as lanes THP-1). The hACAT1 proteins stably expressed in CHO cells were used as controls (marked as lanes CHO). Lanes: 1, direct modification by IA (40 mM) only; 2, direct modification by PEG-mal (4 mM) only; 4, indirect modification; 3, without DTT treatment, the negative control of lane 4. The strategies for modification are depicted in Figure 1, and the detailed procedures are described in Materials and Methods. Results represent one of two independent experiments.

mobility rates on SDS−PAGE after PEG-mal modification. After blocking the free Cs with IA and with the DTT treatment, two PEGs can be attached to the hACAT1 in THP-1 cells (Figure 3, lane 4). Additional results show that when the 50 kDa hACAT1 in THP-1 cells was modified by a mixture of IA and PEG-mal, a total of eight ACAT1 bands on SDS−PAGE were observed (data not shown). Therefore, the 50 kDa ACAT1 expressed in both human THP-1 cells and CHO cells has seven free Cs and two bonded C residues.

Determining the Location of the Bonded Cs in hACAT1. We next set out to determine the position of the bonded cysteines in hACAT1. For this purpose, we employed various ACAT1 mutants with single C to A mutations (C92A, C333A, C345A, C365A, C387A, C467A, C516A, C528A, C546A). Each of these mutants was produced using the WT hACAT1 as the template. If the mutated C exists in free form, then that particular ACAT1 mutant should exhibit six free Cs that can be modified without DTT treatment and two bonded Cs that can be modified with the DTT treatment. If the mutated C exists in bonded form, there are two possible results: if the two Cs form a disulfide, that particular ACAT1 mutant should exhibit eight free Cs but no bonded C residues; if the two Cs are blocked by small molecules, that particular ACAT1 mutant should exhibit seven free Cs and one bonded C. As shown in Figure 4A, when the C92A mutant was employed, there were a total of seven ACAT1 bands on SDS−PAGE when modified by a mixture of IA and PEG-mal (Figure 4A, lanes 1−6). Additional experiments showed that two PEGs can be attached to this mutant after blocking the free Cs with IA and following with DTT treatment (Figure 4A, lane 7). Therefore, the C92A ACAT1 mutant has six free Cs and two bonded Cs. We carried out additional experiments using the C333A, C345A, C365A, C387A, C467A, and C516A ACAT1 mutants and obtained the same results as those using the C92A ACAT1 mutant (data not shown). Thus, in the wild-type ACAT1, C92, C333, C345, C365, C387, C467, and C516 all exist as free Cs. We next carried out direct modification experiments using the C528A mutant. The results showed that seven or eight PEG-mal-

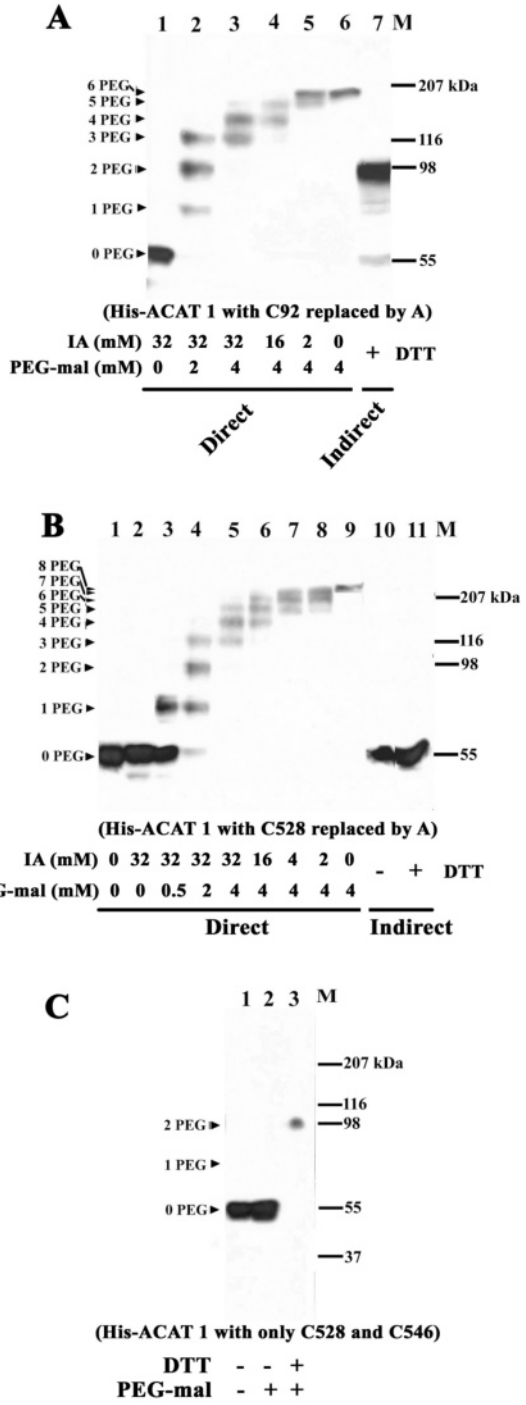

FIGURE 4: Direct and indirect PEG-mal modifications of various His-ACAT1 mutants transiently expressed in CHO cells. (A) Lanes 1−6 are results of direct modification of the C92A mutant; the ratio of IA to PEG-mal (mM/mM) used is 32/0, 32/2, 32/4, 16/4, 2/4, and 0/4, respectively. Lane 7 is the result of indirect modification of the C92A mutant by PEG-mal. (B) Lanes 1−9 are results of direct modification of the C528A mutant; the ratio of IA to PEG-mal (mM/mM) is 0/0, 32/0, 32/0.5, 32/2, 32/4, 16/4, 4/4, 2/4, and 0/4, respectively. Lane 11 is the result of indirect modification of the C528A mutant; lane 10, without DTT treatment, is the negative control of lane 11. The strategies for direct modification and for indirect modification are depicted in Figure 1. (C) Lanes: 1, lysates of cells expressing the C2(528/546)-His-ACAT1 mutant without modification; 2, same lysates as used in lane 1 but treated with 4 mM PEG-mal; 3, same lysates as used in lane 1 but treated with 8 mM DTT at 37 °C for 30 min. Afterward, an equal volume of 20 mM PEG-mal was added to conduct the PEG-mal modification. Results are representative of two independent experiments.

modified ACAT1 bands were detectable; however, the separation of these bands was incomplete, which prevented us from determining the exact number of free Cs present in the C528A mutant (Figure 4B, lanes 1−9). We thus performed the indirect modification experiment using the C528A mutant and found that no PEG can be attached to this mutant after blocking the free Cs with IA and following with the DTT treatment (Figure 4B, lane 11). The same result was obtained when the C546A mutant was employed to perform the indirect modification experiment (data not shown). These results suggest that C528 and C546 form a disulfide bond, rather than being blocked by small molecules. To test this interpretation, we employed an additional His-hACAT1 mutant containing only C528 and C546, but no other Cs (*26*). As shown in Figure 4C, this mutant cannot be modified by PEG-mal without the DTT treatment; however, after the DTT treatment, two PEGs could be attached, supporting the conclusion that the two Cs, C528 and C546, are bonded as a disulfide in hACAT1.

Lys-C Cleavage of C-Terminal HA-Tagged ACAT1. The above results suggest that the two Cs, C528 and C546 near the C-terminus, form a disulfide bond in hACAT1. To further examine this possibility by taking a different approach, we carried out endoproteinase Lys-C digestion of ACAT1. As shown in Figure 8, there are two predicted endoproteinase Lys-C cleavage sites near the C-terminus of ACAT1: Lys495 and Lys531. We reason that Lys-C digestion should produce, among many fragments, two peptide fragments that are near the C-terminus of ACAT1: Lys496−Lys531 and Asn532−Phe550. If a disulfide forms between C528 and C546 in WT ACAT1, the two peptide fragments would be linked together by the disulfide bond after the Lys-C cleavage; however, treating the digests with DTT would cause separation of these two peptide fragments. In contrast, if C528 and C546 are blocked by small molecules and therefore do not form a disulfide bond with each other, the two fragments (Lys496−Lys531 and Asn532−Phe550) produced after Lys-C digest would remain separate from each other, with or without the DTT treatment. To date, no specific antibodies that recognize the C-terminal end of ACAT1 are available. To circumvent this deficiency, we need to insert an antigenic tag at the ACAT1 C-terminus. In our earlier work, we have introduced the nine amino acid HA tag (with amino acid sequence YPYDVPDYASL) at the C-terminus of ACAT1; this construct is designated as hACAT1-HA. We had shown that the HA tag attached to the C-terminus of hACAT1 did not significantly alter the ACAT enzyme activity (*28*). Here, using the hACAT1-HA construct expressed in CHO cells as the enzyme source, we showed that eight ACAT1 bands were observed after direct modification by the mixture of IA and PEG-mal, and two PEGs could be attached to the ACAT1-HA after blocking the free thiols with IA and after the DTT treatment (Figure 5A,B). Thus, the hACAT1-HA also has seven free Cs and two bonded Cs. We next performed Lys-C digestion on hACAT1-HA, treated the digests with or without DTT, and subjected the digests to SDS−PAGE and Western blotting analysis using the anti-HA antibody as the probe. As shown in Figure 5C, without the DTT treatment, a single peptide band with molecular mass of about 7 kDa on SDS−PAGE after Lys-C digestion could be observed (Figure 5C, lane 1). Its size is consistent with the size of the two predicted C-terminal peptide

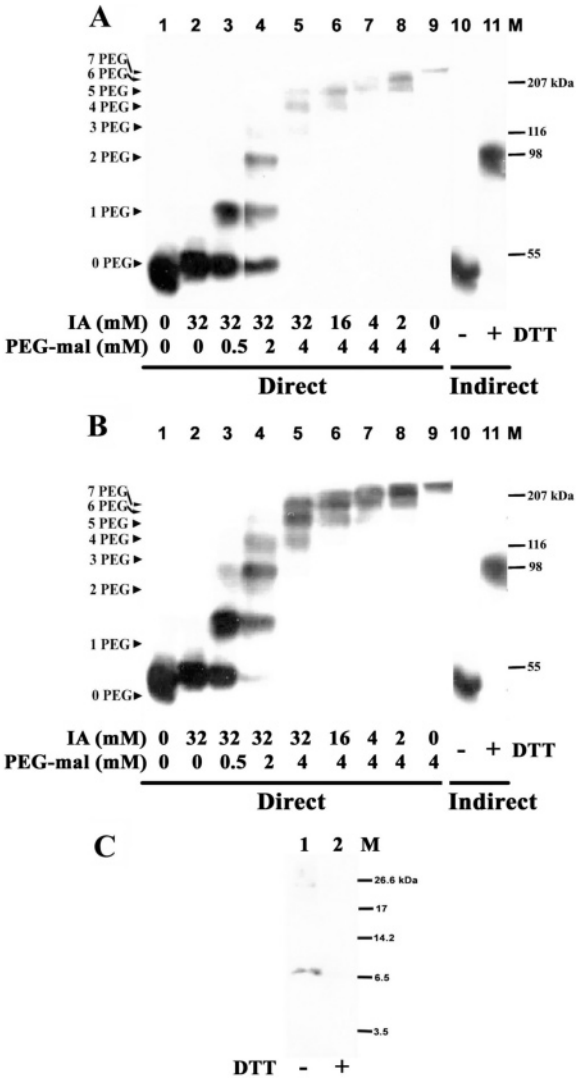

FIGURE 5: Direct and indirect PEG-mal modifications and the Lys-C cleavage of C-terminal HA-tagged hACAT1 (hACAT1-HA). Panels A and B show the direct and indirect modifications of hACAT1-HA transiently expressed in CHO cells. Lanes 1−9 are results of direct modification; the ratio of IA to PEG-mal (mM/mM) used was 0/0, 32/0, 32/0.5, 32/2, 32/4, 16/4, 4/4, 2/4, and 0/4, respectively. Lane 11 shows results of indirect modification; lane 10, without DTT treatment, is the negative control of lane 11. The cell lysates were analyzed by SDS−PAGE, and the hACAT1-HA bands were visualized by (A) anti-HA antibody or by (B) anti-ACAT1 antibodies (DM10). Strategies for direct and indirect modifications are depicted in Figure 1. (C) Analysis of the Lys-C digested mixture by tricine SDS−PAGE and Western blot using the anti-HA antibody. The details are described in Materials and Methods. Results are representative of two independent experiments.

fragments: Lys496−Lys531 and Asn532−Phe550−HA tag. When the digest was treated with DTT, the intensity of the 7 kDa band was greatly reduced (Figure 5C, lane 2). These results provide the additional evidence that the C528 and C546 indeed form a disulfide bond in hACAT1. When the Lys-C digest was treated with DTT, the HA tag should be present as the C-terminal fragment Asn532−Phe550−HA tag with an expected molecular mass of about 3 kDa. Unfortunately, in three separate attempts, we were unable to verify this prediction by Western blot analysis. This C-terminal fragment is small in size and is hydrophilic; these properties might have caused the fragment to be lost during

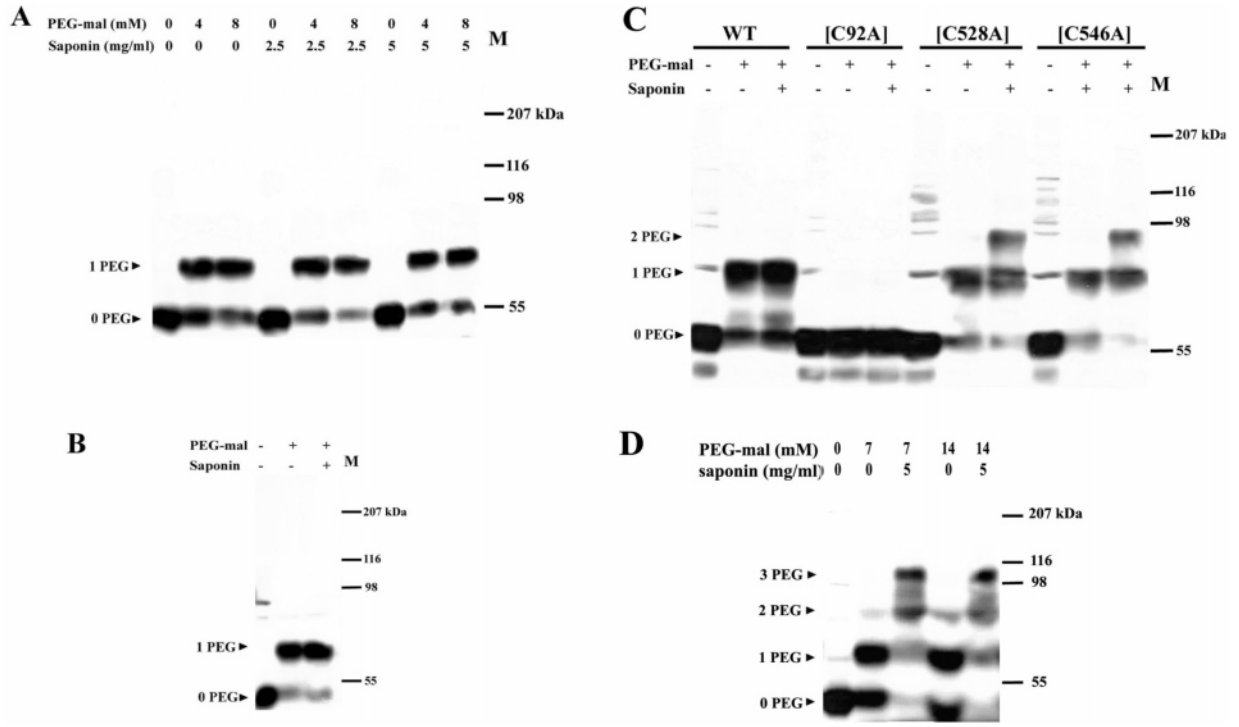

FIGURE 6: PEG-mal modification of WT and various mutant hACAT1s under native condition. (A) PEG-mal modification of WT hACAT1 stably expressed in CHO cells under native condition. The supernatants of the cell lysates were modified by PEG-mal at various concentrations as indicated, in the presence or absence of saponin as indicated, at 4 °C for 1 h. The modification reactions were terminated by adding ME at 10 mM and continuously incubated at 4 °C for 30 min. Thereafter, an equal volume of the lysate buffer plus 60 mM IA was added per sample to modify the unreacted Cs under denatured condition. The modified mixtures were analyzed by SDS−PAGE, and the ACAT1 bands were visualized by Western blot using anti-ACAT1 antibodies. (B) PEG-mal modification of hACAT1 expressed in differentiated human THP-1 cells under native condition. The modification procedure was the same as described in (A). The final PEG-mal concentration for modification is 4 mM; the final saponin concentration is 5 mg/mL. (C) PEG-mal modifications of WT and various mutant His-hACAT1s, with single C to A mutations as indicated. The modification procedure is the same as described in (A). The final PEG-mal concentration in modification is 4 mM; the final saponin concentration is 5 mg/mL. (D) PEG-mal modification of DTT-reduced hACAT1. The CHO cells stably expressing wild-type hACAT1 was treated with 100 mM DTT in cold PBS (to reduce the disulfide linkage); then the cells were lysed by homogenization, and the supernatant was used to carry out PEG-mal modification with or without saponin as indicated. The concentrations of PEG-mal were as indicated. The details are described in Materials and Methods. Results are representative of two independent experiments.

the transfer and washing procedures that were used in the Western blot analysis.

PEG-mal Modification of ACAT1s under Native Condition. As shown above, all of the free Cs of hACAT1 can be modified by both PEG-mal and IA under denatured condition. However, the accessibility of these free Cs under native condition is unknown. PEG-mal is a membrane-impermeant reagent and has been used to study the topology of membrane proteins (*33*). To gain information on the environment of various cysteines of hACAT1, we used PEG-mal to probe the environment of the free cysteines as well as that of the disulfide of ACAT1 present in microsomal vesicles. We have previously shown that microsomes prepared by the isolation procedure used here (described in Materials and Methods) are nonleaky, right-side out vesicles; we also showed that treating these microsomes with the mild detergent saponin at 2.5 mg/mL causes these microsomes to become permeable to various large molecules (*25*). As shown in Figure 6A, when recombinant hACAT1 expressed in CHO cells was tested under native condition, only one PEG could be attached, with or without microsome permeabilization by saponin. This result suggests that only one free cysteine is located on the cytoplasmic side of the ER, where the redox potential favors free cysteines (*34*); the other six free cysteines are buried in or near the transmembrane domains or at the hydrophobic interior of the protein. We next repeated the same experiment, using the microsomes prepared from the PMA-treated THP-1 cells as the hACAT1 source, and obtained the same result (Figure 6B). According to the previous ACAT1 membrane topology models (*15*, *16*), the N-terminal region of ACAT1 is located in the cytoplasm, while the C-terminal region is located in the lumen of the ER. To test this prediction, the C92A ACAT1 mutant, the C528A mutant, and the C546A mutant were used to carry out the PEG-mal modifications under native condition. As shown in Figure 6C, no PEG can be attached to the C92A-ACAT1 mutant either with or without saponin treatment, supporting the conclusion that C92 of hACAT1 is indeed on the cytoplasmic side of the ER membrane. For the C528A and the C546A mutants, one PEG can be attached without saponin treatment, while two PEGs can be attached with saponin treatment. Thus, both the C546 in the C528A mutant and the C528 in the C546A mutant are located in the lumen of the ER. We next tested the validity of this interpretation by using the WT ACAT1 as the enzyme source. As shown in Figure 6D, only one PEG can be attached to the DTT-treated hACAT1 without saponin treatment; this PEG is predicted to be attached to C92. However, with saponin, three PEGs can be attached to the DTT-treated ACAT1. These results support the conclusion that both C528 and

C546 are located in the ER lumen [where the redox potential favors disulfide bond formation as catalyzed by enzyme(s) (*34*−*37*)].

Contribution of the Disulfide Bond to ACAT1 Activity and Its Expression Level in CHO Cells. Previously, the possible contribution of each C residue to ACAT1 activity has been investigated by using the ACAT1 mutants expressed in insect cells (*26*). The drawback of this system is that the high overexpression may cause the ACAT1 proteins to contain various misfolded species; thus, the activity of ACAT1 could not be accurately determined. Here, we measured the activity of the various single C to A ACAT1 mutants in intact CHO cells. To normalize the enzyme activities, we determined the ACAT1 protein expression levels by Western blots. As shown in Figure 7, all of the ACAT1 mutants tested in the current work are essentially fully active. Therefore, none of the free Cs, or the disulfide between C528 and C546, is necessary for ACAT activity. We did note that, unlike the other single C to A mutants, the expression levels of the two mutants lacking the disulfide bond (i.e., the C528A mutant and the C546A mutant) decreased significantly, to approximately 25% of that for the WT ACAT1, as shown in Figure 7B. These results suggest that the disulfide bond may contribute to the stability of ACAT1 in intact cells.

DISCUSSION

ACAT1 contains nine Cs. Because of the enzyme's low expression level and low yield in purification, it has been very difficult to use conventional protein chemical methods to elucidate the disulfide linkage. In the present work, we describe a new method to quantify and map the disulfide linkage. The strategy mainly includes three steps: (1) determining the number of free and/or bonded Cs by PEG-mal with various concentrations of IA, with or without DTT under the denatured condition; (2) determining the position of those bonded Cs by analyzing the PEG-mal modification patterns of various single C to A mutants under denatured condition; (3) confirming the presence of the disulfide through appropriate endoproteinase digestion. The results demonstrated that there is only one disulfide bond, formed between C528 and C546, in hACAT1. The method described in our current work can also be used to investigate the disulfide linkage in other proteins with low expression levels, as long as the number of disulfide bonds is limited to one or two. For those proteins without specific antibodies available, a small peptide tag (i.e., HA, c-myc) can be inserted at the appropriate site to aid in detection using Western blot analysis.

ACAT1 contains multiple transmembrane domains. Previously, ACAT1 membrane topology has been investigated by using two different methods: either by insertion of a small antigenic peptide tag (HA) at various hydrophilic segments of the protein (*15*) or by successive truncation from the C-terminus (*16*). These two methods led to two similar but different topology models: both models predicted that the N-terminal fragment is located in the cytoplasm and the C-terminal is located in the lumen of the ER, and both models support the existence of four TMDs near the N-terminal half and the existence of one TMD near the C-terminal (*15*, *16*). However, the HA tag method detected two more TMDs in the middle section of the protein and

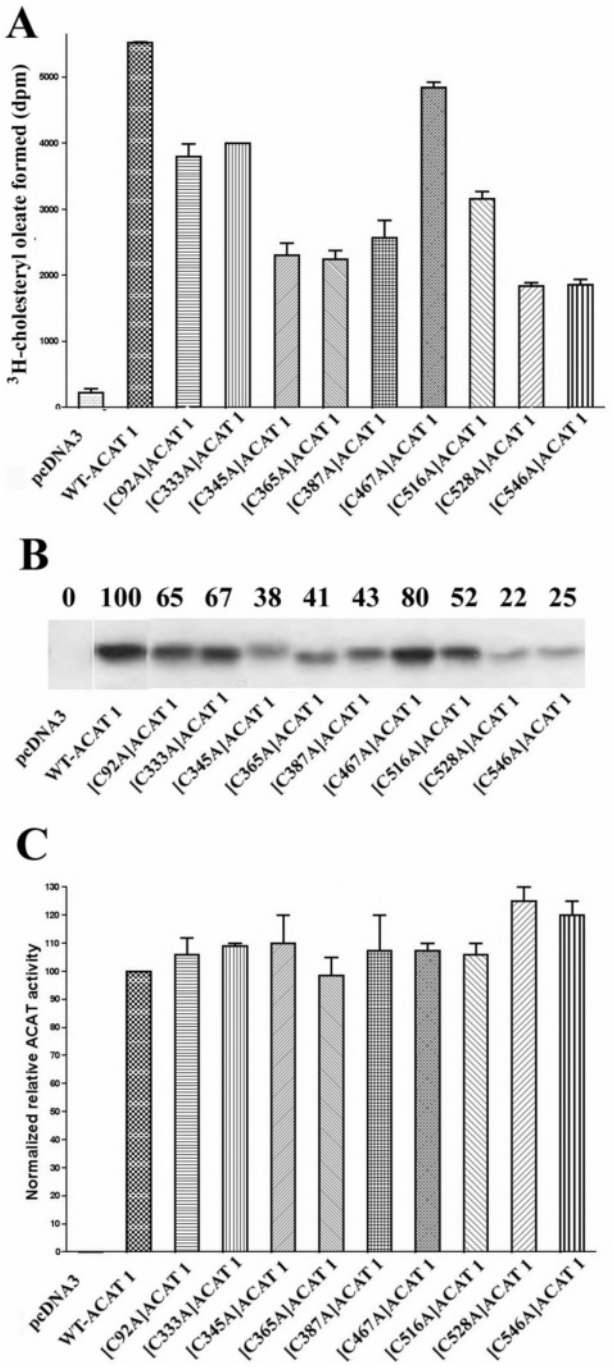

FIGURE 7: Activities and expression levels of various single C to A hACAT1 mutants as indicated, transiently expressed in CHO cells. (A) Total ACAT activities. (B) Expression levels. The CHO cells transiently expressing ACAT1 mutants were lysed by lysate buffer containing 10 mM IA and analyzed by SDS−PAGE and Western blots. (C) Normalized relative ACAT activities. The dpm value of the cells transfected by the pcDNA3 vector only was used as the blank value. The value found in cells expressing WT His-hACAT1 was used as the 100% value. The details are described in Materials and Methods. Results are representative of two independent experiments.

supported a seven-TMD model (15), while the truncation method supported a five-TMD model (16). Both methods have certain drawbacks. For instance, the HA tag inserted at certain regions caused partial loss in ACAT1 enzyme activity (*15*), while the truncation method invariably led to total inactivation in ACAT enzyme activity. More recently, the PEG-mal modification method together with C-scanning

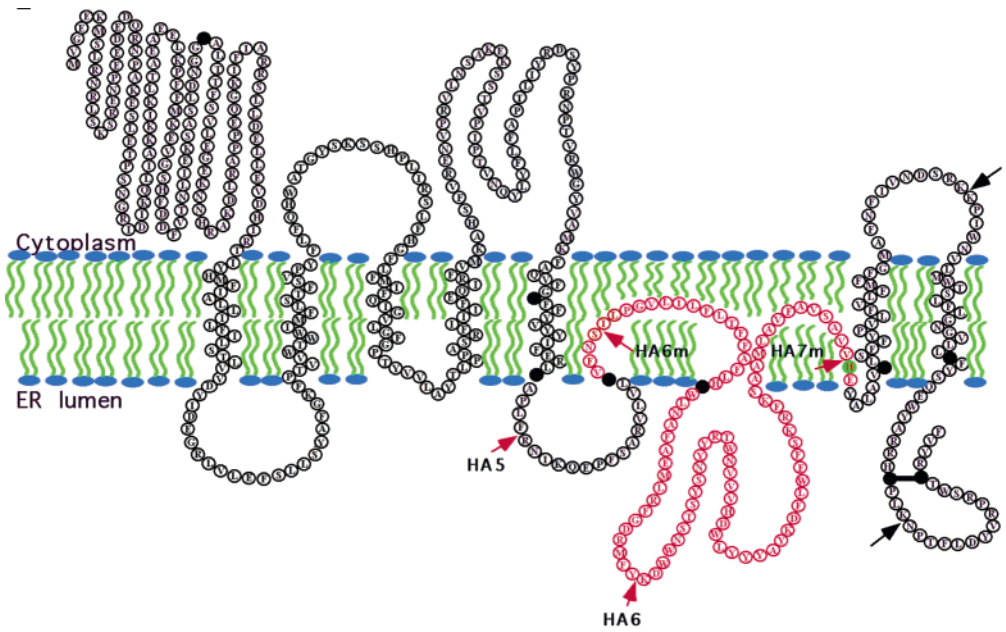

FIGURE 8: Revised 7-TMD topology model for ACAT1. The free Cys are shown as filled circles; the disulfide bond is shown as a bar. Black arrows indicate the Lys-C cleavage sites at the C-terminus. This model is a revision of our previous model which was deduced by using the HA tag insertion method (*15*). The sites for inserting the tags HA5, HA6, HA6m, and HA7m are indicated by red arrows. On the basis of the results in ref *15*, the HA5 tag and the HA6 tag are in the ER lumen; the HA6m tag and the HA7m tag may be embedded in the ER membrane. Our current result cannot exclude the possibility that the regions between TMD 5 and TMD 6 (depicted in red color) may form two additional TMDs.

mutagenesis has been used successfully to investigate the membrane topology of various membrane proteins (*33*, *38*, *39*). The major advantage of this method is that it only introduces point mutations on nonconserved residues and is expected to produce minimal disturbance on the topology of native membrane proteins. Indeed, the present data show that single C mutagenesis at nonconserved amino acid residues of hACAT1 has produced minimal perturbations on its enzyme activity. Using this approach, our current results show that C92 is on the cytoplasmic side of the ER membrane, while all other remaining Cys are either buried within the ER membrane or folded within various regions of the ACAT1 protein itself. Hydropathy analysis shows that each of the remaining Cys is located within a long stretch of hydrophobic peptide with high α-helical propensity. We thus hypothesize that all of the remaining Cs are parts of the hydrophobic peptides located within the ER membrane. Our additional results show that the disulfide bond, formed between C528 and C546, is located in the lumen of the ER membrane. Together, these results support a revised seven-TMD model for ACAT1, as shown in Figure 8. This model deviates from the original seven-TMD model, which placed two Cys (C333 and C516) in the ER membrane and four other Cys (C345, C365, C387, C467) in the ER lumen (*15*). Our previous and current results are not compatible with the five-TMD model (*16*), which would place all of the following Cs, C333, C345, C365, C387, and C467, on the cytoplasmic side of the ER membrane.

ACAT1 is the prototypic member of a multimembrane spanning acyltransferase family with more than 20 members that include ACAT1, ACAT2, and diacylglycerol acyltransferase 1 (DGAT1). Within this family, an invariant His residue (His460 in hACAT1) may be part of the enzyme's active site (*23*, *25*). Our current model predicts that His460 is located within the ER membrane. This configuration would allow ACAT1 to catalyze the formation of cholesteryl esters (CEs) in the membrane and serve a dual function: CEs can be removed from the cytoplasmic leaflet of the membrane for cytosolic lipid droplet formation; alternatively, CEs can be removed from the lumenal leaflet of the membrane and enter the lipoprotein assembly process, which occurs in the ER lumen (*24*). Our current results cannot exclude the possibility that the hydrophobic segments located between TMD 5 and TMD 6, depicted in red color in Figure 8, may form two additional TMDs. In the future, this issue can be addressed by employing C-scanning mutagenesis, producing specific single Cs at selective amino acid residues within these regions, and then probing the environment of these residues by using PEG-mal modification under native condition.

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