Characterization of the lipid-binding properties and lipoprotein lipase inhibition of a novel apolipoprotein C-III variant Ala23Thr

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Abstract **We have identified a G-to-A transition in exon 3 of the *APOC3* gene resulting in a novel Ala23Thr apolipoprotein (apo) C-III variant, associated with apoC-III deficiency in three unrelated Yucatan Indians. The Ala23Thr substitution modifies the hydrophobic/hydrophilic repartition of the helical N-terminal peptide and hence could disturb the lipid association. In vitro expression in *Escherichia coli* of wild-type and mutant apoC-III enabled the characterization of the variant. Compared with wild-type apoC-III-Ala23, the mutant apoC-III-Thr23 showed reduced affinity for dimyristoylphosphatidylcholine (DMPC) multilamellar vesicles with higher amounts of free apoC-III. Displacement of apoE from discoidal apoE:dipalmitoylphosphatidycholine (DPPC) complex by apoC-III-Thr23 was comparable to wild type but the less efficient binding of the apoC-III-Thr23 to the discoidal complex resulted in a higher apoE/apoC-III (mol/mol) ratio (34%) than with wild-type/apoE:DPPC mixtures. The inhibition of lipoprotein lipase (LPL) by apoC-III-Thr23 was comparable to that of wild type, and therefore effects on LPL activity could not explain the lower triglyceride (Tg) levels in Thr-23 carriers. Thus, these in vitro results suggest that in vivo the less efficient lipid binding of apoC-III-Thr23 might lead to a faster catabolism of free apoC-III, reflected in the reduced plasma apoC-III levels identified in Thr-23 carriers, and poorer competition with apoE, which might enhance clearance of Tg-rich lipoproteins and lower plasma Tg levels seen in Thr-23 carriers.** — Liu, H., C. Labeur, C-F. Xu, R. Ferrell, L. Lins, R. Brasseur, M. Rosseneu, K. M. Weiss, S. E. Humphries, and P. J. Talmud. **Characterization of the lipid-binding properties and lipoprotein lipase inhibition of a novel apolipoprotein C-III variant Ala23Thr. *J. Lipid Res.* 2000.** 41: **1760–1771.**

Supplementary key words genetic variation • LPL inhibition • lipid binding • DMPC • apoE displacement

The major function of apolipoproteins is the transport and distribution of lipids among various tissues in the body and the control of enzyme activity. Although the roles of apolipoprotein (apo) B, apoE, and apoA-I and apoC-II in lipid metabolism are well defined, this is not true for apoC-III. ApoC-III is a 79-amino acid glycoprotein, accounting for 26% of the protein in very low density lipoprotein (VLDL) and 2% in high density lipoprotein (HDL) (1). Plasma apoC-III levels are positively correlated with plasma triglyceride (Tg) and cholesterol levels (2–4), and elevated apoC-III levels have been found in hypertriglyceridemic individuals (5) and in patients with coronary artery disease (CAD) (6). Furthermore, apoC-III acts as a marker of Tg-rich lipoprotein (TGRL) metabolism and the apoC-III HDL:VLDL ratio has been found to be negatively associated with the progression of atherosclerosis in a number of studies (7, 8).

The mechanisms by which apoC-III regulates the catabolism of TGRL is complex, involving both the inhibition of lipoprotein lipase (LPL), thus reducing Tg hydrolysis (9–11), and the displacement of apoE on the VLDL particles, thus retarding apoE-mediated uptake of TGRL by receptors on hepatocytes. This is supported by in vivo studies showing a rapid conversion of VLDL to LDL in individuals with combined apoA-I and apoC-III

Abbreviations: apo, apolipoprotein; BMI, body mass index; CAD, coronary artery disease; CD, circular dichroism; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; HDL, high density lipoprotein; IPTG, isopropyl-β-D-thiogalactopyranoside; LCAT, lecithin:cholesterol acyltransferase; LPL, lipoprotein lipase; MHP, molecular hydrophobicity potential; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; Tg, triglycerides; TGRL, triglyceride-rich lipoprotein; TRP, tryptophan; VLDL, very low density lipoprotein.

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deficiency (10). Liver perfusion studies have suggested that apoC-III directly inhibits the hepatic uptake of chylomicrons and VLDL (12, 13), possibly by impairing the interaction of apoE on the lipoproteins, with LDL receptors (14). Confirmation of this role of apoC-III in the clearance of TGRL comes from transgenic mouse studies. Disruption of the *apoc3* gene in mice results in protection from postprandial hypertriglyceridemia (15), while the overexpression of human *APOC3* results in hypertriglyceridemia, with a positive correlation between apoC-III levels and Tg concentration (16). This elevated Tg in human *APOC3* transgenic mice is due to an increased number of VLDL particles in the circulation, which contain more Tg and apoC-III and less apoE, thus diminishing apoE-mediated lipoprotein uptake (17). ApoC-III overexpression also reduces the ability of VLDL to bind to glycosaminoglycans, thus decreasing efficient lipolysis at the cell surface (18).

The *APOC3* gene is located on chromosome 11q23, in a cluster with *APOAI* and *APOA4* (19). Genetic variation at the *APOAI-C3-A4* gene cluster has been found to be associated with differences in plasma lipid, lipoprotein, and apo levels (20, 21). Several common variants of the *APOC3* gene have been shown to be associated with high plasma Tg levels in some samples (17, 22–24), with elevated levels of apoC-III in healthy individuals (2), and with premature CAD (25, 26). However, deficiency of apoC-III is rare. A 6.5-kilobase inversion in the *APOAI-C3-A4* gene cluster and a complete deletion of the *APOAI-C3-A4* gene cluster have been reported to cause combined deficiency of apoA-I and apoC-III (27) and familial apoA-I–C-III–A-IV deficiency, respectively (28). In both cases, plasma HDL levels were undetectable, Tg levels were low, and homozygous individuals had premature atherosclerosis. To date, four rare structural variants of apoC-III have been identified: Gln38Lys, Asp45Asn, Lys58Glu, and Thr74Ala (29–32).

In a previous study of the Mayan population of the Yucatan peninsula, an *APOC3* allele was reported to be associated with low plasma apoC-III levels (33). Molecular analysis revealed no gross DNA rearrangements or deletions in the *APOAI-C3-A4* locus. In the current study, we report the identification of a novel apoC-III mutation in these Mayan individuals and the use of recombinant technology to express wild-type and mutant apoC-III for structure-function analysis.

SUBJECTS AND METHODS

Subjects

The lowland Mayan Indians inhabit the Yucatan peninsula and other parts of Central America. Samples were collected in 1985 and 1987 from a series of local villages, as part of a study of chronic disease risk factors in Amerindians (33). All known first- and second-degree relatives were excluded. Ten to 20 ml of ethylenediaminetetraacetic acid (EDTA)-anticoagulated whole blood were drawn in vacutainers, maintained on wet ice, and returned to the laboratory within 3 to 5 days. Sixteen individuals from one remote village of the original sample were included in this study. The 192 Caucasian control males (aged 51–60 years), free from CAD, were recruited from a general practitioner practice in Camberley, south London (21).

Lipid and apolipoprotein measurements

Plasma apoC-III was quantified in triplicate by single radial immunodiffusion (34), using commercial plates and control standards from Daiichi Pharmaceuticals (Fort Lee, NJ). Total plasma cholesterol and triglyceride levels were determined manually by enzymatic methods (35).

DNA amplification by polymerase chain reaction

Genomic DNA was isolated from frozen buffy coats. The *APOC3* gene was amplified with primer pairs 1 and 5, 2 and 5, and 6 and 8 covering the 5′ promoter from −345 to 3′ bp 3545, which included all exons and the 3′ untranslated region (**Table 1**). All the primers, W-1 detergent, and DNA *Taq* polymerase were obtained from GIBCO-BRL (Gaithersburg, MD). The polymerase chain reaction (PCR) was performed in a reaction mixture (total volume, 50 μl) including 250 ng of primers, one of which was biotinylated, 200 ng of genomic DNA, and 1 U of *Taq* polymerase. The reactions were performed on an Intelligent Heating Block (Cambio, Cambridge, UK) at 95°C for one cycle of 5 min, 55°C for 1 min, and 72°C for 2 min, and subsequently for 35 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 2 min, in the reaction buffer provided by the manufacturer, with $MgCl_2$ ranging from 0.10 to 0.15 mM.

Direct sequencing for mutation detection

PCR products were purified with streptavidin-coated beads (Dynal, Bromborough, UK) to separate the double-stranded DNA. Sequencing was carried out according to the Sequenase version 2 protocol (United States Biochemical, Cleveland, OH).

*Aci*I digestion

Fifteen microliters of the PCR products amplified with primers 4 and 5 was incubated at 37°C with 10 units of *Aci*I (New England BioLabs, Hitchin, UK) for 16 h in a total volume of 20 μl, using the buffer recommended by the manufacturer. DNA fragments were separated by electrophoresis on a 10% acrylamide gel, stained with ethidium bromide, and viewed under ultraviolet light.

TABLE 1. Oligonucleotides used for PCR and direct sequencing for mutation detection

5′ Oligonucleotide	Sequence	3′ Oligonucleotide	Sequence
1	5′-CCAGTGTGAAAGGCTGAGATGG	2	5′-CTACCTTAGGGGCCACGCCAC
3	5′-GGTCCTGCTGACCACCCATTG	4	5′-CAATGGGTGGTCAAGCAGAAGC
5[a]	5′-GAGCACCTCCATTCCATTGTTGG	6	5′-CATGGTTGCCTACAGAGGATG
7	5′-ATGGATAGGCAGGTGGACTTG	8[a]	5′-TGACCTTCCGCACAAAGCTGT
9	5′-GAGAAGTTTVTVTGAGTTCTG		

[a] Indicating biotinylated primers.

DNA polymorphisms

Briefly, the genotypes of the *Sst*I and *Pvu*II polymorphisms of the *APOC3* gene were determined as reported previously (36), the −75G→A substitution of the *apoAI* promoter region and the 1100C→T transition of the *APOC3* gene were genotyped by PCR in combination with allele-specific oligonucleotide melting (21).

Molecular hydrophobicity potential calculations; three-dimensional construction of the peptide

Construction of the peptides was carried out as previously described (37). The method accounted for the contribution of the lipid-water interface, the concomitant variation of the dielectric constant, and the transfer energy of atoms from a hydrophobic to a hydrophilic environment (38). The calculation of the molecular hydrophobicity potential (MHP) along the peptide was carried out by assuming that the hydrophobic interaction between an atom and a point M decreases exponentially with the distance according to the equation (39):

$$MHP = Etr_i \exp(r_i - d_i) \qquad Eq.\ 1)$$

where Etr_i is the energy of transfer of atom i, r_i is the radius of the atom i, and d_i is the distance between atom i and a point M.

Calculations were made in a plane perpendicular to the mean orientation of the long axis of the helix, which was moved every 2 Å along this axis.

Secondary structure prediction

The secondary structure prediction was carried out at the NPS@ (Network Protein Sequence @nalysis) web site http://pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_seccons.html. Various predictive methods are used to obtain the consensus: SOPMA (40), PHD (41, 42), Predator (43), GORIV (44), DPM (45), DSC (46), SIMPA96 (47, 48), and HNNC (49).

Generation of pET23b/*APOC3* construct

The human *APOC3* cDNA (a kind gift from J. Taylor, San Francisco, CA) in the pBSSK vector (Stratagene, La Jolla, CA) was used as a template for the amplification of the *APOC3* gene by PCR. This was performed in a 50-μl reaction containing 50 ng of plasmid DNA pBSSK/*APOC3* as template, 250 ng of each primer, and 1 unit of DNA *Taq* polymerase in a buffer recommended by the manufacturers, including dNTPs (0.2 mM each) and 1.5 mM $MgCl_2$. Two primers, the 5′ forward primer with an internal *Nde*I (underlined) site (5′-GGGAATTCCATATGTCAGAGGCCGAGGAT-3′) and a 3′ reverse primer designed to append the sequence for a 3′ *Xho*I restriction site (underlined) (5′-GTGGTGCTCGAGGGCAGCCACGGCTGAAG-3′), were used for PCR, under the following conditions: the initial cycle of 94°C for 5 min, 66°C for 1 min, and 72°C for 2 min was followed by 35 cycles of 94°C for 30 sec, 66°C for 1 min, and 72°C for 1 min. Reactions were performed on an automated Omigene PCR machine (Hybaid, Middlesex, UK). The resulting 264-bp human *APOC3* fragment was digested with *Nde*I and *Xho*I, and subcloned into a similarly digested pET23b vector (Novagen, Cambridge, UK) according to standard methodology. The ligation mixtures were transformed into competent cells of the XL1Blue strain of *Escherichia coli* (Promega, Southampton, UK) and the transformed cells were selected with ampicillin (75 μg/ml) on LB-agar plates. The entire *APOC3* cDNA sequence was sequenced in both directions, using an ABI 377 prism DNA sequencer (Perkin-Elmer Biosystems, Warrington, UK).

Generation of pET23b/A23T*APOC3* mutation

pET23b/A23T was generated by using a QuikChange™ site-directed mutagenesis kit (Stratagene). In a 50-μl reaction, 50 ng of plasmid DNA pET23b/*APOC3* was used as template and two synthetic oligonucleotide primers, the forward primer (5′-CCACCAAGACCACCAAGGATGCA-3′) and the reverse primer (5′-TGCATCCTTGGTGGTCTTGGTGG-3′) with the G-to-A base change (underlined) and complementary to opposite strands of the vector, were extended by *Pfu* Turbo DNA polymerase (Stratagene). For PCR there was an initial cycle of 95°C for 30 sec, followed by 16 cycles of 95°C for 30 sec, 55°C for 1 min, and 68°C for 12 min. After *Dpn*I digestion of the parental deoxyadenosine methylase-methylated template, the synthesized mutated DNA was transformed into *E. coli* XL1Blue supercompetent cells. Candidate clones were screened by sequencing the *APOC3* insert on an ABI 377 prism DNA sequencer, both to confirm the presence of the mutation and to ensure no alterations at other sites.

Expression and purification of the recombinant wild-type and mutant apoC-III-Thr23

Plasmid DNA of pET23b/*APOC3*-Ala23 (wild type) and pET23b/*APOC3*-Thr23 (mutant) was transformed into competent *E. coli* B834 and BL21 (Novagen), respectively, for optimum expression and used to inoculate an overnight culture in SOC medium containing ampicillin at 75 μg/ml (Sigma, Irvine, UK). Thirty milliliters of the overnight culture was used to inoculate 1 liter of SOC medium (ampicillin at 75 μg/ml) and shaken (220 rpm/min) at 37°C. Cells were induced with isopropyl-β-D-thiogalactopyranoside (IPTG, 0.5 mM; Sigma) when the optical density at 600 nm (OD_{600}) of the culture reached 0.6. The cells were harvested (3,000 rpm for 15 min at 4°C) after 2 h of induction for wild-type apoC-III and after 1 h for mutant apoC-III. Cells were lysed in 15 ml of 8 M urea, 50 mM NaH_2PO_4, 10 mM Tris-HCl, 500 mM NaCl (pH 8.0) buffer (by sonication on ice; three times, 1 min each), and then centrifuged at 18,000 rpm (30 min at 4°C), and the supernatant was then incubated with 1 ml of Talon™ cobalt metal affinity resin (2 h at 4°C) (Clontech, Hampshire, UK). The resin was pelleted by centrifugation (3,000 rpm for 5 min), and batch washed (four times). The recombinant human apoC-III fusion proteins were eluted from the column with a buffer consisting of 8 M urea, 50 mM NaH_2PO_4, 20 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), and 500 mM NaCl (pH 6.0–6.3). The fractions containing recombinant apoC-III were identified by electrophoresis on Tricine-sodium dodecyl sulfate (SDS) polyacrylamide gels (17.5% precast gels; Bio-Rad, Hemel Hempstead, UK), and dialyzed overnight at 4°C against 4 M urea, 5 mM NH_4HCO_3 (pH 8.0). This solution was loaded on an anion-exchange column, Mono Q® (Pharmacia, Uppsala, Sweden) and eluted with a linear NaCl gradient (from 0.10 to 0.25 M) in 4 M urea, 5 mM NH_4HCO_3 (pH 8.0) buffer. The fractions containing the pure protein (elution at an NaCl concentration of approximately 0.15 M) were pooled and the final purity of the product was verified as described above. Both apoC-III variants were at least 95% pure. The protein content in the samples was determined by OD measurement at 280 nm, using a molar extinction coefficient of 19,630 ($M^{-1}\ cm^{-1}$).

Lipid-binding properties of apoC-III

Association of recombinant apoC-III variants with lipid was followed by monitoring the turbidity decrease of dimyristoylphosphatidylcholine (DMPC) multilamellar vesicles (Sigma) at 325 nm as a function of the temperature. The DMPC vesicles were obtained as previously described (50) and 40 μg of apoC-III protein was added to 80 μg of DMPC vesicles in a 0.01 M Tris-HCl buffer containing 150 mM NaCl (pH 8.0), 8.5% KBr, 0.01% NaN_3, and 0.01% EDTA, in a Uvikon 931 spectrophotometer (Kontron, Milan, Italy). The kinetics study of lipid/apoC-III association was followed by monitoring the rate of clearance of the turbidity of DMPC liposomes at 325 nm, as a function of time at 20 and 30°C. The rate constant $k = -\ln(0.5)/t_{1/2}$, where $t_{1/2}$ is

the time needed to obtain a 50% reduction of the optical density signal at 325 nm.

Preparation and isolation of phospholipid: apoC-III complexes

ApoC-III:DMPC complexes were prepared by incubation of the recombinant apoC-III with DMPC vesicles, at DMPC-to-protein ratios of 2:1 (w/w) at 25°C for 16 h. The complexes were isolated by gel filtration on a Superose 6HR (Amersham Pharmacia Biotech UK, Little Chalfont, UK) column in 0.01 M Tris-HCl buffer containing 150 mM NaCl (pH 7.6) and NaN_3 (0.2 g/l) on a fast protein liquid chromatography system. Complexes were detected by measuring the absorbance at 280 nm and the tryptophan (Trp) fluorescence emission at 330 nm (excitation at 295 nm). The Superose 6HR column was calibrated with a set of protein standards [between molecular masses of 668 kDa (thyroglobulin) and 65 kDa (cytochrome *c*)]. The resulting calibration curve was used for the determination of the molecular mass or Stokes radius of the particles eluting from the column.

Circular dichroism measurements

Circular dichroism (CD) spectra of each recombinant apoC-III protein and their complexes with DMPC are measured on a Jasco (Easton, MD) 600 spectropolarimeter at room temperature. Measurements were carried out at a protein concentration of 200 μg/ml in 5 mM NH_4HCO_3 buffer, pH 8.0. Nine spectra were collected and averaged for each sample. For the protein and protein:DMPC complexes the secondary structures were obtained by curve fitting on the entire ellipticity curve between 184 and 260 nm, using software available on the Internet (http://bioinformatik.biochemtech.ini-halle.de/cdnn/).

Displacement of apoE from the apoE:DPPC complexes

The apoE:dipalmitoylphosphatidylcholine (DPPC) complexes were prepared by the sodium cholate dialysis method as described (51), using DPPC (Sigma) at a 2:1 (w/w) ratio and using recombinant apoE3 purified from *E. coli* as described (52). The protein-lipid mixtures were incubated overnight at 43°C and then dialyzed against 10 mM Tris-HCl buffer, pH 8.0, containing 150 mM NaCl, disodium-EDTA (0.1 g/l), and 1 mM azide, each time for 24 h at 43°C, at room temperature, and at 4°C. The homogeneity of the apoE:DPPC complexes was verified by gel filtration on a Superose 6 PG column, from which the complexes eluted in one homogeneous peak. The protein concentration in the complexes was determined by optical density measurement at 280 nm and the phospholipid concentration was determined enzymatically (Biomérieux, Rockland, MD). The concentration of the complexes is expressed as its protein content in all following experiments. ApoE complexes were mixed with the apoC-III proteins at a 1:1 (w/w) ratio and incubated at room temperature for 2 h. The apoE:DPPC-apoC-III mixture was then separated by gel filtration on a Superose 6 PG column. The protein elution profile was recorded by measuring the Trp emission at 330 nm in each fraction. The content of apoE (53)and apoC-III (54) was measured in each fraction by sandwich enzyme-linked immunosorbent assay (ELISA), using affinity-purified polyclonal antibodies. Briefly, antibodies were coated on to 96-well ELISA plates; residual binding sites were blocked and samples or standards were incubated on the plates for 24 h at 37°C. After washing away excess antigen the peroxidase-labeled antibody was incubated for 2 h at 37°C. After washing, the amount of bound peroxidase was revealed with a chromogenic substrate. The plates were read and the standard curve and samples were calculated with the software provided with the reader (Biotek Reader adapted with KC4 software). The sensitivity of the assay is approximately 2 ng.

Detection of apoC-III and apoE proteins by Western blotting

Samples were separated on a Tricine-SDS polyacrylamide gel (17.5%), or on a 15% SDS polyacrylamide gel. Proteins were transferred onto a wet blot system (Bio-Rad) in a Tris (5 mM)-glycine (192 mM) buffer, pH 8.3, with 20% methanol. Proteins were visualized with rabbit anti-apoC-III or rabbit anti-apoE polyclonal antibodies and a secondary peroxidase-labeled goat anti-rabbit antibody (Sigma). The bound peroxidase was visualized with BM chemiluminescence luminal substrate (Boehringer-Mannheim, Mannheim, Germany).

LPL inhibition by apoC-III variants

Bovine LPL (EC 3.1.1.34) (Sigma) catalytic activity, in the presence of apoC-III wild type and mutant, was measured with an emulsified [^{3}H]triolein substrate, Intralipid™ (100 mg/ml; Pharmacia Laboratories, Milton Keynes, UK) (55). The incubation medium contained 2% (v/v) of the emulsion, 6% (w/v) bovine serum albumin (BSA; Sigma), 1.2% NaCl (w/v), 6.3% Tris-HCl (w/v) pH 8.5, and 3 IU of heparin (Sigma). As a source of apoC-II, lyophilized human plasma apoC-II was dissolved in 5 M urea, 10 mM Tris-HCl (pH 8.5) at 2 mg/ml. The experiments were optimized for both apoC-II and LPL, to give optimum hydrolysis with minimum apoC-II concentration. The emulsion was preincubated with each apoC-III sample with or without 40 ng of apoC-II for at least 15 min, after which time the bovine LPL (10 μl of a 1.5-μg/ml solution) was added, in a final volume of 200 μl. The mixture was shaken in a water bath at 25°C for 30 min. The reaction was stopped, the fatty acids were extracted, and the radioactivity was counted (55).

Statistical analysis

To test whether there was a statistically significant difference in the kinetic measures, a Wilcoxon signed rank test was used. Time was used to pair the variables. A P value of <0.05 was taken to be significant.

RESULTS

Identification of the novel Ala23Thr-*APOC3* mutation

The nucleotide sequences of the *APOC3* gene including the 5′ flanking region, all exons, and the 3′ flanking region (−345 to 3545), were examined in four DNA samples from Mayan Indians with plasma apoC-III levels of 1.6, 1.9, 8.4, and 18.4 mg/dl, respectively, using PCR and direct sequencing. In the two individuals with apoC-III deficiency (ID57 and ID67; **Table 2**), a G1125-to-A transition in exon 3 of the *APOC3* gene, changing amino acid 23 from alanine to threonine, was identified (results not shown). Both subjects were heterozygous for the sequence change. In addition, a number of other sequence differences were detected among the four individuals accounting for known polymorphisms of the *APOC3* gene (56). No other novel sequence difference was detected. The G1125-to-A sequence change abolishes an *Aci*I cutting site (CCGC) and this enabled confirmation of the sequence change by PCR and restriction enzyme digestion. This method was used to screen the additional 12 Mayan Indian DNA samples and a sample of healthy men from south London (21). None of the 192 Caucasians were carriers of the sequence change, but Mayan sample ID103 was a carrier of the mutation. This individual's plasma

TABLE 2. Plasma apoC-III and lipid levels in Mayan Indian sample

ID	Age	Sex	BMI	ApoC-III	Tg	Chol	HDL	G-75/A	*Sst*I	C1100/T	*Pvu*II
	yrs		*kg/m²*	*mg/dl*	*mg/dl*	*mg/dl*	*mg/dl*				
57	21	F	29.6	1.9	86	119	44	GA	S−S−	CT	V−V+
67	23	F	19.7	1.6	45	85	44	GA	S−S−	CT	V−V−
103	60	M	19.1	5.5	204	159	43	AA	S−S−	TT	V−V−
42	20	M	24.8	8.4							
44	30	F	22.8	9.0							
50	25	F	24.7	4.7	80	196	70				
59	35	F	25.8	18.4	306	241	111				
65	20	F	19.9	3.0	117	110	53				
79	44	F	31.2	9.8	255	139	42				
84	27	F	21.9	9.0	218	171	61				
87	27	F	23.5	5.2	160	130	44				
88	70	F	23.1	6.0	106	141	49				
93	30	M	24.1	6.9	186	151	51				
95	36	M	27.6	9.2	242	120	33				
102	26	M	23.4	5.5	132	104	39				
106	28	M	24.0	5.2	160	169	52				
Mean[a]	32	—	24.0	7.7	178	152	55				
(±SD)	(13)	—	(3)	(3.8)	(70)	(40)	(21)				

[a] Mean(±SD) from the other 13 Mayans (5 males and 8 females) included in the study.

apoC-III levels (5.5 mg/dl) were lower than the sample mean (7.7 mg/dl) but was not among those with the lowest apoC-III levels. The available lipid and lipoprotein data for the three *APOC3*-Thr23 carriers and 13 noncarriers are presented in Table 2. The two individuals with the lowest plasma apoC-III had levels that were 20 and 25% of the sample mean, with Tg and total cholesterol levels that were correspondingly low (reduced to 25 and 48% and 56 and 78%, respectively). Levels of apoC-III in the third carrier were 71% of the sample mean and plasma lipid levels were slightly above that of the noncarriers. This subject was male, older than the female carriers, and had a low body mass index (BMI) of 19.1 kg/m².

To identify the haplotype on which the A1125 allele (*APOC3*-Thr23) occurred, DNA polymorphisms of the *APOA1-C3-A4* gene cluster (the −75G→A substitution of the gene encoding apoA-I, the *Sst*I polymorphism, the 1100C→T transition, and the *Pvu*II polymorphism of the *APOC3* gene) were used. The data are compatible with the mutation for Thr-23 being present on a rare A−75, S−, T1100, V− haplotype in all three individuals (Table 2).

Molecular modeling

Molecular modeling was used to predict the significance of the Ala23Thr substitution on the secondary structure and thus its effect on the function of the protein. The N-terminal domain of apoC-III is helical, as suggested by the secondary structure predictions (**Fig. 1A** and **B**). The region from residue 15 to 33, corresponding to the core of the predicted α-helical N-terminal domain, was examined in detail. The distribution of the hydrophobic and hydrophilic envelopes suggests that this segment is amphipathic, because there is a good segregation between the hydrophobic and hydrophilic faces of the α helix (**Fig. 2A**). The Ala23Thr substitution induces a modification of the hydrophobic distribution around the α helix (Fig. 2B and C). Analysis of the isopotential surfaces showed that Thr-23 induces a hydrophilic domain into the hydrophobic potential that is likely to influence the affinity of apoC-III-Thr23 for a lipid surface.

Expression of recombinant apoC-III

To obtain large quantities of apoC-III proteins to investigate the structure-function relationship, an *E. coli* expression system was developed that yielded milligram quantities of the recombinant C-terminus histidine-tagged apoC-III proteins. For optimal expression wild-type apoC-III was expressed in *E. coli* B834 and apoC-III-Thr23 was expressed in *E. coli* BL21. For reasons of stability different induction times were used, 2 h for wild type and 1 h for apoC-III-Thr23. After induction by IPTG at 37°C the fusion proteins reached about 5% of the total cellular protein (as determined by Coomassie blue staining of SDS-polyacrylamide gels). Because the fusion proteins were contained in inclusion bodies they were purified in the presence of urea, using a combination of Talon affinity agarose and anion-exchange chromatography. The resulting apoC-III fusion proteins were shown to be more than 95% pure and approximately 2 mg of the pure recombinant fusion protein was obtained from 1 liter of culture medium.

Lipid-binding properties of the recombinant apoC-III proteins

DMPC-binding experiments were performed on three separate occasions to assess the effect of wild-type and mutant apoC-III on the kinetics of the interaction with DMPC multilamellar vesicles. Both wild-type and mutant apoC-III-Thr23 bound to DMPC rapidly and maximally around the transition temperature of the lipid (23°C), as indicated by the dramatic decrease in turbidity of the DMPC dispersions (data not shown). The rate of interaction between the apoC-III proteins and DMPC was monitored by monitoring the decrease in turbidity at 325 nm. Experiments were performed below the transition temperature at 20°C and above the transition temperature at 30°C. At

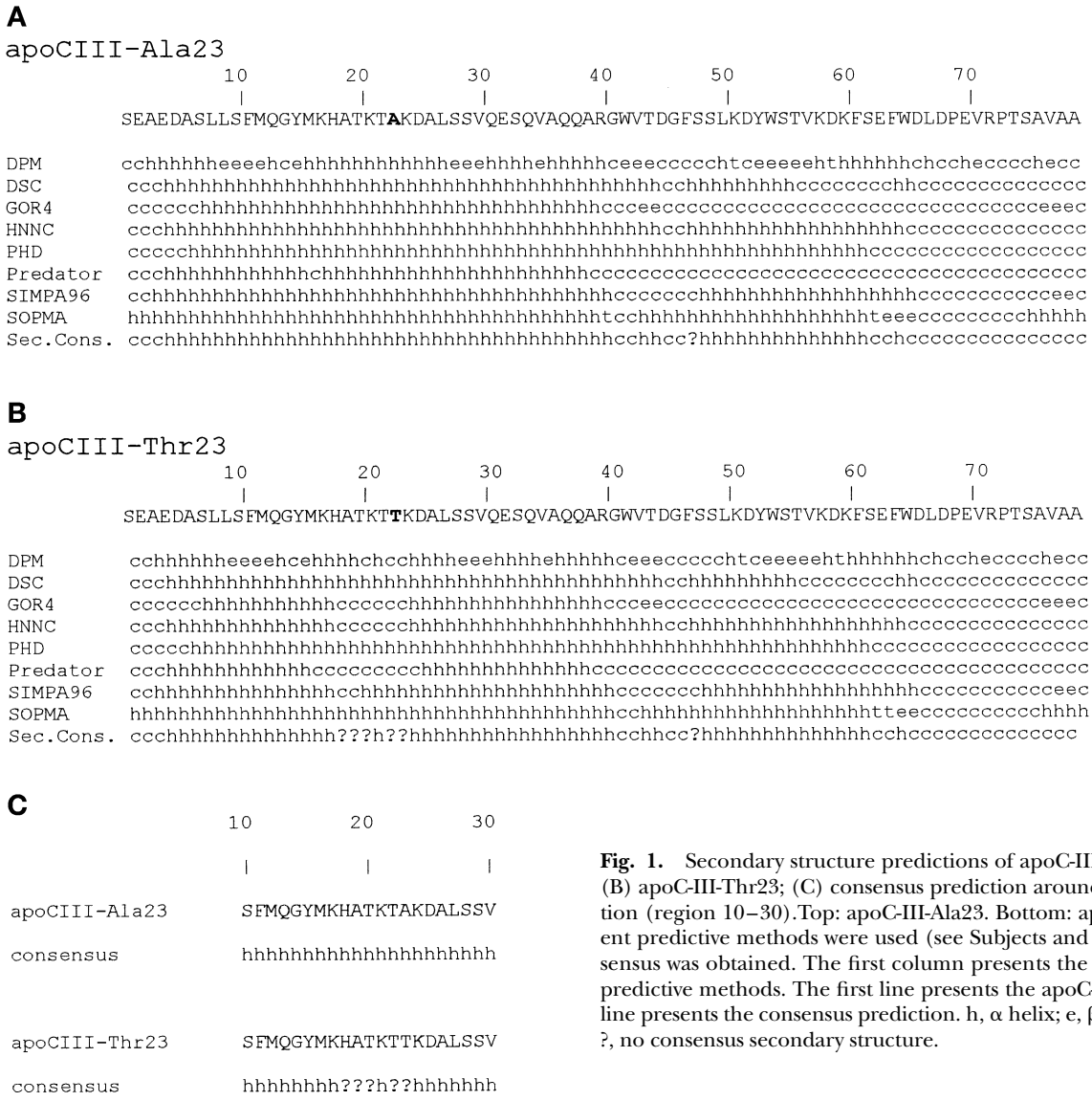

Fig. 1. Secondary structure predictions of apoC-III. (A) apoC-III-Ala23; (B) apoC-III-Thr23; (C) consensus prediction around the Ala23Thr mutation (region 10–30).Top: apoC-III-Ala23. Bottom: apoC-III-Thr23. Different predictive methods were used (see Subjects and Methods) and a consensus was obtained. The first column presents the names of the various predictive methods. The first line presents the apoC-III sequence; the last line presents the consensus prediction. h, α helix; e, β sheet; c, coil; t, turn; ?, no consensus secondary structure.

both temperatures tested, the rate constant of the binding of the mutant apoC-III (the k value) was slower as compared with the wild type (**Fig. 3A** and **B** and **Table 3**), suggesting a less efficient binding of the mutant apoC-III. These values at 20°C were 0.43 ± 0.03 and 0.28 ± 0.03 min^{-1} for wild-type and mutant apoC-III, respectively ($P < 0.0001$) (Fig. 3A) and at 30°C the values were 0.17 ± 0.02 and 0.07 ± 0.01 min^{-1}, respectively ($P < 0.0001$) (Fig. 3B). The resulting DMPC:apoC-III discoidal particles (formed at 23.5°C) were fractionated on a Superose 6 PG gel-filtration column and the apoC-III content of these fractions was monitored by comparing the Trp emission intensity at 330 nm. These results were reproducible, and results from a representative experiment are presented in **Fig. 4**. Although both apoC-III proteins were able to clarify the DMPC solution, the amount of unbound apoC-III and the size of the discoidal complexes formed were quite different for each protein. In the gel-filtration run no free lipid, which would typically elute within the void volume of the column (below elution volume of 20 ml), could be detected. Both complexes eluted as homogeneous peaks although at different maxima; wild-type apoC-III eluted at 29.0 ml or with a size corresponding to a Stokes radius of 61 Å, while the mutant C-III eluted at 27.3 ml, corresponding to larger particles with a Stokes radius of 72 Å. Moreover, the amount of unbound apoC-III eluting at 38 ml is higher for the mutant apoC-III than for the wild-type apoC-III despite the fact that identical amounts of protein and incubation ratios of protein:lipid were used for the preparation of the complexes. Although the differences are small, in terms of molecular weight these represent a large difference.

Physicochemical characteristics of the native and lipid-bound apoC-III

The secondary structure of the native and lipid bound proteins was determined by CD measurements and the results are presented in Table 3. Both proteins showed a sim-

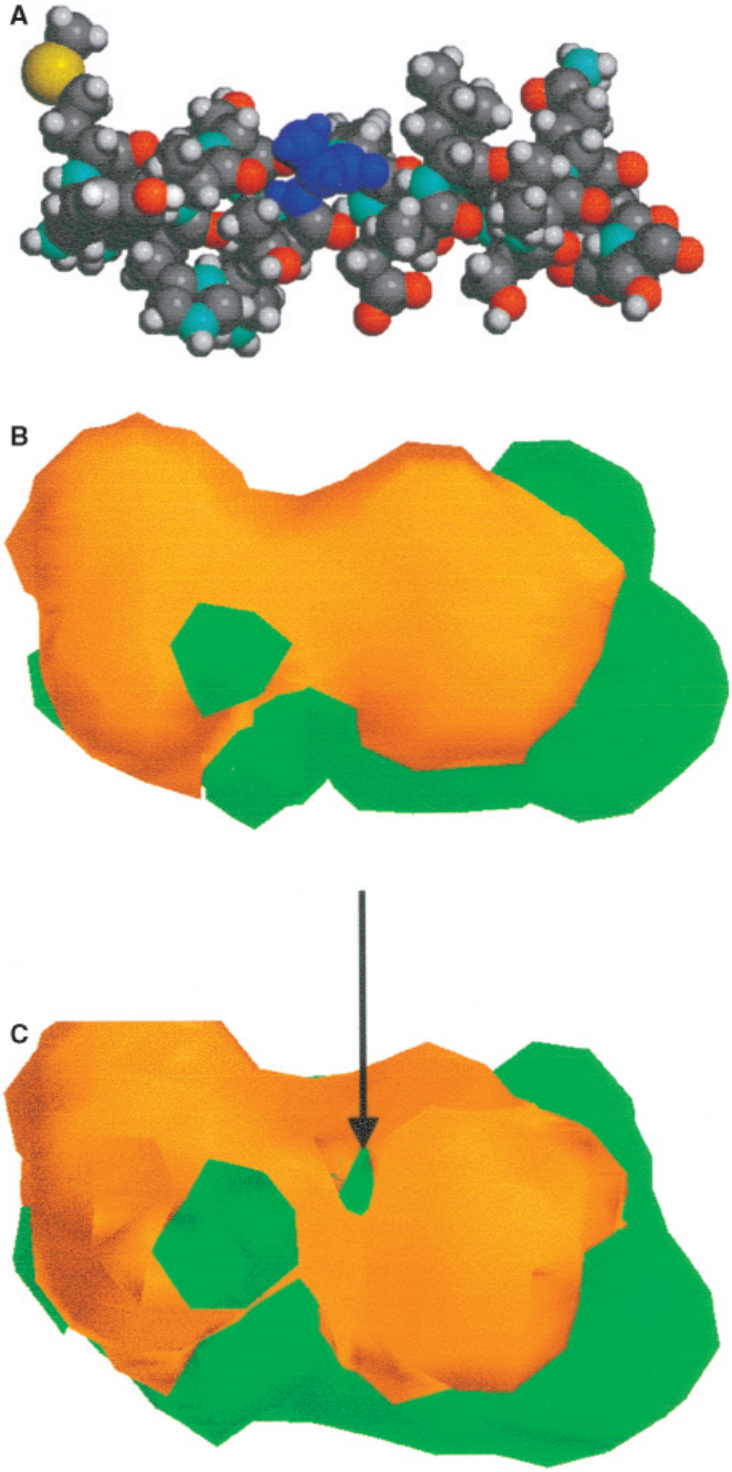

Fig. 2. MHP surfaces around segment 15–33. (A) Corey Pauling Kolton representation of peptide 15–33. The N terminus is on the left, and the C terminus is on the right. Ala-23 is highlighted in blue. (B) apoC-III-Ala23 and (C) apoC-III-Thr23. MHP surfaces around segment 15–33 in the same orientation. Green surfaces represent hydrophilic domains whereas orange surfaces represent hydrophobic domains. The surfaces are cut with a plane to visualize the mutated residue (in blue), identified with an arrow.

ilar α-helical content both in native and lipid-bound form. The percentage of α-helical structure increased by 17% for the wild type and by 15% for the mutant apoC-III on binding to DMPC.

Displacement of apoE by apoC-III variants from discoidal apoE:DPPC complexes

The capacity of apoC-III to displace apoE from the reconstituted apoE:DPPC complexes was monitored by analyzing gel-filtration profiles of apoE:DPPC-apoC-III mixtures. Briefly, as described in Subjects and Methods, the apoE: DPPC complexes were incubated in vitro with the apoC-III proteins at a 1:1 (w/w) ratio for 2 h. The mixture was then gel filtrated on a Superose 6 PG column to check the integrity of the original apoE:DPPC complexes and to identify the distribution of apoE and apoC-III on the particles. The apoE:DPPC complex eluted at its original elution volume and any displaced apoE or unbound apoC-III, not

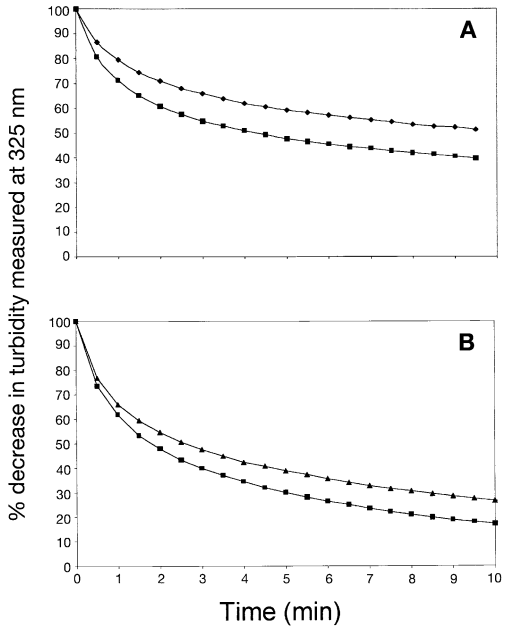

Fig. 3. Kinetics of clarification of DMPC liposomes by apoC-III. Decrease in turbidity of DMPC multilamellar vesicles mixed with 40 μg of recombinant protein of wild-type apoC-III-Ala23 (solid squares) and mutant apoC-III-Thr23 (solid triangles), at a lipid-to-protein ratio of 2:1 (w/w) over 10 min at (A) 20°C and (B) 30°C. Results are expressed as a percentage of the original optical density signal of DMPC multilamellar vesicles measured at 325 nm. These results are representative of three repeat experiments.

associated with the complex, eluted at higher elution volumes of 34 ml (data not shown). To determine the exact distribution of both apoE and apoC-III, samples from all the individual fractions were separated on either Tricine-SDS-polyacrylamide gels for the analysis of apoC-III or on 15% SDS-polyacrylamide gels for the analysis of apoE, combined with Western blotting for apoC-III and apoE. ApoE displaced from its original complex was detected at higher elution volumes, as expected. Both apoC-III-Ala23 and apoC-III-Thr23 were found associated with the apoE:DPPC complex. These qualitative analyses illustrated that a fraction of the apoE from the complex had been displaced by apoC-III, and that apoC-III had been incorporated into the apoE:DPPC complex. A more accurate quantification of the distribution of both apoC-III and apoE was also determined by measuring both proteins in the individual fractions by specific and sensitive ELISA methods. Displacement by apoC-III-Ala23 resulted in 87% of the original apoE remaining in the apoE:DPPC complex while 81% of original apoE remained after displacement by apoC-III-Thr23. The apoC-III concentrations determined in the complex were 62 μg/ml for apoC-III-Ala23 and 43 μg/ml for apoC-III-Thr23. The resulting complexes revealed that the ratio (mol/mol) of apoE/apoC-III-Thr23 was a modest 34% higher in the apoE/apoC-III-Ala23 complexes.

LPL inhibition by recombinant apoC-III proteins

The 2.5-fold difference in LPL activity in the presence and absence of apoC-II is low and reflects the fact that the experiment was optimized to be performed at the lowest concentration of apoC-II that activated LPL. The ability of increasing concentrations of recombinant apoC-IIIs (range, 1.5–15 μM) to inhibit bovine LPL was estimated in the presence (**Fig. 5A**) or absence (Fig. 5B) of apoC-II. Both wild type and the apoC-III variant showed a similar ability to inhibit LPL and were more potent inhibitors than human plasma apoC-III (results not shown). These results represent the means of three experiments.

DISCUSSION

We have identified a novel apoC-III Thr-23 for alanine substitution associated with apoC-III deficiency, identified in three Mayan Indians from the Yucatan Peninsula, with two of the three carriers having low plasma Tg levels. Because samples from relatives were not available, cosegregation of the phenotype with Thr-23 could not be confirmed. The 1125G→A transition, creating the amino acid substitution, occurs at a CpG dinucleotide, a "hot spot" for mutational events, raising the possibility that the mutation could have occurred more than once. However, this is unlikely in view of the rarity of apoC-III deficiency and *APOC3* gene mutations. Using DNA polymorphisms of the *APOAI-C3-A4* gene cluster, all three individuals with the mutation were carriers of a haplotype defined by −75A, S−, 1100T, V−. Although samples were not available from sufficient unrelated Yucatan subjects to determine the frequency of this haplotype, the haplotype common to all three carriers is defined by the rare alleles of the −75G→A,

TABLE 3. Physicochemical characteristics of recombinant wild type and apoC-III-Thr23 and the interaction with apoE:DPPC complexes

	k (% of Wild Type) [a]		Bound/ Free[b]	% Increase in α Helix on Binding to DMPC	ApoE/ApoC-III Molar Ratio in DPPC Complex (% of Wild Type)
	20 °C	30°C			
apoC-III-Ala23	0.43 ± 0.03 (100)	0.17 ± 0.02 (100)	3.6	17	100
apoC-III-Thr23	0.28 ± 0.03 (65)	0.07 ± 0.01 (41)	2.1	15	134

[a] The kinetics study of DMPC:apoC-III association was performed by monitoring the rate of clearance of the turbidity of DMPC liposomes at 325 nm, as a function of time at different temperatures. The rate constant *k* was determined as described in the Subjects and Methods section.

[b] Ratio of bound apoC-III on the complex/unbound apoC-III, estimated from the relative tryptophan fluorescence emission intensity of the gel-filtered samples.

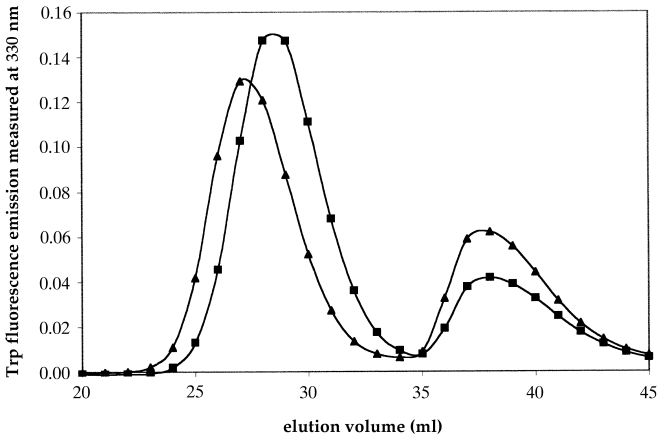

Fig. 4. Gel filtration on a Superose 6 HR column of apoC-III:DMPC complexes. ApoC-III-Ala23 (solid squares); apoC-III-Thr23 (solid triangles). Tryptophan fluorescence emission was measured at 330 nm as a function of the elution volume in milliliters.

1100C→T and *Ssi*I in studies of Caucasians (57, 58), suggesting that in these individuals the mutation, occurring on this rare allele, is identical by descent.

By comparison with the C-terminal domain, the N terminus, and particularly residues 12–40 of apoC-III, are well conserved among species (59), increasing the probability that this mutation is functionally important. We therefore carried out molecular modeling and expression studies to explore the possible mechanisms by which the Ala23Thr mutation leads to apoC-III deficiency.

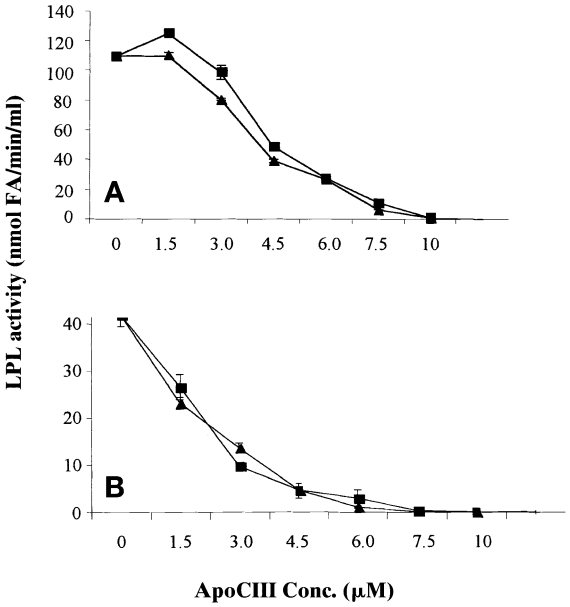

Fig. 5. Effect of recombinant apoC-III proteins on LPL activity, using Intralipid™ as substrate. Bovine LPL (15 ng) was incubated for 30 min at 25°C in a total volume of 200 μl with ^{3}H-labeled Intralipid™ in the presence (A) and in the absence (B) of 40 ng of human apoC-II protein with apoC-III-Ala23 (solid squares) and apoC-III-Thr23 (solid triangles). Each data point is the mean of triplicate samples. LPL activity is expressed as nanomoles of fatty acid released per minute per milliliter.

All the soluble apolipoproteins, including apoC-III, have a similar gene arrangement of repeated blocks of functionally identical amino acids, which have the characteristics of amphipathic α helices, with hydrophobic residues segregated on one side of the helix and polar residues on the other (60, 61). These amphipathic helices are involved in the lipid-binding properties of the apolipoproteins, and the amphipathicity of apolipoprotein is correlated with the ability of the peptide to interact with phospholipid. On the basis of the consensus secondary structure predictions the N-terminal domain of apoC-III is predicted to fold as a typical amphipathic lipid-binding α helix, especially between residues 5 and 40 (37, 62). Calculations of the hydrophobicity potentials around segment 15–33 of this N-terminal helix suggest that the hydrophobic isopotential envelope, which is undisturbed and prominent for the wild-type protein, is disturbed in the Thr-23 mutant. The Thr-23 for alanine substitution introduces a hydrophilic patch in the center of the hydrophobic isopotential envelope (Fig. 2B and C). This substitution would be predicted to decrease the energy of interaction with lipids at the N terminus. The secondary structure prediction shows that the mutation induces a local structural variability that would be predicted to reduce the stability of the first helical domain (Fig. 1A and B). Because the amphipathic helical structure appears to be the lipid-interacting unit in apolipoproteins (37, 63), the two modeling methods suggest a decreased lipid-binding affinity for the Thr-23 mutant.

These predictions are borne out by the in vitro experiments, which were carried out with recombinant wild-type and mutant apoC-III. Both recombinant proteins have a terminal histidine tag, but all the physicochemical data point to the similar behavior of the histidine-tagged and natural wild-type apoC-III, confirming that function is not affected by its presence. Functional characterization of uncleaved N-terminal histidine-tagged recombinant apoA-I (64, 65) and C-terminal recombinant histidine-tagged lecithin: cholesterol acyltransferase (LCAT) (57) has been performed, and the tagged proteins are reported to be no different when compared with plasma apoA-I or LCAT, respectively. In addition, the extra six histidine residues are unlikely to be well structured and therefore are unlikely to affect function. For the lipid moiety, DMPC was used. DMPC is commonly used in in vitro experiments examining, for example, apoA-I and E lipid-binding properties (66, 67). We chose to analyze the apoE displacement by apoC-III on discoidal apoE complexes because these particles are more homogeneous than VLDL or HDL isolated from individual donors. Furthermore, apoC-III-deficient plasma was not available and endogenous apoC-III would affect the experiments.

With respect to the lipid-binding properties, data from both the kinetics of DMPC binding and the analysis of the gel filtration of the DMPC complexes demonstrate that, although apoC-III-Thr23 does bind to phospholipids, the affinity of this mutant apoC-III is statistically significantly lower than that of the wild-type apoC-III-Ala23, although these differences are modest. Wild-type apoC-III formed a

smaller complex with DMPC compared with the apoC-III-Thr23:DMPC complex, which had a reduced bound/free apoC-III ratio, suggesting that apoC-III-Thr23 has a lower affinity for the phospholipid than the wild-type apoC-III. The secondary structure analyses by CD revealed that for both proteins the α-helical content of apoC-III after binding to DMPC increased by about 15–17%, illustrating that as a result of the binding to the phospholipid the secondary structure of both proteins was stabilized.

In vivo, *APOC3* transgenic mice display severe hypertriglyceridemia as a result of a dramatic reduction in hepatic uptake of TGRL (68, 69). The mechanism for this has been suggested to be due to the inhibition of VLDL binding to the LDL receptor due to the high plasma levels of apoC-III, and could be corrected by breeding these mice with apoE transgenics (70). These counteracting effects of apoC-III and apoE are confirmed by in vitro studies showing that apoC-III decreases and apoE increases lipoprotein binding to low density lipoprotein receptor-related protein (71). Taken together these data suggest that apoC-III interferes with the apoE-mediated hepatic uptake of lipoproteins by the displacement of apoE, thus reducing TGRL clearance.

Cardin, Jackson, and Johnson (72) reported that a common binding site existed on DMPC vesicles for various apolipoproteins and thus apoE and apoC-III may share common binding sites on such lipid particles. Their binding to lipid particles is competitive, reversible, and in equilibrium. In the present study displacement among the apolipoproteins occurred without the concomitant dissociation of lipid from the lipid particles, because no free lipid was seen when apoC-III displacement of apoE from the complex was analyzed by gel filtration. The displacement of apoE by apoC-III depends on the lipid-binding affinity of apoC-III; in other words, it is dependent on the amphipathicity of the α helix of each protein. The displacement of apoE from the apoE:DPPC complex by apoC-III-Thr23 was comparable with wild-type apoC-III, but binding of apoC-III on the complex was decreased for the mutant apoC-III. This is probably due to the reduced lipid-binding ability of the mutant apoC-III, resulting in a higher apoE/apoC-III-Thr23 complex, compared with wild-type apoC-III.

In the current study, in vitro effects on LPL hydrolysis by recombinant apoC-III-Thr23 protein were comparable to that of wild-type recombinant apoC-III, suggesting that this amino acid change, located in the N terminus of apoC-III, does not affect LPL inhibition.

To date, four rare naturally occurring amino acid variants have been reported (29–32). A Gln38Lys substitution was associated with higher apoC-III levels and Tg levels (32). This amino acid substitution results in an additional charge on the protein that might enhance lipid binding and/or alter the effect on LPL, which could explain the raised plasma apoC-III and Tg levels; however, no in vitro studies were carried out to confirm this. A Lys58Glu change has been reported, associated with 30–40% lower apoC-III levels and with reduced apoC-III-Glu58 on VLDL and HDL resulting in apoE-enrichment of HDL, creating atypically large HDL particles (31). Neither the Asp45Asn variant (29), nor the Thr74Ala variant, which disrupts a glycosylation site, is associated with dyslipidemia (30). Both Lys58 and Ala23 are conserved among several mammalian species (59, 62).

Extrapolation of in vitro data to the in vivo situation must be carried out with caution. The molecular modeling and the in vitro data, using recombinant apoC-III, demonstrate that residue 23 lies in an amphipathic helix that is important in lipid binding but not for the direct inhibition of LPL. We can only speculate that in vivo, in Thr-23 carriers, apoC-III-Thr23, with its lower lipid-binding affinity, might be catabolized more rapidly in plasma, resulting in low plasma apoC-III levels. This could results in TGRL particles with a higher apoE/apoC-III ratio that might be cleared faster by receptors and thus result in low Tg levels. The lower plasma Tg levels seen in two of the three carriers of the variant might reflect this increased cellular uptake of the TGRL particles mediated by apoE because the apoC-III-Thr23 competes less well for lipid binding. The third Thr-23 carrier had raised plasma Tg and cholesterol levels when compared with the mean values for the Mayan control sample and a low BMI. Difference in lipid levels could not be explained by apoE genotype because all three carriers had the same *apoE3* genotype. This suggests that other unidentified genetic and/or environmental factors (e.g., diet or smoking) may be modulating the effect of the Ala23Thr variant.

It is surprising that in the heterozygous state this apoC-III-Thr23 variant appears to act in a dominant manner. This might reflect the coinheritance of other unidentified mutations affecting the clearance of TGRL. In vitro studies using short peptides of apoE suggest that apoE may function as a dimer when acting as a ligand for lipoprotein binding to the LDL receptor (73). Thus, by analogy, an alternative hypothesis is that apoC-III normally acts as a multimer and heteromultimers containing both apoC-III-Ala23 and apoC-III-Thr23 may be unstable or their associations with lipids may be weak, which would result in a dramatic increase in apoC-III catabolism and cause apoC-III deficiency in heterozygous individuals. Unfortunately, because of the geographic location of the only identified carriers of this variant, it is not possible to confirm these predictions in vivo by detailed turnover studies and lipid profiles. However, the findings shed light on the functional domains of this important apolipoprotein and the modeling and *E. coli* expression system will allow other functional regions to be examined.

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