Functional analysis of the missense *APOC3* mutation Ala23Thr associated with human hypotriglyceridemia

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Abstract **We have shown that expression of apolipoprotein (apo) C-III promotes VLDL secretion from transfected McA-RH7777 cells under lipid-rich conditions. To determine structural elements within apoC-III that confer to this function, we contrasted wild-type apoC-III with a mutant Ala23Thr originally identified in hypotriglyceridemia subjects. Although synthesis of [^{3}H]glycerol-labeled TAG was comparable between cells expressing wild-type apoC-III (C3wt cells) or Ala23Thr mutant (C3AT cells), secretion of [^{3}H]TAG from C3AT cells was markedly decreased. The lowered [^{3}H]TAG secretion was associated with an inability of C3AT cells to assemble VLDL$_1$. Moreover, [^{3}H]TAG within the microsomal lumen in C3AT cells was 60% higher than that in C3wt cells, yet the activity of microsomal triglyceride-transfer protein in C3AT cells was not elevated. The accumulated [^{3}H]TAG in C3AT microsomal lumen was mainly associated with lumenal IDL/LDL-like lipoproteins. Phenotypically, this [^{3}H]TAG fractionation profiling resembled what was observed in cells treated with brefeldin A, which at low dose specifically blocked the second-step VLDL$_1$ maturation. Furthermore, lumenal [^{35}S]Ala23Thr protein accumulated in IDL/LDL fractions and was absent in VLDL fractions in C3AT cells.** **These results suggest that the presence of Ala23Thr protein in lumenal IDL/LDL particles might prevent effective fusion between lipid droplets and VLDL precursors. Thus, the current study reveals an important structural element residing within the N-terminal region of apoC-III that governs the second step VLDL$_1$ maturation.—** Sundaram, M., S. Zhong, M. B. Khalil, H. Zhou, Z. G. Jiang, Y. Zhao, J. Iqbal, M. M. Hussain, D. Figeys, Y. Wang, and Z. Yao. **Functional analysis of the missense APOC3 mutation Ala23Thr associated with human hypotriglyceridemia. *J. Lipid Res.* 2010.** 51: **1524–1534.**

Supplementary key words triacylglycerol • VLDL assembly • apolipoprotein C-III • lumenal lipid droplets

Apolipoprotein (apo) C-III is a small exchangeable apolipoprotein (79 amino acids) and a major protein constituent of plasma VLDL and HDL (1). Elevated plasma apoC-III level is positively correlated with plasma triacylglycerol (TAG) concentration in hypertriglyceridemia subjects (2, 3). The strong correlation between plasma apoC-III and TAG levels has also been suggested in a genome-wide association studies with the Lancaster Amish population, which showed that individuals with an apoC-III null allele (R19X) had lower fasting and postprandial plasma TAG level as well as significantly reduced incidence of coronary artery calcification, which suggested that the deficiency of apoC-III could also exert an effect on cardioprotection (4). In addition, transgenic mice overexpressing human apoC-III showed severe hypertriglyceridemia and, in some cases, even hepatosteatosis (5, 6). The positive link between plasma apoC-III and TAG levels has been attributed to apoC-III's inhibitory effect toward the activity of lipoprotein lipase (LPL) (7, 8) and interference with binding/uptake of TAG-rich lipoproteins through receptor-dependent (9) and receptor-independent (10) endocytic pathways. Recently, cumulative in vivo and in vitro evidence suggests that the impact of apoC-III on plasma TAG concentration may result from its function in promoting hepatic VLDL secretion. An in vivo link between

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Abbreviations: apo, apolipoprotein; ARF1, ADP ribosylation factor 1; C3AT, Ala23Thr; C3wt, wild-type apoC-III; ER, endoplasmic reticulum; IDL, intermediate density lipoprotein; iPLA$_2$, calcium-independent phospholipase A$_2$; LPL, lipoprotein lipase; MTP, microsomal triglyceride-transfer protein; PC, phosphatidylcholine; PLD, phospholipase D; TAG, triacylglycerol.

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apoC-III levels and VLDL production was reported in overweight individuals with reduced insulin sensitivity (11) and was also demonstrated in human subjects under different metabolically stressed conditions (12–16). We have recently provided in vitro evidence that hepatic apoC-III expression augments VLDL assembly/secretion from cultured hepatic cells under lipid-rich conditions (17).

The mechanism by which increased apoC-III expression promotes VLDL assembly and secretion is unclear. Structural analysis of apoC-III has shown that the protein is composed of six amphipathic helices with several flexible linker regions (18). Each helix is approximately 10 amino acids in length and in close contact with the surface of lipid micelles. Several missense mutations of human *APOC3* have been described (19–22); one of which, the Ala23Thr mutation (A23T) was found in Mayan Indian subjects with hypotriglyceridemia (19). Two of the heterozygous A23T carriers had plasma TAG concentrations that were 25% and 48%, respectively, of normal (19). The Ala23Thr mutant displayed reduced affinity to phospholipid liposomes with normal inhibitory effect on in vitro LPL activity (19). Residue Ala23 is located on the hydrophobic side of helix 2, a region that shares structural homology with lipoprotein receptor binding motifs found in apoE and apoB-100 (18). Our recent transfection studies have suggested that the Ala23Thr mutant of apoC-III lost its ability to stimulate hepatic VLDL secretion under lipid-rich conditions (17). The mechanism responsible for the loss of function of A23T mutation in promoting hepatic VLDL assembly/secretion has not been defined.

Hepatic VLDL assembly takes place during apoB-100 translation and translocation across the endoplasmic reticulum (ER) membrane. However, maturation of VLDL, especially TAG-rich $VLDL_1$, is accomplished through a multiple-lipidation process that occurs when nascent VLDL particles (VLDL precursors) traverse through the ER/Golgi secretory compartments (23–25), although some denser VLDL particles (e.g., $VLDL_2$) may reach maturation within the ER (26, 27). The lipid substrates (mainly TAG) for apoB-100 lipidation are partitioned into the microsomal lumen, probably in the form of lumenal lipid droplets (28), through a process that requires the activity of microsomal triglyceride-transfer protein (MTP) (29–31). Kinetic analysis of apoB-100 and TAG assembly has suggested that while the MTP activity is required for the early stage of apoB-100 lipidation, the recruitment of lipid droplets into VLDL at the late stage is achieved via an MTP-independent process (commonly known as second-step lipidation) (25, 32). The second-step lipidation is sensitive to brefeldin A (33, 34), a fungal metabolite that potently interferes with ER vesiculation mediated through formation of coatomers. Treatment with brefeldin A at low dose (0.2 μg/ml) does not compromise general ER/Golgi trafficking, but it effectively blocks the assembly/secretion of TAG-rich $VLDL_1$ containing apoB-100 or apoB-48 without affecting their denser precursor lipoproteins (31, 33, 34). In addition, the activities of two phospholipases have been shown to play a role in VLDL maturation, the calcium-independent phospholipase A_2 ($iPLA_2$) and phospholipase D (PLD1) (35, 36). Expression of aberrant ADP ribosylation factor 1 (ARF1 that activates PLD1) (37) or treatment with brefeldin A (inhibitor of ARF1) (31, 33) effectively prevented TAG-rich VLDL secretion with little effect on the denser particles. Thus, maturation of VLDL probably not only requires sufficient lipid substrate (mainly TAG) availability, but it also depends on the protein factors that effectively mediate the fusion of lipid droplets with apoB to form TAG-rich VLDL.

Using a pulse-chase approach, we showed that apoC-III expression could promote $VLDL_1$ maturation even under the conditions that MTP activity was transiently suppressed, which suggests that apoC-III may play an important role in second-step lipidation through an MTP-independent mechanism (17). The current work aims to define mechanisms whereby the A23T mutation in apoC-III cause hypotriglyceridemia. Our data suggest that the A23T mutant expression prevented bulk TAG incorporation during the second-step VLDL maturation.

MATERIALS AND METHODS

Materials

Medium and reagents used for cell culture studies were obtained from Invitrogen (Burlington, ON). [^{35}S]methionine/cysteine (1,000 Ci/mmol) and protein A-Sepharose™ CL-4B beads were obtained from GE Healthcare (Mississauga, ON). [2-^{3}H]glycerol (9.6 Ci/mmol), brefeldin A, and horseradish peroxidase-linked anti-goat antibody were obtained from Sigma-Aldrich (Oakville, ON). Oleate, TAG, and phospholipid standards were from Avanti Polar Lipids (Albaster, AL). Antibody against human apoC-III (used for Western blot analysis) was obtained from Academy Biomedical Co., Inc. (Houston, TX). Polyclonal antisera against rat VLDL (used for immunoprecipitation of apoB-100) or human apoC-III (used for immunoprecipitation of apoC-III) were generated in our laboratory. Protease inhibitor cocktail, horseradish peroxidase-linked anti-mouse and anti-rabbit IgG antibodies, and chemiluminescent substrates were obtained from Roche Diagnostics (Laval, PQ). MTP inhibitor (BMS-197636) was a gift from Dr. David Gordon (31).

Cell culture

Stably transfected McA-RH7777 cells expressing wild-type human apoC-III or the Ala23Thr mutant was generated as described previously (17). The cells were maintained in DMEM containing 10% fetal bovine serum, 10% horse serum, and 200 μg/ml G418.

Subcellular fractionation

C3wt and C3AT cells ($\sim 6 \times 10^6$ cells/100-mm dish, in duplicates) were labeled with [^{3}H]glycerol (15 μCi/ml) in DMEM supplemented with 20% FBS and 0.4 mM oleate for 30 or 60 min. At each time point, medium was collected for lipid analysis. Cells were harvested in 2 ml of Tris-sucrose buffer (10 mM Tris-HCl, pH 7.4, and 250 mM sucrose), which was supplemented with protease inhibitor cocktail, and homogenized in a ball-bearing homogenizer (20 passages). The post-nuclear supernatant was obtained by centrifugation (12,000 *g*, 4°C, 10 min) and was loaded into a quick-seal centrifuge tube. Cytosol and total microsomes were separated by centrifugation using a Beckman TLA-100.3 rotor (500,000 *g*, 4°C, 16 min). The total microsomes were rinsed twice with Tris-sucrose buffer to minimize cytosol

contamination. The lumenal contents were released from total microsomes with 0.1 M sodium carbonate (pH 11.3) by gentle mixing using a nutator for 30 min at room temperature. The lumenal contents were separated from microsomal membranes by ultracentrifugation (500,000 *g*, 4°C, 30 min). Lumenal contents were further subjected to cumulative rate flotation ultracentrifugation (31). Lipids were extracted from subcellular fractions (i.e., cytosol, total microsomes, microsomal lumen, and membrane) and lumenal lipoprotein fractions, separated by TLC, and the radioactivity associated with [^{3}H]TAG and [^{3}H]phosphatidylcholine (PC) was quantified by scintillation counting as previously described (17).

Metabolic labeling of apoB-100

Cells (100-mm dishes) were labeled with [^{35}S]methionine/cysteine (100 μCi/ml) for 60 and 90 min using 5 ml methionine/cysteine–free DMEM supplemented with 20% FBS and 0.4 mM oleate. Cells were harvested and homogenized using ball-bearing homogenizer. Microsomes were obtained by ultracentrifugation, and the lumenal contents were released by treatment with Na_2CO_3 as described above. The lumenal contents were separated by cumulative rate flotation ultracentrifugation, and [^{35}S]apoB-100 and [^{35}S]apoC-III were immunoprecipitated from each fraction using appropriate antibodies and analyzed by SDS-PAGE followed by fluorography. Radioactivity associated with these apolipoproteins was determined by scintillation counting. In some experiments, cells were treated with brefeldin A (0.2 μg/ml) and/or the MTP inhibitor BMS-197636 (0.2 μM) for 30 min, and labeled with [^{35}S]methionine/cysteine for 60 min prior to isolation of microsomes as described previously (31, 33).

Co-immunoprecipitation of apoB and apoC-III

McA-RH7777 cells expressing both human apoB-100 and apoC-III were cultured in DMEM containing 20% FBS for 1 h, and lysed in NP-40 buffer (37). The cell lysates were incubated with preimmune rabbit serum and Sepharose CL-4B beads for 2 h, followed by pelleting the beads by centrifugation (13,000 *g*, 10 min, 4°C). The supernatant was subjected to immunoprecipitation with anti-human apoB or anti-human apoC-III antibody, respectively. The immunocomplexes were resolved by SDS-PAGE to detect apoB and apoC-III by Western blot analysis.

Other assays

The activity of MTP was determined as described previously (17). Protein concentration was quantified by the Bradford method (38).

RESULTS

Human apoC-III A23T mutation leads to loss of function in promoting $VLDL_1$ assembly

Compared with neo control cells, expression of wild-type apoC-III in McA-RH7777 cells (designated C3wt cells) pro-

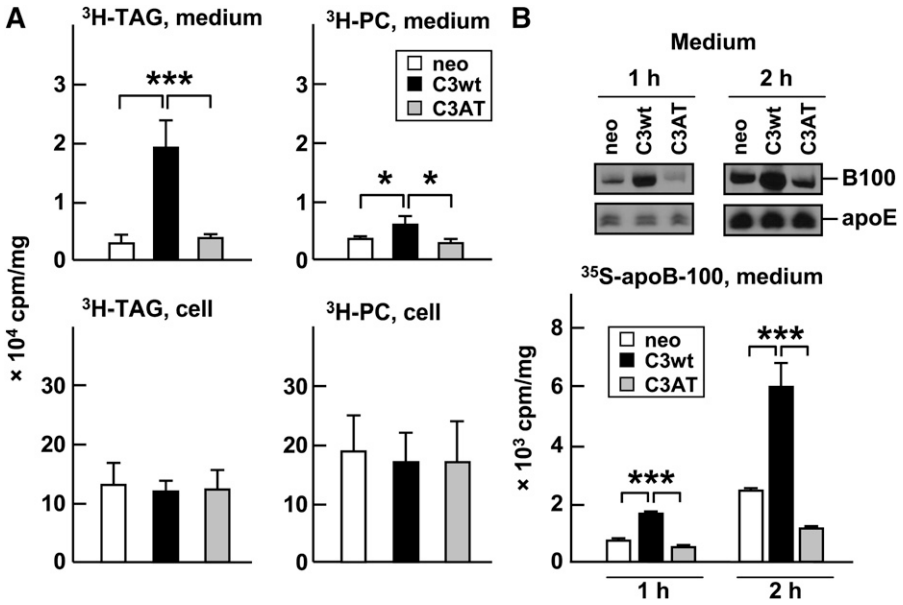

Fig. 1. Expression of Ala23Thr mutant in McA-RH7777 cells fails to stimulate [^{3}H]TAG or [^{35}S]apoB-100 secretion under lipid-rich conditions. A: Cells expressing wild-type apoC-III (C3wt) or Ala23Thr mutant (C3AT) or *neo* control were labeled with [^{3}H]glycerol for 2 h in DMEM containing 20% FBS and 0.4 mM oleate. Lipids were extracted from cells and media, respectively, at the end of labeling and separated by TLC. Radioactivity associated with [^{3}H]TAG and [^{3}H]PC was quantified by scintillation counting. The values are expressed as cpm/mg of cell protein. Statistical significance *** $P < 0.001$, *$P < 0.05$ (Student *t*-test of C3wt versus Neo, or C3wt versus C3AT as indicated). Error bars indicate average ± SD (n = 4 dishes of cells). B: The cells were continuously labeled with [^{35}S]methionine/cysteine for 1 h or 2 h with methionine/cysteine-free DMEM supplemented with 20% FBS and 0.4 mM oleate. [^{35}S]apoB-100 and [^{35}S]apoE were recovered from the medium by immunoprecipitation, and subjected to SDS-PAGE. Representative fluorograms of [^{35}S]apoB-100 and [^{35}S]apoE are shown. Quantification of radioactivity associated with [^{35}S]apoB-100 was achieved by scintillation counting (bottom panels) and the values are expressed as cpm/mg of cell protein. Statistical significance *** $P < 0.001$ (Student *t*-test of C3wt versus neo or C3wt versus C3AT as indicated). Error bars indicate average ± SD (n = 3 dishes of cells). apo, apolipoprotein; C3AT, Ala23Thr; C3wt, wild-type apoC-III; PC, phosphatidylcholine; TAG, triacylglycerol.

moted the secretion of [^{3}H]glycerol–labeled TAG or PC in the presence of 0.4 mM oleate, and the stimulatory effect of apoC-III in lipid secretion was abolished by the expression of the A23T mutant (designated C3AT cells) (**Fig. 1A**, top panels). The incorporation of [^{3}H]glycerol into cell-associated TAG and PC was comparable among the three groups of cells (Fig. 1A, bottom panels). As reported previously, the enhanced lipid secretion was accompanied by an increase in ^{35}S-labeled-apoB-100 secretion from C3wt, but not from C3AT, cells. Secretion of [^{35}S]apoE was not affected by apoC-III expression (Fig. 1B). Pulse-chase experiments showed that while expression of wild-type apoC-III resulted in increased secretion efficiency of [^{35}S]apoB-100, expression of the Ala23Thr mutant had no effect compared with neo control (supplementary Fig. I, left panel). The amount of cell-associated [^{35}S]apoB-100 during chase showed little difference between C3AT cells and the neo control (supplementary Fig. I, right panel). These results, consistent with what was reported previously (17), indicate that A23T is a loss-of-function mutation in human apoC-III that fails to promote secretion of TAG and apoB-100.

To determine whether the decreased apoB-100 and TAG secretion was attributable to impaired TAG-rich $VLDL_1$ assembly, we monitored newly synthesized [^{35}S] apoB-100 in association with lipoproteins within the microsomal lumen. Our previous kinetic studies showed that it took at least 35–40 min for the newly synthesized apoB-100 to become associated with $VLDL_1$, and the apoB-100–containing $VLDL_1$ assembly (i.e., the second step) was achieved in a post-ER compartment (25, 31). Thus, in the present study we labeled the cells with [^{35}S]methionine/cysteine for 60 and 90 min to allow full assembly of $VLDL_1$ within the microsomes. In C3wt cells, [^{35}S]apoB-100 was readily detectable in the $VLDL_1$ fraction at 60-min labeling time point and became prominent at 90 min (**Fig. 2A**). On the contrary, [^{35}S]apoB-100 was not detectable in the $VLDL_1$ fraction in C3AT cells; rather, [^{35}S]apoB-100 was found in denser lipoproteins such as $VLDL_2$, IDL, and LDL at 60 min (Fig. 2B). There was an insignificant amount of [^{35}S]apoB-100 associated with $VLDL_1$ in C3AT cells at 90 min labeling. These results suggest that while A23T mutation abolished apoC-III's ability to promote $VLDL_1$ assembly, it did not interfere with the assembly of denser $VLDL_2$, IDL, or LDL particles.

Accumulation of [^{3}H]TAG as IDL/LDL in the lumen of C3AT microsomes

We have shown previously that maturation of VLDL through the *cis*-most to the *trans*-most elements of Golgi saccules was associated with incremental enlargement of the particle diameters: 31 nm in the *cis* saccules and 43 nm in the *trans* end (25). The increase in VLDL diameter represents approximately 3-fold enlargement in volume, suggesting recruitment of core lipids within the Golgi lumen. To determine whether the impaired $VLDL_1$ formation in C3AT cells was attributable to the lack of lumenal lipid substrate, we compared lumenal distribution of newly synthesized lipid between C3wt and C3AT cells. To this end, we performed metabolic labeling with [^{3}H]glycerol in conjunction with subcellular fractionation at 30- and 60-min labeling time points. Distribution of the newly

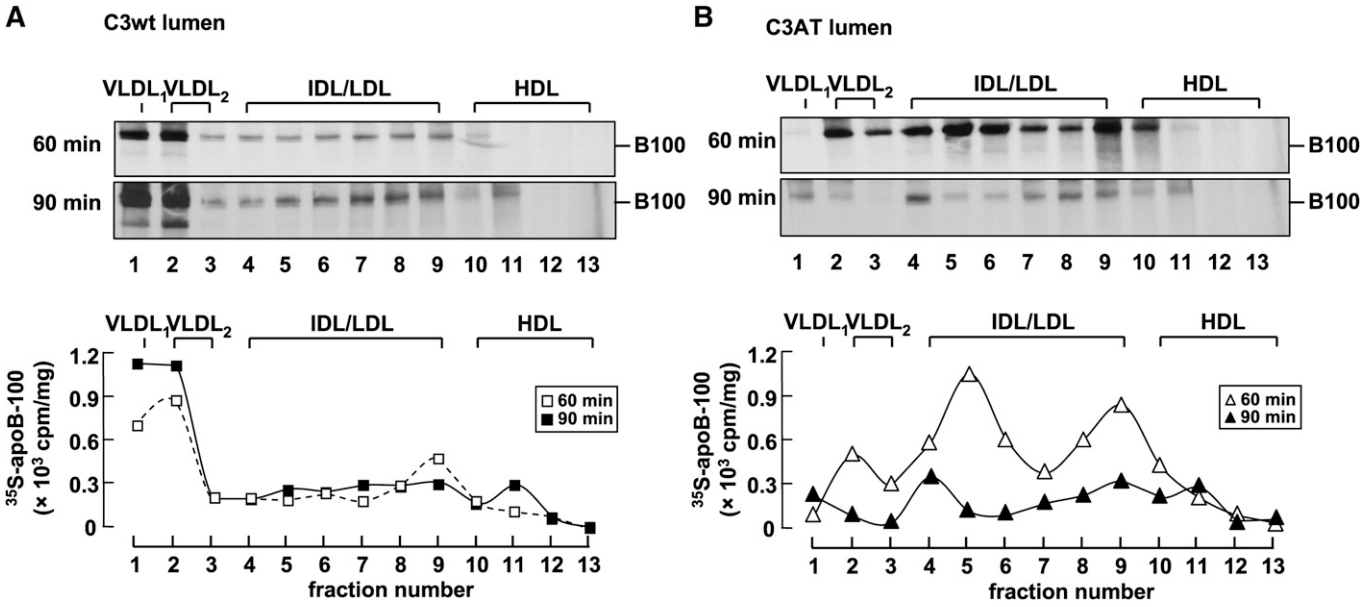

Fig. 2. Expression of Ala23Thr mutant in McA-RH7777 cells fails to promote $VLDL_1$ assembly. Cells expressing wild-type apoC-III (panel A) or Ala23Thr mutant (panel B) were labeled with [^{35}S]methionine/cysteine for 60 or 90 min in the presence of 20% serum and 0.4 mM oleate. Cells were homogenized at the end of labeling, and the microsomes were isolated from respective cells by ultracentrifugation. The microsome vesicles were treated with Na_2CO_3 to release the lumenal content, which was further separated into the indicated lipoprotein fractions by cumulative rate flotation centrifugation. The [^{35}S]apoB-100 in each fraction was recovered by immunoprecipitation and subjected to SDS-PAGE. Representative fluorograms of [^{35}S]apoB-100 in the lipoprotein fractions are shown on top. Radioactivities associated with [35S]apoB-100 were quantified by scintillation counting (bottom panels). apo, apolipoprotein; C3AT, Ala23Thr; C3wt, wild-type apoC-III.

synthesized TAG and PC between total microsomes (**Fig. 3A**), microsomal membrane (Fig. 3B), microsomal lumen (Fig. 3C), and cytosol (Fig. 3D) was determined. Results showed that microsome-associated [^{3}H]TAG in C3AT cells was increased compared with that in C3wt cells and that such an increase (by 110%) became prominent at the end of 60-min labeling (Fig. 3A, top). Separating the microsomes further into membrane and lumenal fractions revealed that the amount of [^{3}H]TAG at 60-min labeling time was increased in both membrane (by 180%) (Fig. 3B, top) and lumen (by 60%) (Fig. 3C, top) in C3AT cells. The level of [^{3}H]PC in lumenal content was also increased in C3AT cells (Fig. 3C, bottom). A slight but insignificant decrease in cytosol [^{3}H]TAG was consistently observed in C3AT cells compared with C3wt cells (Fig. 3D, top). Distribution of cytosolic [^{3}H]PC was comparable between C3wt and C3AT cells (Fig. 3D, bottom). These results suggest that C3AT cells had no deficiency in driving newly synthesized TAG into microsomes.

Further fractionation of the lumenal content showed a skewed distribution of newly synthesized [^{3}H]TAG toward IDL/LDL and HDL fractions in C3AT cells at 30 min, and IDL/LDL-associated [^{3}H]TAG became more prominent at 60 min (**Fig. 4A**). In contrast, the [^{3}H]TAG in IDL/LDL fractions in C3wt cells was significantly lower than that in C3AT cells (Fig. 4A). High concentration of [^{3}H]PC in the IDL/LDL fractions was also observed in the microsomal lumen of C3AT cells (Fig. 4B). As expected, secretion of [^{3}H]TAG (Fig. 4C) and [^{3}H]PC (Fig. 4D) was dramatically reduced in C3AT cells. Accumulation of lumenal [^{3}H]TAG and [^{3}H]PC in VLDL fractions was minimal in both C3wt and C3AT cells, as secretion of mature VLDL particle occurs rapidly after its assembly (25). The increased accumulation of TAG in lumenal IDL/LDL fractions in C3AT cells, together with its diminished TAG secretion, implies that the defect of Ala23Thr mutant in promoting VLDL assembly may be a result of impaired utilization of lumenal TAG for $VLDL_1$ maturation.

Expression of Ala23Thr mutant does not increase MTP activity

MTP plays a role in facilitating TAG partitioning into microsomes, and increased MTP activity is associated with increased TAG accumulation within the microsomal lumen (29–31). We then determined whether the increase in lumenal [^{3}H]TAG accumulation in C3AT cells was also a consequence of enhanced MTP activity. Comparison of the *Mttp* expression (by real-time RT-PCR) showed that while *Mttp* expression was increased in C3wt cells compared with neo-control cells, the enhanced *Mttp* expression was significantly diminished in the presence of C3AT mutant (**Fig. 5**A). Likewise, the in vitro TAG transfer activity of MTP was reduced in C3AT cells compared with C3wt cells (Fig. 5B). These data suggested that the increase in [^{3}H]TAG accumulation within the microsomal lumen of C3AT cells was not due to enhanced MTP activity in these cells.

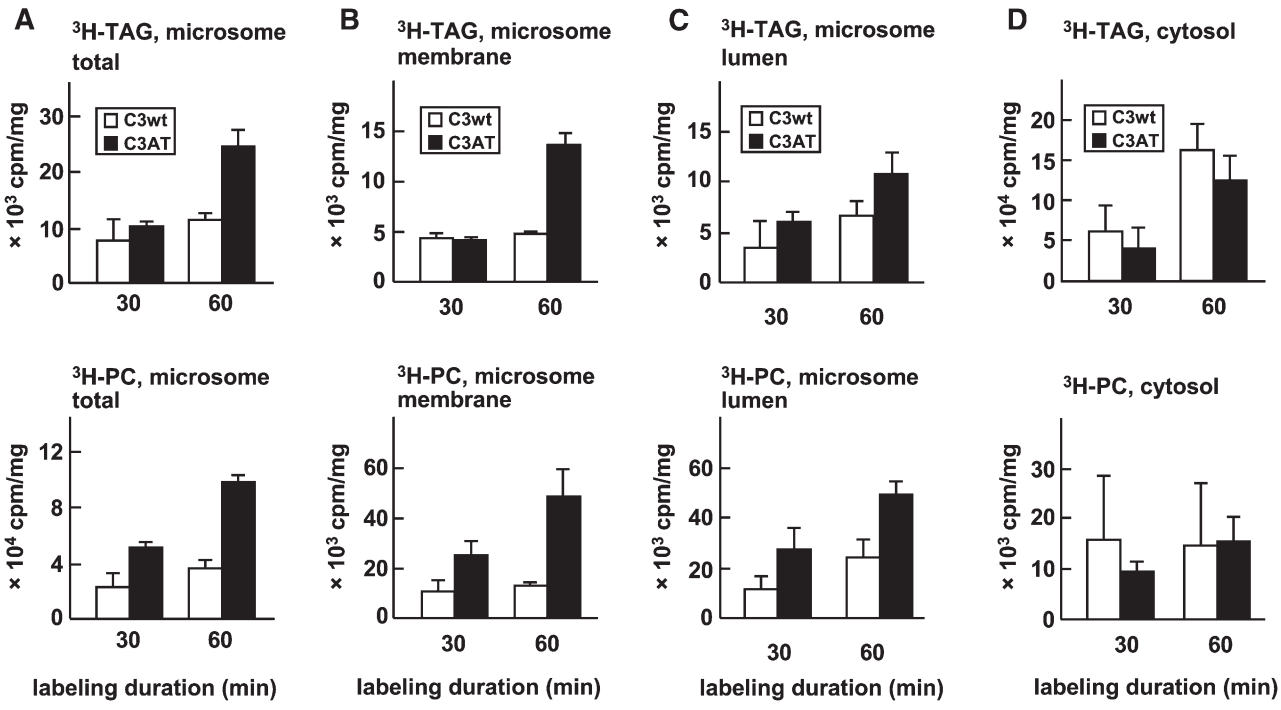

Fig. 3. Expression of Ala23Thr mutant in McA-RH7777 cells does not affect lipid partitioning into microsomes. The C3wt and C3AT cells were labeled with [^{3}H]glycerol (15 μCi/ml medium) for 30 min or 60 min. At the end of labeling, cells were homogenized and separated into cytosol and microsomes. The microsome vesicles were treated with Na_2CO_3, followed by ultracentrifugation to separate the membranes and lumen. Lipids were extracted from the respective samples, resolved by TLC, and the radioactivity associated with [^{3}H]TAG (top) and [^{3}H]PC (bottom) of microsomes (panel A), microsomal membranes (panel B), microsomal lumen (panel C), and cytosol (panel D) was quantified by liquid scintillation counting. Each bar represents average values from two dishes of cells. apo, apolipoprotein; C3AT, Ala23Thr; C3wt, wild-type apoC-III; PC, phosphatidylcholine; TAG, triacylglycerol.

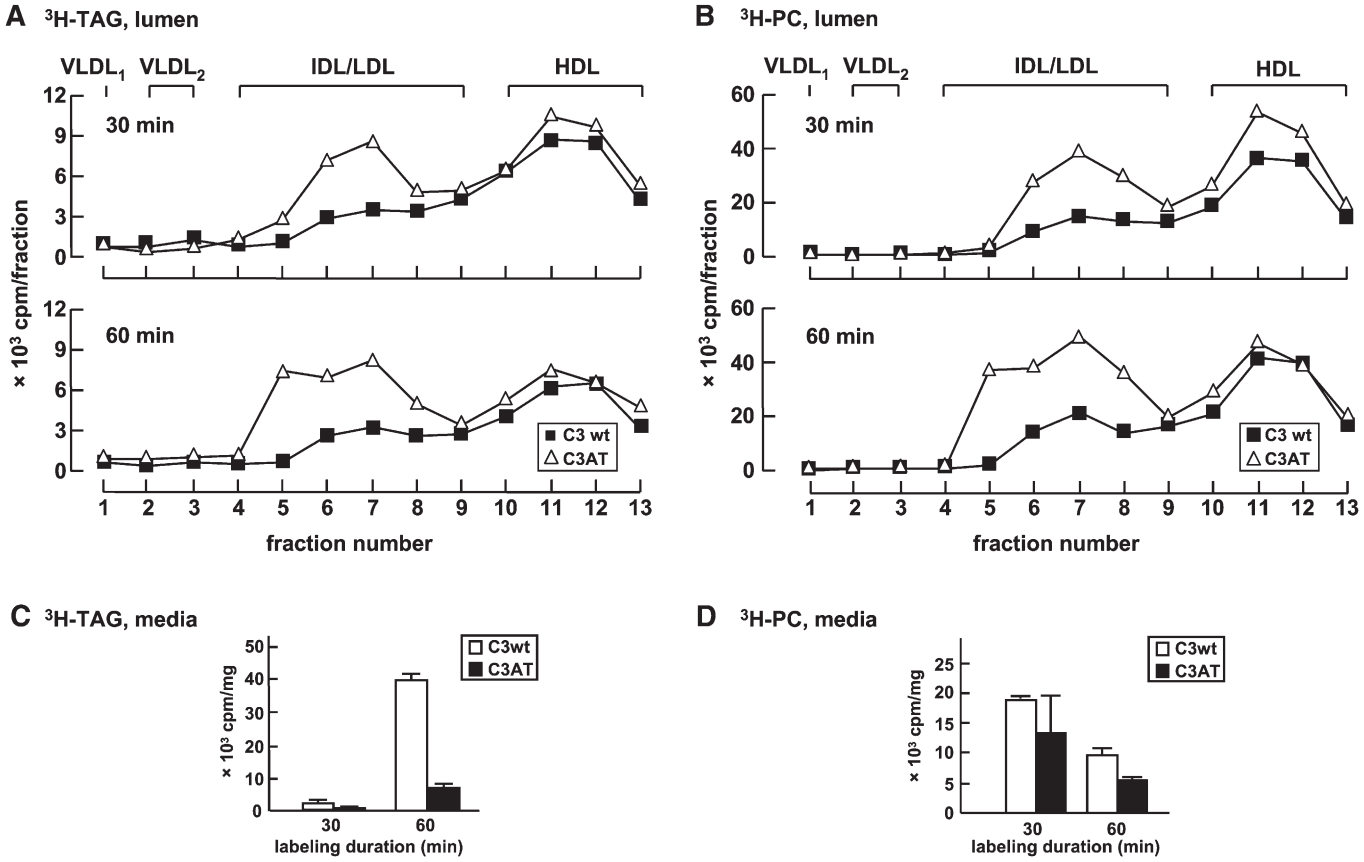

Fig. 4. Expression of Ala23Thr mutant results in accumulation of TAG and PC in IDL/LDL fractions in microsomal lumen. The lumenal content obtained in Fig. 3C was further separated into the indicated lipoprotein fractions by cumulative rate flotation centrifugation. Lipids were extracted from each lipoprotein fraction, and the radioactivity associated with [^{3}H]TAG (panel A) and [^{3}H]PC (panel B) was quantified. At each time point, [^{3}H]TAG and [^{3}H]PC that was secreted into the medium was also quantified as shown in panel C and panel D, respectively. C3AT, Ala23Thr; C3wt, wild-type apoC-III; IDL, intermediate density lipoprotein; PC, phosphatidylcholine; TAG, triacylglycerol.

Together, the data above suggest that the inability of C3AT cells to assemble $VLDL_1$ was not attributable to the lack of partitioning of lipid substrates into the microsomes. Rather, the utilization of lipid substrate for $VLDL_1$ assembly was impaired, leading to marked accumulation of newly synthesized TAG associated with IDL/LDL within the microsomal lumen in C3AT cells.

The Ala23Thr mutant prevents second-step VLDL assembly similar to brefeldin A

The second-step lipidation, involving the fusion of lipid droplets to VLDL precursors, is sensitive to brefeldin A treatment (33, 34). We then determined lumenal TAG distribution in cells treated with brefeldin A or with both brefeldin A and MTP inhibitor BMS-197636. As shown previously, assembly of TAG-rich apoB-100-VLDL, but not its denser precursor particles, was inhibited by low-dose brefeldin A (**Fig. 6A**). On the contrary, assembly of all apoB-containing lipoproteins (ranging from VLDL to LDL), as expected (31), was drastically suppressed by MTP inhibition (Fig. 6B). Under conditions with low-dose brefeldin A where the second-step VLDL maturation was blocked, [^{3}H]TAG (Fig. 6C) and [^{3}H]PC (Fig. 6D) accumulated in the microsomal lumen in the forms of IDL/LDL. This lumenal lipid profiling in brefeldin A–treated cells is almost identical to that obtained in C3AT cells (Fig. 4). Lumenal [^{3}H]TAG and [^{3}H]PC contents were also drastically suppressed by MTP inhibition (Fig. 6C, D), reinforcing the notion that the MTP activity is required for partitioning of lipid into microsomal lumen.

Accumulation of Ala23Thr mutant in lumenal IDL/LDL

The above results suggest that the Ala23Thr mutant protein might interfere with fusion of lipid substrates with VLDL precursors. Structural analysis of human apoC-III by NMR (18) has located residue Ala23 on the hydrophobic surface of helix 2 (**Fig. 7A**). Thus, it is expected that the A23T mutation would disrupt amphipathicity of α helices by introducing a polar residue on the hydrophobic surface (Fig. 7B). To test whether A23T mutation affects apoC-III protein interaction with lipids, we contrasted Ala23Thr mutant protein distribution among lumenal lipoproteins with that of wild-type apoC-III protein. Fractionation of the lumenal content revealed that while wild-type [^{35}S]apoC-III was distributed among all of the lipoprotein fractions (including VLDL fractions), the Ala23Thr mutant protein was absent in $VLDL_1$ and $VLDL_2$ fractions but accumulated in IDL/LDL fractions (Fig. 7C). This abnormal distribution of Ala23Thr mutant protein within the lumen coincided with that of [^{3}H]TAG in the

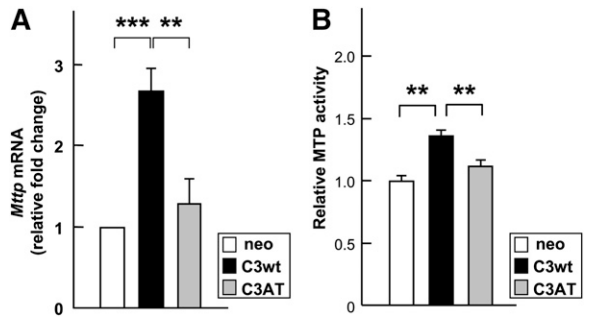

Fig. 5. Expression of Ala23Thr mutant does not increase *Mttp* expression or MTP activity. A: The relative *Mttp* mRNA concentrations (with respect to 18s rRNA) were quantified by real-time RT-PCR. Data are presented as fold changes between C3wt and C3AT cells in comparison to that in neo control (set as 1). B: The TAG transfer activity of MTP was determined using cell lysate obtained from C3wt, C3AT, or neo control and the MTP activity is expressed relative to neo control. Each bar represents average values ± SD obtained from triplicate samples. ***$P < 0.001$; **$P < 0.01$ (Student *t*-test of C3wt versus neo or C3wt versus C3AT as indicated). C3AT, Ala23Thr; C3wt, wild-type apoC-III; MTP, microsomal triglyceride-transfer protein; PC, phosphatidylcholine; TAG, triacylglycerol.

same cells (shown as dashed lines in Fig. 7C, bottom panel), suggesting that the mutant protein probably was associated with lipid droplets as IDL/LDL-like entities. The presence of the mutant proteins on the surface of these lipid droplets may curtail incorporation of lipid droplets into VLDL. Co-immunoprecipitation (co-IP) experiments showed that only trace amounts of apoC-III proteins could be detected by Western blot analysis of the sample pulled down using an anti-apoB antibody (Fig. 7D, left two lanes). When coIP experiments were performed using anti-apoC-III antibody, there was no detectable apoB-100 by Western blot analysis (Fig. 7D, middle two lanes). Thus, interaction between apoC-III and apoB-100 or apoB-48 within the secretory pathway was minimal. These results suggest that the Ala23Thr mutant, through its association with TAG, may interfere with VLDL maturation by preventing second-step fusion.

DISCUSSION

Consistent with the notion that apoC-III plays a stimulatory role in second-step lipidation, our current study shows that the defect of Ala23Thr mutant resides at the final stage of the $VLDL_1$ maturation, leading to impaired utilization of lumenal TAG. A series of metabolic labeling experiments in conjunction with subcellular fractionation were designed to trace the path through which the newly synthesized TAG was distributed among various compartments within the cells. Our data indicate that the Ala23Thr mutant expression has no impact on TAG partitioning

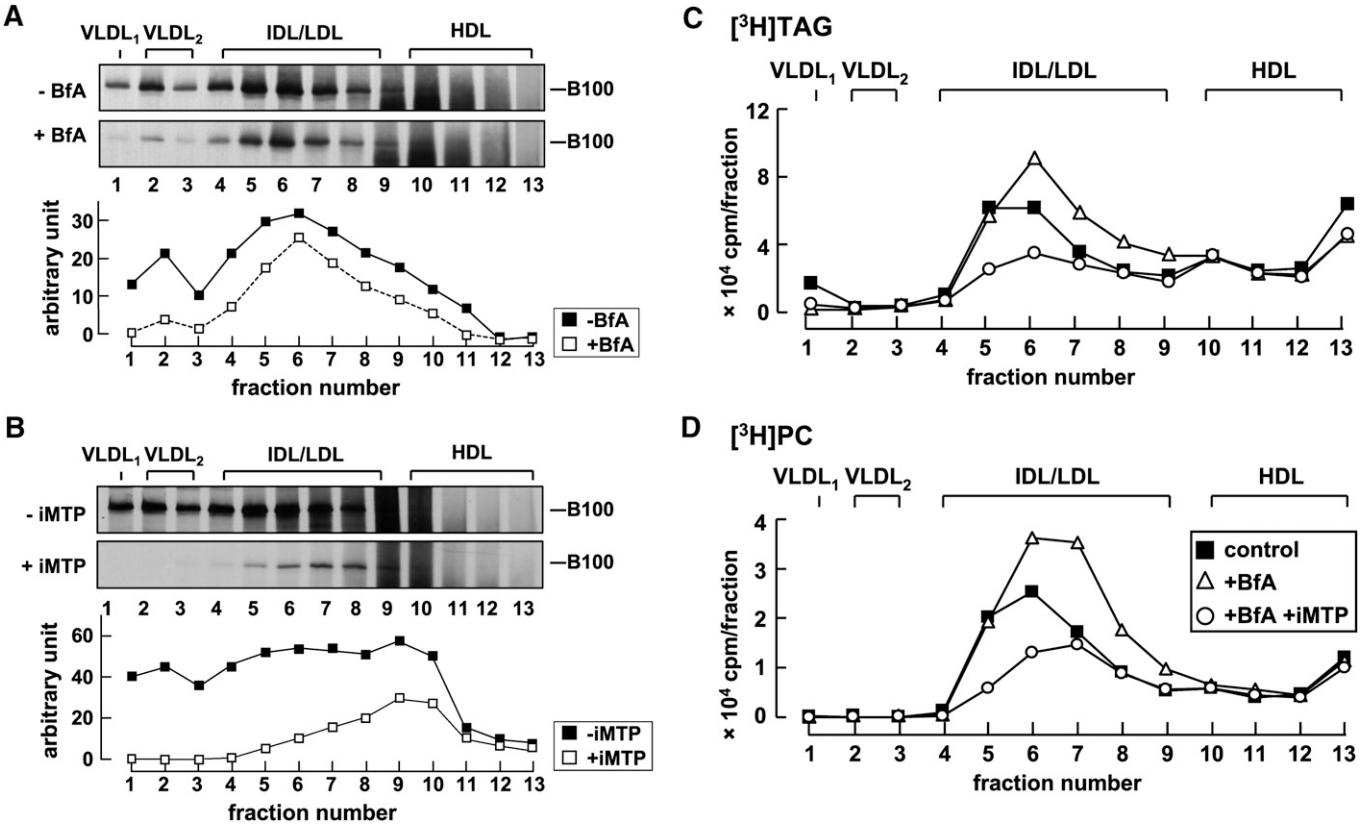

Fig. 6. Treatment with low dose brefeldin A blocks second step $VLDL_1$ assembly and results in accumulation of TAG and PC in IDL/LDL fractions in microsomal lumen. A and B: Cells were pretreated with brefeldin A (Bfa; 0.2 μg/ml) or Bfa + MTP inhibitor (iMTP; 0.2 μM) for 30 min prior to labeling with [^{35}S]methionine/cysteine for 60 min. Both pretreatment and labeling media contained 20% FBS and 0.4 mM oleate. C and D: Cells were treated exactly the same as in A and B, and labeled with [^{3}H]glycerol for 60 min. C3AT, Ala23Thr; C3wt, wild-type apoC-III; IDL, intermediate density lipoprotein; MTP, microsomal triglyceride-transfer protein; PC, phosphatidylcholine; TAG, triacylglycerol.

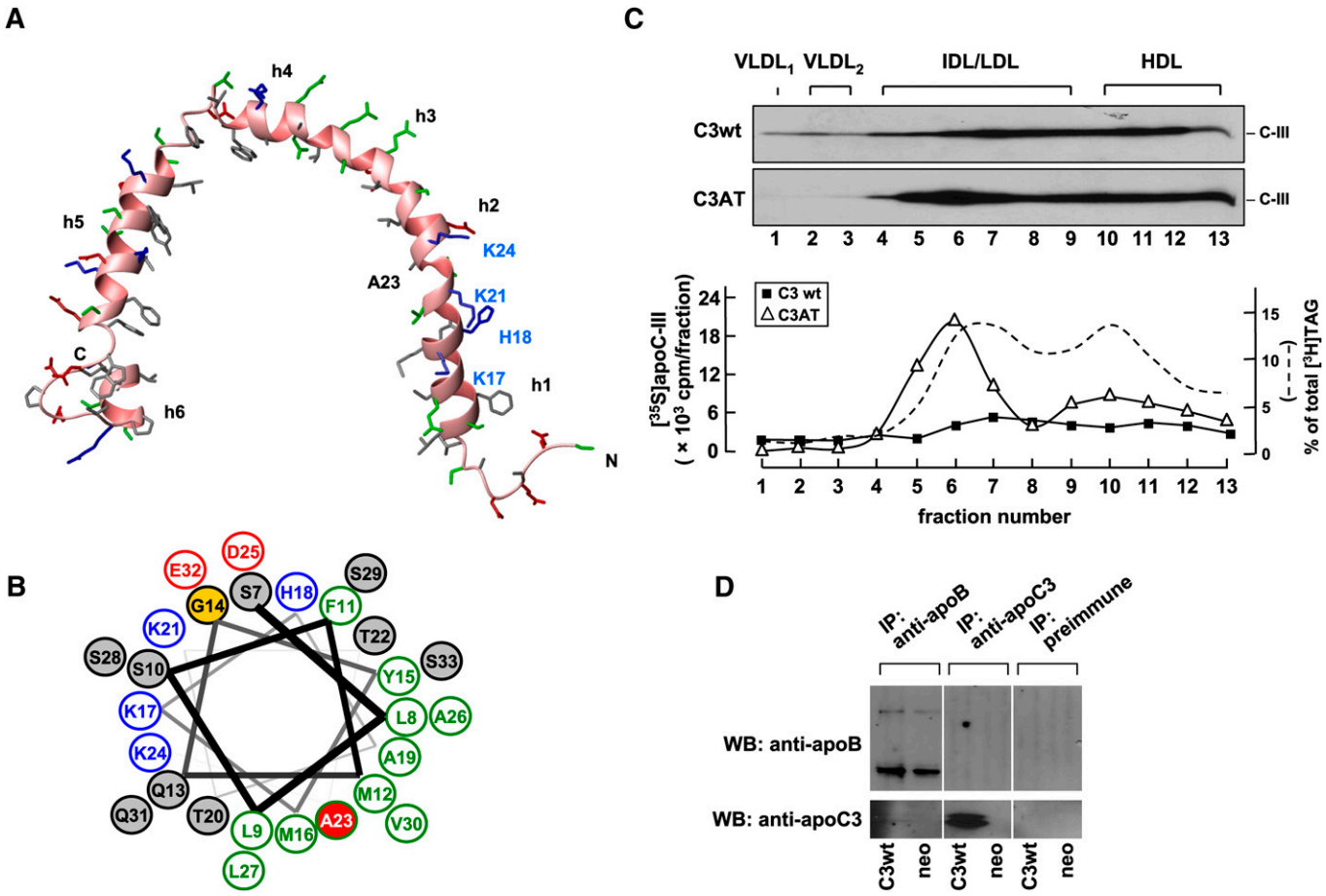

Fig. 7. Model of Ala23 location in apoC-III peptide and lumenal distribution of Ala23Thr mutant. A: Schematic representation of human apoC-III (adopted from Cohn et al. (14), PDB 2jq3) with six segments of α helices (labeled with h1 through h6) that form an arch with hydrophobic residues, including Ala23, facing inward. Positive residues Lys-17, Lys-21, Lys-24, and His-18 on the polar surface of h1 and h2 are also shown. B: A helical wheel diagram of residues 7-33, with hydrophobic residues highlighted in green circles, acidic residues in red, basic residues in blue, hydrophilic neutral residues in gray, and the helix destabilizing residue Gly in yellow. Residue Ala23 is highlighted in red. C: Lumenal [^{35}S]apoC-III distribution coincides with that of [^{3}H]TAG. The experiments were performed essentially the same as that described in Fig. 2, except that [^{35}S]apoC-III in microsomal lumen of C3wt and C3AT cells was analyzed. Data of [^{3}H]TAG derived from Fig. 4A are plotted as % of total lumenal [^{3}H]TAG for comparison. D: Two-way co-immunoprecipitation analysis of apoB and apoC-III. Cell lysates were subjected to immunoprecipitation with either anti-apoB or anti-apoC-III antibody. The immunocomplexes were resolved by SDS-PAGE, and probed for apoB or apoC-III by Western blot analysis, respectively. Rabbit preimmune serum was used as a negative control. apo, apolipoprotein; C3AT, Ala23Thr; C3wt, wild-type apoC-III; IDL, intermediate density lipoprotein; TAG, triacylglycerol.

into the microsomes, nor does it affect the formation of TAG-rich entities within the microsomal lumen (i.e., the IDL/LDL-like particles). These TAG-rich particles, presumably present in the form of lipid droplets within the lumen (28), might represent the lipid substrates utilized for VLDL maturation. However, in the presence of the Ala23Thr mutant, these lumenal lipid droplets were unable to be incorporated into apoB to form mature $VLDL_1$. Therefore, we postulate that apoC-III normally acts as a fusogen that facilitates the fusion between apoB and the lipid droplets.

We and others have previously shown that the VLDL assembly intermediates are largely associated with the microsomal membranes (and not present in the microsomal lumen), and these membrane-attached species are the precursor of secreted VLDL (25, 39). However unlike apoB, apoC-III was present predominantly in the microsomal lumen and not in the membranes (data of the fractionation experiments are not shown). Data from the current co-immunoprecipitation experiment also suggest that there is little physical interaction between apoB (apoB-100 and apoB-48) and apoC-III (Fig. 7D). Rather, the apoC-III protein probably resides on the lipid droplets and mediates fusion. We propose a working model depicting the potential role of apoC-III in the formation of lumenal lipid droplets and facilitating fusion in the second step in the Golgi compartment (**Fig. 8**). The apoC-III–facilitated fusion does not require the MTP activity, nor does the fusion process necessarily entail the transfer of apoC-III onto the resulting $VLDL_1$ particle. Rather, apoC-III proteins might be shed from the lipid droplets after fusion and secreted predominantly as HDL. These events have been observed experimentally (17).

Why does the Ala23Thr mutant fail to promote fusion in the second-step lipidation? Ala23 is the first residue of helix 2 (h2 in Fig. 7A) (18). The Thr for Ala substitution in-

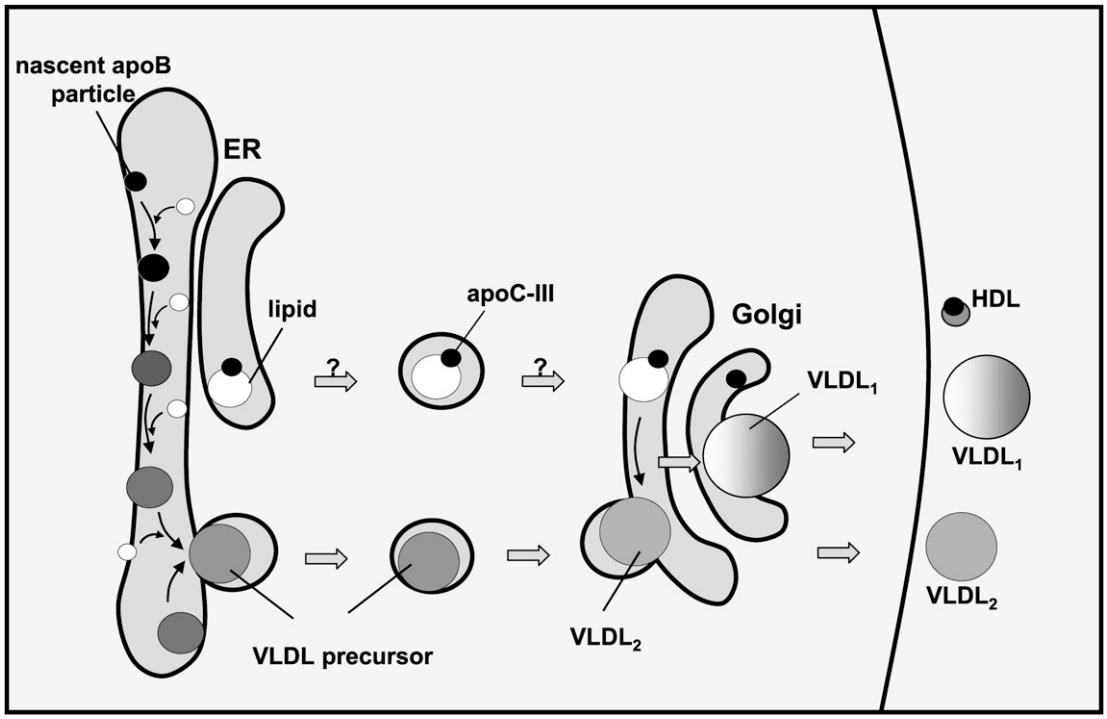

Fig. 8. Model of a possible role of apoC-III in the formation of lipid droplets and in promoting the second step VLDL assembly. The nascent apoB-containing lipoprotein particles are assembled within the ER lumen co- or posttranslationally, and exit ER through coatomer-mediated vesiculation. Lipid substrates synthesized in the ER probably also exit ER through vesiculation, and apoC-III may play a role in the formation of lipid droplets and eventual fusion with VLDL assembly intermediates. The Ala23Thr mutant is unable to promote fusion, resulting in accumulation of lipid substrates similar to the brefeldin A treatment (not shown in the model). apo, apolipoprotein; ER, endoplasmic reticulum.

evitably alters the overall hydrophobic moments of h2, which may explain the observed less-efficient binding of apoC-III to dimyristoylphosphatidylcholine multilamellar vesicles (19). In an amphipathic α-helix, such changes often perturb the secondary structure because binding of hydrophobic lipids is an integral component of protein folding. Moreover, Ala23 is also adjacent to a putative oblique peptide (residues 6-20) of human apoC-III (40). Unlike normal amphiphathic α-helix, whose helical axis is parallel to the membranes or surface of vesicles, the helical axis of an oblique peptide adopts an angle (or is tilted) relative to the membrane. The oblique insertion into the lipid bilayer often causes membrane disruption by promoting highly curved hemifusion intermediates, leading to fusion. Tilted peptides have been discovered in viral fusion proteins as well as in many proteins involved in lipid/lipoprotein metabolism, such as apoB-100 signal peptide (41), LPL and hepatic lipase (42), cholesteryl ester transfer protein (42), lecithin cholesterol acyltransferase (43), apoA-II (44), and MTP (45). In the case of apoC-III, the predicted oblique peptide has a tilted angle of 40° with the interface plane (40). The oblique peptide of apoC-III had relatively lower lipid-binding efficiency and formed looser and less stable complexes with lipids (46). These properties of the oblique peptide located at the N-terminal region of apoC-III would hypothetically confer lipid-destabilizing capabilities and exchangeabilities in vivo. Regions immediately downstream of the oblique peptide could be as critical, as they may define distances between donor and acceptor lipid entities when fusion is taking place. Whether the A23T mutation would affect the function of this putative oblique peptide in mediating fusion between lipid droplets and apoB needs to be determined experimentally. However, our preliminary experimental data suggest that the presence of this putative oblique peptide at the N terminus of apoC-III could function as a signal peptide. We found that expression of a construct that encoded only the 79 amino acids of mature apoC-III (without sequences encoding the signal peptide) resulted in normal secretion of the recombinant protein (M. Sundaram and Z. Yao, unpublished observation). Moreover, expression of the apoC-III–lacking signal peptide resulted in enhanced VLDL-TAG secretion as effectively as the normal apoC-III protein possessing the signal peptide (M. Sundaram and Z. Yao, unpublished observation). The significance of these unique structural features associated with human apoC-III merits further investigation.

CONCLUSION

We have determined the molecular mechanisms by which the A23T mutation leads to the loss of function of apoC-III in promoting the assembly and secretion of TAG-rich $VLDL_1$ from hepatic cells under lipid-rich conditions. Our study suggests that an important structural element of human apoC-III, residing within the N-terminal region of this protein, may govern its function in the fusion be-

tween lipid droplets and apoB in the second-step $VLDL_1$ assembly.

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