Missense Mutations in APOB within the βα1 Domain of Human APOB-100 Result in Impaired Secretion of ApoB and ApoB-containing Lipoproteins in Familial Hypobetalipoproteinemia

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Familial hypobetalipoproteinemia (FHBL) is associated with mutations in the APOB gene. We reported the first missense APOB mutation, R463W, in an FHBL kindred (Burnett, J. R., Shan, J., Miskie, B. A., Whitfield, A. J., Yuan, J., Tran, K., Mc- Knight, C. J., Hegele, R. A., and Yao, Z. (2003) J. Biol. Chem. 278, 13442–13452). Here we identified a second nonsynonymous APOB mutation, L343V, in another FHBL kindred. Heterozygotes for L343V (n = 10) had a mean plasma apoB at 0.31 g/liter as compared with 0.80 g/liter in unaffected family members (n = 22). The L343V mutation impaired secretion of apoB-100 and very low density lipoproteins. The secretion efficiency was 20% for B100wt and 10% for B100LV and B100RV. Decreased secretion of mutant apoB-100 was associated with increased endoplasmic reticulum retention and increased binding to microsomal triglyceride transfer protein and BiP. Reduced secretion efficiency was also observed with B48LV and B17LV. Biochemical and biophysical analyses of apoB domain constructs showed that L343V and R463W altered folding of the α-helical domain within the N terminus of apoB. Thus, proper folding of the α-helical domain of apoB-100 is essential for efficient secretion.

Apolipoprotein (apo) B, a large amphipathic glycoprotein, plays a central role in human lipoprotein metabolism (1, 2). The human apoB gene (APOB) is located on chromosome 2 and produces, via a unique mRNA editing process (3), two forms of apoB, namely apoB-48 (2152 amino acids) and apoB-100 (4536 amino acids) (4, 5). ApoB-48 is the truncated form of apoB-100 consisting of the N-terminal 48% of full-length apoB-100. ApoB-48 is synthesized in the intestine and is essential for the formation and secretion of chylomicrons. ApoB-100 is synthesized in the liver and is an essential structural component of very low density lipoprotein (VLDL) and its metabolic products, intermediate density lipoprotein (IDL) and low density lipoprotein (LDL), and is also a ligand for the LDL receptor. Unlike humans, the rat liver produces both apoB-100 and apoB-48, and both forms can assemble VLDL (6).

A pentatetrapartite model for human apoB-100 has been proposed, which depicts a five-domain structure composed of alternating amphipathic α-helices and amphipathic β-strands, namely βα1-βα1-α2-βα2-α3 (7). The βα1 domain is a mixture of 13 amphipathic β-strands and 17 amphipathic α-helices, whose amino acids share extensive sequence homologies to the yolk protein lipovitellin (7–9). The apoB βα1 domain has been modeled on the structure of silver lamprey lipovitellin, in which the 13 β-strands (amino acids 21–263) form a β-barrel, whereas the 17 α-helices (amino acids 440–592) form a two-layered helical bundle (10). There is an interface between the α-helical bundle

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6 The abbreviations used are: apo, apolipoprotein; DMEM, Dulbecco’s modified Eagle’s medium; ER, endoplasmic reticulum; FBS, fetal bovine serum; FHBL, familial hypobetalipoproteinemia; IDL, intermediate density lipoproteins; LDL, low density lipoprotein; mAb, monoclonal antibody; MTP, microsomal triglyceride transfer protein; VLDL, very low density lipoprotein; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; LV, B100LV.
and the extended amphipathic β-sheets (termed C-sheet and A-sheet in the lipovitellin structure). Recent studies have shown that the ability of apoB to initiate lipid binding resides in a region within the amphipathic C-sheet (i.e. between apoB-19 (residues 1–862) and apoB20.1 (residues 1–912)) (11, 12).

Several lines of evidence indicate that the βα1 domain of apoB is of particular importance in lipoprotein assembly. Transfection studies show that apoB segments lacking the βα1 domain are either unable to be secreted (13) or poorly secreted (14). Mutagenesis experiments show that correct disulfide bond formation within the βα1 domain is required for efficient secretion of apoB (15–17), and this requirement is independent of the lipidation state of apoB (18). The sequence elements involved in the physical interaction between apoB and its molecular chaperone, the microsomal triglyceride transfer protein (MTP), have been located to the βα1 domain (19). MTP, the product of the abetalipoproteinemia (OMIM 200100) gene (20, 21), is known to transfer triglyceride, cholesteryl esters, and phospholipids and is essential for apoB-containing lipoprotein assembly and secretion (22). In vitro experiments suggest the presence of multiple MTP-binding sites within apoB (9, 23, 24).

Segrest et al. (25) have proposed that the interaction of MTP with the apoB βα1 domain forms a lipid pocket that facilitates lipid recruitment during lipoprotein formation. Recently, the same group has postulated a hairpin-bridge mechanism for lipid pocket completion (26).

Familial hypobetalipoproteinemia (FHBL; OMIM 107730) is a genetically heterogeneous autosomal co-dominant disorder characterized by low levels (<5th percentile for age and sex) of plasma apoB-containing lipoproteins (27–30). It has been suggested that FHBL represents a longevity syndrome (31) and might be associated with cardiovascular protection because of resistance to atherosclerosis (32). Heterozygotes are usually asymptomatic with LDL cholesterol and apoB-100 concentrations <50% of those in normal plasma. Homozygotes have undetectable plasma LDL cholesterol and apoB, and their clinical presentation, depending on the specific mutation, varies from no symptoms to severe gastrointestinal and neurological dysfunction, similar to that in abetalipoproteinemia (27, 28, 30). Phenotyping and Genotyping of the L343V Kindred—The proband (Fig. 1A, subject II:1) was referred to a lipid disorders clinic with marked hypocholesterolemia detected on routine lipid screening. The subject was also noted to have iron deficiency anemia secondary to gastrointestinal bleeding. A pedigree was constructed, and the kindred extended to a total of 32 members, including 10 heterozygotes and 22 unaffected subjects. Fasting blood (10 ml) samples obtained from each individual were collected into a plain tube and a tube containing EDTA (1 mg/ml) to separate serum and plasma, respectively, by centrifugation (2,500 rpm, 15 min, 20 °C). The protease inhibitor aprotonin (100 kilounits/ml) was added to plasma. Plasma total cholesterol, high density lipoprotein cholesterol, triglyceride, LDL cholesterol, and the serum activities of alanine aminotransferase (EC 2.6.1.2), aspartate aminotransferase (EC 2.6.1.1), and γ-glutamyltransferase (EC 2.3.2.2) were measured enzymatically using reagents from Roche Diagnostics on a Hitachi 917 analyzer. Plasma apoB-100 and apoA-I were measured using Behring reagents (Lane Cove, Australia) on a Behring BN-II nephelometer. Serum α-tocopherol and retinol were determined by reverse phase-high performance liquid chromatography using a C-18 column. Serum 25-dihydroxyvitamin D was determined using the INCSTAR/DiaSorin radioimmunooassay (Stillwater, MN). In some experiments, proteins in delipidated plasma were subjected to PAGE (5% gel) containing 0.1% SDS (SDS-PAGE), and transferred to a nitrocellulose membrane (Bio-Rad) as described previously (36). The membrane was incubated with monoclonal antibody (mAb) 1D1 (a gift of Drs. R. W. Milne and Y. L. Marcel, University of Ottawa Heart Institute, Ottawa, Canada) that recognizes an epitope of human apoB in amino acids 401–582. Genomic DNA was extracted from peripheral blood leukocytes by a standard Triton X-100 procedure. The 29 exons of APOB, including intron-exon boundaries, were amplified using PCR. Each reaction contained 100–200 ng of genomic DNA, 1× PCR Buffer, 2 mM MgCl₂, 5 pmol of each of forward and reverse primer (M13-tagged), and 1 unit of TaqDNA polymerase (Roche Diagnostics) in a total volume of 25 μl. DNA was amplified under the following conditions: 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 58 °C for 30 s, 72 °C for 45 s, and a final extension for 2 min at 72 °C. BigDye Terminator sequencing reactions were then carried out in both directions using PCR product as template and M13 primers. Sequencing reactions were purified and detected on an ABI Prism 3730 sequencer (Applied Biosystems, Foster City, CA). Sequences were visually checked and then edited and aligned to control sequences using the program Bio-Edit (37). The L343V mutation was confirmed by sequenc-
ApoB L343V Mutation

ing of a second PCR of APOB exon 9 of the proband. As the L343V mutation creates an HphI restriction site, family members were screened by restriction fragment length polymorphism assay. Genotype analysis of APOE was performed by PCR amplification followed by digestion with the restriction enzyme HhaI as described previously (38).

Preparation of Expression Plasmids—The expression plasmids encoding human apoB-100 (39), B-48 (40), and B-17 (41) cDNA, respectively, were prepared as described previously. Numbers following apoB represent the percent of full-length apoB (apoB-100). The resulting pB48wt and pB17wt were used as templates to prepare pB48RW and pB17RW, respectively, using the QuikChange™ mutagenesis kit (Stratagene, Ann Arbor, MI) as described previously (35). For the preparation of pB100RW, a NotI-ClaI fragment (the NotI site was located at the 5′ upstream of the apoB cDNA and the Clai site at nucleotide 5849 of the apoB cDNA) was excised from pB48 and inserted into pB100wt that had been digested with NotI and Clai. Expressing plasmids encoding variants of apoB-100, apoB-48, and apoB-17 that contained the L343V mutation were similarly constructed. The mutagenic primers used to introduce the L343V mutation were as follows: CA TCT CTC TTG and GCT GGA CAC CTC AAT CAC CTG TGG CAA GAG AGA (forward) and GCT GGA CAC CTC AAT CAC CTG TGG CAA GAG AGA (reverse). Both primers presented are in the 5′-or 3′-oriented ultracentrifugation. The 35S-apoB proteins in each fraction were recovered by immunoprecipitation, resolved by SDS-PAGE, and visualized by fluorography as described above.

Subcellular Fractionation—Subcellular fractionation of intracellular microsomes was performed as described previously (35). In brief, cells (two 10-cm dishes) were homogenized using a ball-bearing homogenizer (H and Y Enterprise, Redwood City, CA). Postnuclear supernatants were subjected to fractionation by centrifugation in a gradient of Nycodenz (Sigma). The recombinant human apoB in each fraction was probed by immunoblotting using mAb 1D1. The anti-calnexin (StressGen, Ann Arbor, MI) and anti-giantin (Abcam, Hornby, Ontario, Canada) antibodies were used to probe endoplasmic reticulum (ER) and cis/medial Golgi, respectively.

Lipoprotein Fractionation—Cells were labeled with [35S]methionine/cysteine (100 μCi/ml) for 4 h in methionine- and cysteine-free DMEM with or without 20% FBS and 0.4 mM oleate. After labeling, the media were fractionated into VLDL1 (S, >1000), VLDL2 (S, 20–100), and other lipoproteins by rate flotation ultracentrifugation. The 35S-apoB proteins in each fraction were recovered by immunoprecipitation, resolved by SDS-PAGE, and visualized by fluorography as described above.

Co-immunoprecipitation—Cells were treated with MG132 (25 μM) for 1 h, washed twice with cold PBS, and harvested in 1 ml of lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 5 mM EDTA, 20% sucrose, protease inhibitor mixture). The samples were incubated for 1 h at 4 °C by gentle mixing, and the insoluble materials were removed by centrifugation (13,000 rpm, 4 °C, 15 min). Aliquot of the supernatant (500 μg of protein) was mixed with lysis buffer to a final volume of 1 ml. The samples were pre-cleared by incubation with non-immune rabbit serum (2 h, 4 °C) prior to incubation with an anti-human LDL antisera (16 h, 4 °C). Immunocomplexes were captured with protein A-Sepharose CL-4B beads, and proteins were eluted with SDS-PAGE sample buffer, resolved by SDS-PAGE, and analyzed by immunoblotting for apoB, MTP (using anti-MTP antibody that was a gift of Dr. C. C. Shoulders, MRC Clinical Sciences Centre, Hammersmith Hospital, London, UK), and BiP (the anti-Grp78 (BiP) antibody was purchased from StressGen, Ann Arbor, MI).

Assessment of ApoB Polyubiquitination—Cells (~80% confluence in 10-cm culture dishes) were incubated in media ± MG132 (25 μM) for 1 h and lysed with 1% SDS in RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% w/v phenylmethylsulphonyl fluoride, 1 mM dithiothreitol) at 80 °C. The samples were incubated for additional 16 h (4 °C) and diluted to 0.1% SDS with RIPA buffer, and apoB proteins were immunoprecipitated with the rabbit anti-human apoB antisera as described above. After repeated washing with 0.1% SDS in RIPA buffer, the captured proteins were released into SDS-PAGE sample buffer, divided into 2 equal aliquots, and resolved by SDS-PAGE. Proteins were transferred to nitrocellulose membranes, probed by mAb 1D1 or anti-ubiquitin (SPA-203: StressGen, Ann Arbor, MI), and visualized by enhanced chemiluminescence (Roche Diagnostics) using horseradish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad).

Protease Protection Analysis of ApoB-100—Cells (80% confluence in 10-cm dishes) were incubated in media ± MG132 (25 μM) for 1 h. The media were removed, and the cells (combined from two dishes) were harvested in ice-cold PBS. The cells were
collected after centrifugation (50 x g, 2 min), resuspended in microsome buffer (10 mM Tris-HCl, pH 7.4, 250 mM sucrose, 100 μM leupeptin, 100 μM phenylmethysulfonyl fluoride, 25 μM MG132, 10 kilounits/ml aprotinin), and homogenized using ball-bearing homogenizer as described previously (44). Nuclei were pelleted by centrifugation (9,500 rpm, 4 °C, 10 min, in an SS34 rotor), and the postnuclear supernatant was further centrifuged (100,000 rpm, 4 °C, 16 min, in a MLA-130 rotor) to obtain microsomes. The microsomes were resuspended in the microsome buffer (without protease inhibitors) and subjected to protease protection analysis as described previously (44, 45).

Immunoblotting for human apoB (1D1), protein-disulfide isomerase (SPA-891, StressGen, Ann Arbor, MI), Hsp70 (SPA-820, StressGen), p97 (RDI-PRO6527, Research Diagnostics, Inc., Concord, MA), and the N-terminal epitope of calnexin (SPA-865, StressGen) was performed using respective antibodies.

Preparation of Bacterially Expressed ApoB Domain Constructs—For protein expression, plasmids coding for apoB domain constructs, namely B6.4-13, B6.4-17, and B13-17, were transformed into BL21 (DE3) competent cells. Transformed cells were grown at 37 °C to an absorbance of 0.6–0.8 at 600 nm in Luria broth and treated with isopropyl-β-d-thiogalactopyranoside (1 mM) for 3 h. Cell pellets were collected, treated with lysozyme (1 mg/ml, 30 min, 25 °C), and disintegrated with a probe sonicator (Branson, Danbury, CT). Inclusion bodies were dissolved in 8 M urea after washing with 1% Triton X-100 and 1 M urea.

For protein preparation, denatured proteins were loaded onto a nickel-nitrilotriacetic acid-Sepharose column (Qiagen, Valencia, CA) and eluted with 250 mM imidazole in 6 M guanidine hydrochloride (GdnHCl) at pH 8.0. Purified proteins were refolded by slowly adding the denatured protein into a refolding buffer (50 mM Tris, 800 mM arginine, 10 mM reduced glutathione, 2 mM oxidized glutathione, 0.02% sodium azide, pH 8.0). The proteins (1–2 mg/ml in the refolding buffer) were incubated at 4 °C for 16 h and dialyzed extensively against 10 mM Tris, 150 mM sodium chloride, pH 7.5 (TS buffer). The final protein volume and concentration were adjusted using an Amicon Ultra concentrating apparatus (Millipore, Billerica, MA), and the protein concentration was determined by UV absorbance at 280 nm (46).

Limited Proteolysis of ApoB Domain Construct B6.4-17—The wild-type or mutant forms of the apoB domain construct B6.4-17 (1 mg/ml in TS buffer) were mixed with freshly prepared bovine pancreas trypsin (1 mM hydrochloric acid) at the

FIGURE 1. The L343V mutation within human apoB-100. A, pedigree of the LV kindred. The roman numerals designate the generation numbers, and the individual subjects in each generation are identified by arabic numerals. The arrow indicates the proband. B, electropherogram tracings of exon 9 sequence from genomic DNA templates in a normal subject and a heterozygote for APOB L343V. Single letter amino acid codes are shown in boldface along with codon numbers and nucleotide sequences. The position of the mutant nucleotide is indicated by an arrow. C, top line depicts the wild-type (wt) B100, with locations of α helices in the βα1 domain shown. Arrows indicate positions of Leu343 and Arg463. The bottom panels show 35S-labeled human apoB-100 and the “apoB-48 like protein” (39) associated with cells (left) and secreted into the media (right) after 4 h of metabolic labeling with [35S]methionine/cysteine under lipid-poor (-; serum- and oleate-free) or lipid-rich (+; 20% serum plus 0.4 mM oleate) conditions. a.a., amino acids; LV, B100LV; RW, B100RW.
ApoB L343V Mutation

TABLE 1
Plasma lipid and apolipoprotein concentrations and APOE genotype in the L343V kindred

| Subject | Age (years) | Sex, M/F | Lipids | Apoproteins | APOE genotype |
|---------|-------------|----------|--------|-------------|---------------|
|         |             |          | TC    | HDL-C       | TG | LDL-C | ApoB | ApoA-1 |       |
| LV heterozygotes |         |          |       |             | g/liter |        |       |       |       |
| II:1    | 64          | M        | 2.8   | 1.4         | 0.7 | 1.1   | 0.35 | 1.43  | e2/3  |
| II:3    | 60          | F        | 3.5   | 1.3         | 0.9 | 1.8   | 0.59 | 1.32  | e2/3  |
| II:9    | 54          | F        | 2.9   | 2.0         | 0.3 | 0.7   | 0.27 | 1.89  | e2/3  |
| II:11   | 54          | F        | 2.9   | 1.5         | 0.7 | 1.1   | 0.43 | 1.44  | e3/3  |
| III:1   | 41          | M        | 2.1   | 1.1         | 1.4 | 0.4   | 0.29 | 1.31  | e2/3  |
| III:2   | 35          | F        | 2.3   | 1.3         | 0.2 | 0.7   | 0.27 | 1.57  | e2/3  |
| III:3   | 31          | M        | 2.8   | 1.7         | 0.3 | 0.9   | 0.26 | 1.99  | e2/3  |
| III:5   | 32          | F        | 3.3   | 2.3         | 0.4 | 0.8   | 0.29 | 0.93  | e3/3  |
| III:20  | 22          | M        | 1.9   | 0.8         | 0.6 | 0.9   | 0.17 | 0.99  | e2/3  |
| III:4   | 4           | M        | 1.6   | 1.0         | 0.5 | 0.3   | 0.31 | 1.46  |       |

| Mean    |              |          | 2.6   | 1.5         | 0.6 | 0.9   | 0.31 | 1.46  |       |
| S.D.    |              |          | 0.6   | 0.5         | 0.4 | 0.4   | 0.12 | 0.35  |       |

Unaffected

| II:2    | 59          | F        | 4.8   | 1.9         | 0.4 | 2.8   | 0.81 | 1.90  | e3/3  |
| II:4    | 62          | M        | 5.3   | 1.4         | 0.9 | 3.5   | 1.09 | 1.65  | e3/3  |
| II:5    | 57          | F        | 4.2   | 1.1         | 1.7 | 2.3   | 0.84 | 1.39  | e2/3  |
| II:6    | 59          | M        | 5.0   | 1.2         | 1.3 | 3.2   | 1.02 | 1.41  | e3/3  |
| II:7    | 56          | F        | 3.7   | 1.5         | 1.2 | 1.7   | 0.70 | 1.64  | e2/3  |
| II:8    | 60          | M        | 5.5   | 1.1         | 0.7 | 4.0   | 1.23 | 1.35  | e3/3  |
| II:12   | 53          | M        | 4.2   | 1.1         | 0.9 | 2.7   | 0.82 | 1.36  | e3/3  |
| III:6   | 30          | M        | 5.1   | 0.9         | 0.9 | 3.8   | 1.13 | 1.06  | e3/3  |
| III:8   | 30          | M        | 4.6   | 1.6         | 0.6 | 2.7   | 0.80 | 1.47  | e3/3  |
| III:9   | 25          | M        | 4.6   | 1.5         | 0.5 | 2.8   | 0.71 | 1.35  | e2/3  |
| III:10  | 24          | F        | 3.8   | 1.7         | 0.8 | 1.7   | 0.57 | 1.61  | e2/3  |
| III:12  | 30          | F        | 3.5   | 1.6         | 0.3 | 1.8   | 0.56 | 1.42  | e2/3  |
| III:13  | 28          | F        | 5.1   | 1.3         | 0.7 | 3.5   | 1.10 | 1.45  | e3/3  |
| III:14  | 27          | F        | 4.5   | 1.8         | 0.3 | 2.6   | 0.74 | 1.73  | e2/3  |
| III:15  | 24          | F        | 5.0   | 1.4         | 0.7 | 3.3   | 1.02 | 1.57  | e3/3  |
| III:17  | 33          | F        | 5.4   | 1.7         | 0.5 | 3.5   | 0.91 | 1.55  | e3/3  |
| III:19  | 18          | F        | 2.9   | 1.1         | 0.6 | 1.5   | 0.47 | 1.19  | e2/3  |
| III:21  | 21          | M        | 4.2   | 0.8         | 1.5 | 2.8   | 1.02 | 1.02  | e3/3  |
| III:22  | 19          | M        | 2.7   | 1.2         | 0.6 | 1.3   | 0.42 | 1.26  | e3/3  |
| IV:1    | 4           | M        | 3.7   | 1.2         | 1.06| 1.9  | 0.66 | 1.21  | e2/3  |
| IV:4    | 1           | M        | 3.0   | 1.3         | 0.9 | 1.2   | 0.46 | 1.17  | e2/3  |
| IV:5    | 8           | M        | 3.8   | 1.8         | 0.3 | 1.9   | 0.57 | 1.41  | e3/3  |

| Mean    |              |          | 4.3   | 1.4         | 0.8 | 2.6   | 0.80 | 1.42  |       |
| S.D.    |              |          | 0.8   | 0.3         | 0.4 | 0.8   | 0.24 | 0.22  |       |

注：
a. 超氧化物歧化酶被认为是降低LDL-C和apoB的标志物。
b. HDL-C和LDL-C被测量直接。
c. *p < 0.05 compared with unaffected. All other comparisons are not significant.

for background correction. Protein after refolding and concentrating was prepared in 5 mM potassium phosphate using PD-10 desalting column (GE Healthcare), and its concentration was determined by UV absorbance at 280 nm immediately before each experiment. For chemical unfolding experiments, protein samples at 0.1–0.2 μM were prepared in 5 mM potassium phosphate, pH 7.5. Protein unfolding was achieved using an Aviv titration accessory by the addition of the same concentration of protein in 7 M GdnHCl, pH 7.5, in 0.1 M steps into 2.0 ml of native protein solution in a 1-cm cuvette. The sample volume was maintained constant throughout the titration, and protein unfolding was monitored at 222 nm at 25 °C. After each injection of denaturant, the sample was stirred for 3 min and equilibrated for 20 s, and the data were collected with an averaging time of 20 s. Because no unfolding curve appears to have a two-state transition and to avoid the bias in base-line correction, the raw CD data were converted to percentage unfolded values using data points at 0 and 6 M GdnHCl as the folded and unfolded reference points, respectively.

Molecular Modeling — The molecular model of apoB17 was created using the program MODELLER (48). Residues 19–766 of human apoB (49) were aligned with residues 18–758 of silver lamprey lipovitellin (50) with the assistance of the program...
BLAST 2 sequences (51). This alignment, along with the coordinates from the crystal structure of lipovitellin (10), was used as the input for MODELLER to create the homology model as described previously (35, 42). The model does not include residues 1–19 or 676–737 of apoB because of the lack of corresponding lipovitellin coordinates. Molecular graphics were generated with MOLMOL (52).

RESULTS

The L343V Kindred—The proband (II:1) had a plasma total cholesterol of 2.8 mM, LDL cholesterol of 1.1 mM, and apoB-100 of 0.35 g/liter. The presence of similarly reduced plasma concentrations of apoB-containing lipoproteins in first degree relatives confirmed the diagnosis of heterozygous FHBL in the proband (Fig. 1A). Lipid, lipoprotein, and apolipoprotein values for the L343V kindred are shown in Table 1. LV heterozygotes (n = 10) had mean plasma total cholesterol, LDL cholesterol, and apoB concentrations of 2.6 and 0.9 mm and 0.31 g/liter, respectively. LV heterozygotes had a significant mean decrease in total cholesterol (by 40%), LDL cholesterol (by 65%), and apoB-100 (by 61%) concentrations as compared with unaffected family members (n = 22). The apoE2 isoform was similarly present in LV heterozygotes (60%) and unaffected family members (41%), ruling out this variant as a possible contributor to hypobetalipoproteinemia. Despite low lipid and lipoprotein concentrations, none of the LV heterozygotes had developmental problems, malabsorption, or neurological deficits. When compared with unaffected family members, heterozygotes had significant mean increases in the serum liver enzymes alanine aminotransferase, aspartate aminotransferase, and γ-glutamyltransferase of 1.8-, 1.5-, and 2.0-fold, respectively (Table 2). No impact on serum ferritin was observed. Serum α-tocopherol concentrations were lower in LV heterozygotes compared with unaffected family members. Serum α-tocopherol was positively correlated with both plasma total cholesterol (r = 0.86; p < 0.0001), LDL cholesterol (r = 0.77; p < 0.0001) and apoB (r = 0.78; p < 0.0001) concentrations, reflecting the known relationship between vitamin E and plasma lipid concentrations. Serum retinol concentration did not differ between the two subject groups; 25-dihydroxyvitamin D levels were increased by 1.3-fold in LV heterozygotes compared with unaffected family members.

Identification of L343V Mutation—The above plasma lipid and apoB-100 data indicated a co-dominant inheritance in the FHBL kindred. Examination of plasma apoB-100 of the proband by SDS-PAGE and immunoblotting detected only the full-

| Subject | Fat-soluble vitamins | Liver enzymes | Ferritin |
|---------|---------------------|--------------|---------|
|         | α-tocopherol | Retinol | 25-OH D† | ALT | AST | GGT | µg/liter |
|         | µM | µM | µM | units/liter | units/liter | units/liter |         |
| Heterozygotes | | | | | | | |
| II:1 | 20 | 2.4 | 62 | 80 | 59 | 59 | 6 |
| II:3 | 18 | 3.3 | 53 | 76 | 47 | 74 | 172 |
| II:9 | 19 | 2.0 | 48 | 21 | 18 | 17 | 33 |
| II:11 | 15 | 1.9 | 84 | 67 | 49 | 59 | 134 |
| II:12 | 10 | 3.1 | 105 | 81 | 42 | 51 | 484 |
| II:14 | 18 | 2.3 | 101 | 38 | 21 | 24 | 84 |
| II:5 | 17 | 1.8 | 91 | 41 | 38 | 36 | 163 |
| III:20 | 7 | 1.6 | 22 | 29 | 24 | 12 | 491 |
| IV:2 | ND | ND | ND | 136 | 67 | 62 | 246 |
| Mean | 15† | 2.5 | 75† | 63† | 41† | 44† | 201 |
| S.D. | 5 | 0.8 | 30 | 36 | 17 | 22 | 178 |
| Unaffected | | | | | | | |
| II:2 | 33 | 2.9 | 87 | 14 | 18 | 12 | 143 |
| II:4 | 28 | 2.2 | 78 | 18 | 14 | 19 | 426 |
| II:5 | 25 | 3.1 | 65 | 22 | 23 | 26 | 161 |
| II:6 | 31 | 3.3 | 81 | 32 | 34 | 48 | 158 |
| II:7 | 24 | 2.0 | 55 | 26 | 22 | 28 | 178 |
| II:8 | 33 | 2.0 | 44 | 72 | 56 | 23 | 374 |
| II:12 | 23 | 2.5 | 41 | 51 | 26 | 18 | 136 |
| III:6 | 24 | 2.0 | 31 | 17 | 22 | 16 | 131 |
| III:8 | 23 | 2.3 | 54 | 19 | 22 | 19 | 122 |
| III:9 | 23 | 2.5 | 59 | 27 | 24 | 20 | 83 |
| III:10 | 28 | 2.4 | 54 | 22 | 22 | 13 | 29 |
| III:11 | 17 | 2.1 | 73 | 60 | 37 | 36 | 140 |
| III:13 | 22 | 1.9 | 58 | 61 | 34 | 43 | 111 |
| III:14 | 24 | 1.8 | 52 | 17 | 21 | 16 | 88 |
| III:15 | 24 | 2.4 | 39 | 29 | 25 | 18 | 31 |
| III:17 | 25 | 1.9 | 63 | 27 | 22 | 10 | 40 |
| III:19 | 16 | 1.4 | 51 | 108 | 41 | 25 | 112 |
| III:21 | 23 | 2.3 | 33 | 39 | 27 | 16 | 132 |
| III:22 | 14 | 2.1 | 30 | ND | ND | ND | ND |
| IV:1 | ND | ND | ND | ND | ND | ND | ND |
| IV:4 | ND | ND | ND | ND | ND | ND | ND |
| IV:5 | 16 | 1.0 | 67 | 19 | 31 | 9 | 27 |
| Mean | 24 | 2.2 | 56 | 35 | 27 | 22 | 138 |
| S.D. | 5 | 0.5 | 16 | 24 | 10 | 10 | 101 |

*The abbreviations used are as follows: 25-OH D, 25-dihydroxyvitamin D; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, γ-glutamyltransferase; ND, not determined.

†p < 0.05 was compared with unaffected. All other comparisons are not significant.

TABLE 2
Serum fat-soluble vitamins, liver enzymes, and ferritin concentrations in the L343V kindred
length apoB-100 and no truncated forms of apoB (data not shown). DNA sequence analysis of the APOB gene revealed both a cytosine and guanine peak at the first nucleotide of codon 343 in exon 9 in the proband (Fig. 1B), predicting a missense mutation whereby leucine (CTG) was replaced by valine (GTG) at residue 343 of the mature protein (L343V). Obligate heterozygotes also showed both cytosine and thymine peaks at this position. The mutation co-segregated with the clinical phenotype in the original nuclear and subsequently in the extended family (maximal LOD score = 3.70 at 0% recombination fraction). Further screening of APOB in 420 control nondyslipidemic subjects revealed complete absence of this mutation in exon 9.

Reduced Secretion of ApoB-100 Containing L343V or R463W Mutations from Transfected McA-RH7777 Cells—To determine the effect of L343V on apoB secretion, we introduced the mutation into apoB-100 (B100LV) and compared its secretion with that of wild-type apoB-100 (B100wt) and B100RW using stably transfected McA-RH7777 cells cultured in lipid-rich (i.e. 20% serum plus 0.4 mM oleate) or lipid-poor (i.e. serum-free, oleate-free) media. Metabolic labeling experiments showed that the plasmids encoding B100wt, B100RW, or B100LV expressed and secreted the full-length apoB-100 (Fig. 1C). Incorporation of the 35S-labeled amino acids into cell-associated and medium apoB-100 (both the wild type and the mutants) was increased under the lipid-rich conditions as compared with the lipid-poor conditions. However, under both conditions accumulation of 35S-apoB-100 was markedly diminished for the mutants as compared with that for the wild type. These results provide the first indication that the L343V mutation results in impaired secretion. The stably transfected cells also synthesized a C-terminally truncated “apoB-48-like” protein, which was generated in these cells by an unidentified mechanism independent of the apoB mRNA editing as shown previously (39).

Pulse-chase experiments showed that the secretion efficiency was decreased from 20% for B100wt to ~10% for B100LV and B100RW (compare 3rd panels from right, labeled B100(M) in Fig. 2, A–C). In these experiments, the recombinant human apoB-100 proteins were immunoprecipitated by a polyclonal antibody specific for the human proteins. Including the proteasomal inhibitor MG132 in the chase media resulted in an increase in cell-associated apoB-100 secretion, yet MG132 had marginal effect on B100LV and B100RW secretion (3rd panels from right, labeled B100(M)). In a separate experiment where MG132 was included in the
ApoB L343V Mutation

The effect of L343V and R463W mutation on the ability of apoB to assemble lipid-rich lipoproteins was determined by fractionation of medium lipoproteins containing apoB-100. Mca-RH7777 cells have limited capacity to synthesize or secrete VLDL under lipid-poor conditions, thus the majority of B100wt secreted from cells cultured in serum- and oleate-free medium was associated with IDL and LDL (Fig. 3A, B100wt). Under lipid-rich conditions where the media were supplemented with 20% serum and 0.4 mM oleate, B100wt was secreted mostly in association with VLDL1 and VLDL2 (Fig. 3A, B100wt). The effect of L343V and R463W mutation on the ability of apoB to assemble lipid-rich lipoproteins was determined by fractionation of medium lipoproteins containing apoB-100. Mca-RH7777 cells have limited capacity to synthesize or secrete VLDL under lipid-poor conditions, thus the majority of B100wt secreted from cells cultured in serum- and oleate-free medium was associated with IDL and LDL (Fig. 3A, B100wt). Under lipid-rich conditions where the media were supplemented with 20% serum and 0.4 mM oleate, B100wt was secreted mostly in association with VLDL1 and VLDL2 (Fig. 3B). The L343V mutation markedly diminished the secretion of VLDL1-associated apoB-100, whereas the R463W mutant retained the ability to form VLDL1. These results suggest that although both L343V and R463W mutations result in decreased apoB-100 secretion, their effects on VLDL1 secretion are different.

Increased ER Retention, Binding to Molecular Chaperones and Ubiquitination of B100LV and B100RW—To determine whether or not the L343V or R463W mutation affects intracellular trafficking of apoB-100 along the secretory pathway, we fractionated the microsomes into ER, cis/medial Golgi and distal Golgi using Nyodenz gradient ultracentrifugation (Fig. 4A). Unlike B100wt, which was distributed throughout ER/Golgi, B100LV and B100RW were largely confined to the ER, suggesting an impaired ER exit of the mutant proteins. Retention of the “apoB-48-like” protein in the ER was less profound than apoB-100 in the mutant transfected cells.

Impaired ER exit often is associated with increased binding of misfolded proteins with molecular chaperones. We determined the effect of L343V or R463W mutations on apoB (B-100 and B-48) interaction with MTP (an apoB-specific chaperone) and BiP (a general molecular chaperone), respectively, by co-immunoprecipitation experiments under conditions where proteasomal degradation of apoB was inhibited (i.e. +MG132). The recombinant human apoB (B-100 and the “B-48-like”) were immunoprecipitated under nondenaturing conditions, and the associated MTP and BiP was determined by Western blotting (Fig. 4B, top two panels). Semi-quantification of the MTP bands by scanning densitometry revealed that both L343V and R463W mutations resulted in increased MTP binding, as the MTP/B100 ratio was increased by almost 7-fold. The ratio of MTP/B48 was less affected by the L343V or R463W mutation. Likewise, the interactions between BiP and the mutant apoB-100 proteins were also increased as compared with B100wt (Fig. 4B, 2nd panel from bottom). The levels of total MTP (Fig. 4B, 3rd panel from top) or BiP (bottom panel) in the cells were not affected by apoB-100 expression.

The increased binding of MTP with human apoB proteins was specific; control immunoprecipitation experiments using a pre-immune antibody did not pull down detectable MTP (data not shown). Assuming that the amount of the chaperone proteins BiP and MTP was not limiting and that their interaction with apoB does not reach saturation at low levels of apoB, the increased MTP/apoB and BiP/apoB ratios observed for B100LV and B100RW may suggest an enhanced binding of the molecular chaperones to the mutant apoB-100 proteins. In separate experiments in which the endogenous rat apoB proteins were immunoprecipitated, the amount of MTP associated with the rat proteins was identical between B100wt-, B100RW-, and B100LV-
transfected cells (data not shown). These results suggest that the L343V and R463W mutations indeed result in misfolding of apoB, particularly apoB-100.

Increased association of the mutant apoB-100 proteins with MTP and BiP might be attributable to inefficient translocation across the ER membranes and may result in accumulation of polyubiquitinated, translocation-arrested apoB100. To test these possibilities, we evaluated the levels of polyubiquitinated apoB in cells treated with MG132 for 1 h. The cell lysates were subjected to immunoprecipitation (IP) using anti-human apoB antiserum, and apoB, MTP, and BiP were detected by immunoblotting (WB). The amount of MTP and BiP associated with apoB100 and apoB48 was quantified by densitometry, and the ratios of MTP/B100, MTP/B48, BiP/B100, and BiP/B48 are presented below the blots. The ratio obtained from samples derived from the B100wt-transfected cells was set at 1.0. The experiments were performed twice and similar results were obtained. C, cells were incubated in medium containing MG132 for 1 h. Cell lysates were prepared, and the apoB proteins were precipitated with a polyclonal antibody. Aliquots of immunoprecipitate were resolved by SDS-PAGE (5% gel), transferred to nitrocellulose, and visualized by immunoblotting using mAb 1D1 (top) or anti-ubiquitin (bottom) antibody. The locations of apoB100 and polyubiquitinated apoB species (asterisk) are indicated to the right of each panel. Repetition of the experiment yielded identical results. D, cells were incubated with MG132 for 1 h and collected, and microsomes were prepared by ball-bearing homogenization. Aliquots of the microsomes were incubated with trypsin or trypsin/Triton X-100, as indicated. After stopping the protease reaction, aliquots of samples were subjected to SDS-PAGE (5% gel for apoB, 10% gel for others) and immunoblotting. Cxn, calnexin; B48 indicates apoB-48 like protein; PDI, protein-disulfide isomerase.

Translocation status of apoB-100 proteins was determined by protease protection assay. Data presented in Fig. 4D show that B100wt, like B100RW and B100LV, was sensitive to trypsin digestion, suggesting the majority of apoB-100 polypeptides being exposed on the external surface of isolated microsomes. The cytosolic AAA-ATPase p97 and Hsp70 (associated with the cytosolic surface of the ER membrane) were also sensitive to trypsin under the same conditions. No major difference was detected in the translocation status between mutant and wild-type apoB-100. Protein-disulfide isomerase and the luminal N-terminal domain of calnexin were largely protected from

**FIGURE 4.** ER/Golgi distribution, chaperone association, ubiquitination, and protease protection analysis of apoB. A, distribution of apoB-100 and the apoB-48-like proteins that were associated with subcellular compartments. Microsomes obtained from B100wt-, B100LV-, or B100RW-transfected cells were fractionated by Nycodenz gradient ultracentrifugation. Proteins of the fractionated samples were immunoblotted with anti-giantin, anti-calnexin, and mAb 1D1 (anti-apoB) antibodies, respectively. MG, MG132. B, co-immunoprecipitation of MTP, BiP with human apoB. Cells were treated with MG132 for 1 h. The cell lysates were subjected to immunoprecipitation (IP) using anti-human apoB antiserum, and apoB, MTP, and BiP were detected by immunoblotting (WB). The amount of MTP and BiP associated with apoB100 and apoB48 was quantified by densitometry, and the ratios of MTP/B100, MTP/B48, BiP/B100, and BiP/B48 are presented below the blots. The ratio obtained from samples derived from the B100wt-transfected cells was set at 1.0. The experiments were performed twice and similar results were obtained. C, cells were incubated in medium containing MG132 for 1 h. Cell lysates were prepared, and the apoB proteins were precipitated with a polyclonal antibody. Aliquots of immunoprecipitate were resolved by SDS-PAGE (5% gel), transferred to nitrocellulose, and visualized by immunoblotting using mAb 1D1 (top) or anti-ubiquitin (bottom) antibody. The locations of apoB100 and polyubiquitinated apoB species (asterisk) are indicated to the right of each panel. Repetition of the experiment yielded identical results. D, cells were incubated with MG132 for 1 h and collected, and microsomes were prepared by ball-bearing homogenization. Aliquots of the microsomes were incubated with trypsin or trypsin/Triton X-100, as indicated. After stopping the protease reaction, aliquots of samples were subjected to SDS-PAGE (5% gel for apoB, 10% gel for others) and immunoblotting. Cxn, calnexin; B48 indicates apoB-48 like protein; PDI, protein-disulfide isomerase.
ApoB L343V Mutation

Reduced Secretion of ApoB-48LV and ApoB-17LV from Transfected McA-RH777 Cells—Because the Leu\textsuperscript{433} residue is located within the N terminus of apoB, we tested the effect of L343V mutation on the secretion of two C-terminally truncated apoB forms, namely apoB-48 and apoB-17. In cells stably transfected with the apoB48 cDNA, secretion of B48LV mass (Fig. 5A, top) and \(^{35}S\)-labeled B48LV (Fig. 5A, bottom) was \(\sim 25\%\) less than that of B48wt. As observed previously (35) and confirmed here, secretion of B48RW was decreased by \(\sim 50\%\) as compared with that of B48wt. Decreased secretion of \(^{35}S\)-B48LV and -B48RW was further confirmed by pulse-chase experiments using two different stable clones (Fig. 5B, left). The secretion efficiency of B48LV and B48RW was 23 and 49\%, respectively, lower than that of B48wt.

Apolipoprotein B17 has limited lipid-binding ability yet is able to interact with synthetic liposomes (53) and MTP (54) \textit{in vitro}. Expression of apoB-17 transiently in either COS-7 cells or McA-RH777 cells showed decreased accumulation of B17LV and B17RW in the media as compared with B17wt (Fig. 5C, top). Pulse-chase experiments with transiently transfected cells showed that secretion of B17LV, like that of B17RW (35), was significantly decreased as compared with B17wt (Fig. 5C, bottom). Moreover, additional pulse-chase experiments with stably transfected cells expressing respective B17wt, B17LV, and B17RW also showed decreased secretion efficiency of the mutant proteins (Fig. 5D). These results suggest that both L343V and R453W mutations within the \(\alpha\)-helical domain affect apoB secretion through a mechanism that is independent of lipid association.

Structural Changes Induced by the L343V and R453W Mutations—To gain an insight into the effects of L343V and R453W on apoB structure, we introduced the mutations into apoB domain constructs encoding the respective \(\alpha\)-helical and the C-sheet domains (Fig. 6A). All the apoB domain constructs contained a His tag at the C terminus. The N-terminal \(\beta\)-barrel domain was excluded from these constructs because the \(\beta\)-barrel was relatively independent from the \(\alpha\)-helical and C-sheet domains (see Fig. 7A) (42). The limited protease accessibilities of lysine and arginine residues within the apoB domain constructs were determined as an indication of protein folding. Data presented in Fig. 6B show that B6.4-17wt was cleaved into the \(\alpha\)-helical domain (open arrowhead) and the C-sheet domain (arrow); the former was relatively resistant to trypsin during the 2-h protease treatment, whereas the latter was rapidly degraded (Fig. 6B, left panel). Sequencing analysis of the proteolytic products determined that B6.4-17wt was cleaved at amino acid residue 610. A similar proteolytic pattern was observed for B6.4-17RW (Fig. 6B, middle panel). By contrast, the \(\alpha\)-helical domain of B6.4-17LV was further cleaved, resulting in three visible fragments at 30 min and a fragment whose N terminus starts at amino acid residue 330 (determined by sequencing) at 120 min (Fig. 6B, right panel, open arrowhead). According to the homology model of apoB-17, the cleavage at the C terminus of Arg\textsuperscript{329} removes the first two \(\alpha\)-helices (first helical bundle) from the \(\alpha\)-helical domain (see Fig. 7, A and B).

\textit{In vitro}, the \(\alpha\)-helical domain (B4.6-13) and C-sheet domain (B13-17) could be cross-linked to form heterodimers with an apparent molecular weight of B6.4-17 (Fig. 6C, left panel).
However, B6.4-13RW was unable to form a heterodimer with B13-17 under the same conditions (Fig. 5, middle panel). On the contrary, B6.4-17 behaved identical to B6.4-13wt (Fig. 6, black arrow). Notably, substituting Arg463 with Ala in B4.6-17 also resulted in failed dimerization with B13-17 (data not shown). These results suggest that Arg463 is essential for the formation of B6.4-13/B13-17 dimer.

Finally, we probed the structure of the apoB domain construct B6.4-17 by CD. In far-UV CD scans, B6.4-17wt, B6.4-17RW, and B6.4-17LV exhibited similar spectra, indicating that neither R463W nor L343V mutation caused significant changes in secondary structure (Fig. 6D). In the chemical denaturation experiment, B6.4-17RW showed an unfolding curve slightly different from those of B6.4-17wt and B6.4-17LV (Fig. 6E). The guanidine unfolding curve of the B6.4-17wt had two apparent transitions, one at ~2 μM GdnHCl (open arrowhead) and the second at ~4 μM GdnHCl (open arrowhead), respectively. The R463W mutation appeared to decrease the unfolding cooperativity at low GdnHCl concentration while stabilizing at high GdnHCl concentration. The unfolding trajectory of B4.6-17RW suggests an altered stability in tertiary folding despite its unchanged secondary structural content. By contrast, B4.6-17LV did not induce detectable changes in unfolding.

**DISCUSSION**

Four years ago, we discovered the first missense mutation in APOB, namely R463W, in a Christian Lebanese FHBL kindred (35). Here we report a second novel and rare nonsynonymous, nontruncating APOB gene mutation, L343V, in an extended FHBL kindred. The mutation showed co-dominant segregation with the FHBL phenotype with heterozygotes having lower-than-half normal plasma apoB concentrations. Familial ligand-defective apoB-100 (OMIM 144010), an autosomal co-dominant form of hypercholesterolemia, is the only other phenotype to have been associated with naturally occurring missense mutations in APOB (i.e. the R3500Q, R3500W, R3531C, R3480W, and H3543Y variants) (28, 55, 56). The diseases caused by these point mutations within the coding sequences of APOB demonstrate the key role that apoB-100 plays in regulating VLDL assembly/secretion and receptor-mediated endocytosis of LDL.
FIGURE 7. Modeled structural environment of Leu343. A, a proposed homology model of apoB17. The β-barrel domain (green) is excluded in the rotated image at the bottom, which provides a top view of the α-helical (cyan) and the C-sheet (red) domains. Leu343 and Arg463 are shown as yellow spheres. B, ribbon representation of helices 1–6 of the α-helical domain. Four leucine residues are found at the interface between the first helix pair (helix 1 and helix 2) and the second helix pair (helix 3 and helix 4), with Leu343 highlighted in green. C, distribution of hydrophobic residues in the first two helix pairs (helices 1–4). Hydrophobic residues are shown in yellow space-filling models (Leu343 in green) and the protein backbone in cyan ribbon. Most hydrophobic residues are located either at the interface within each helix pair or between the adjacent helix pairs. The exposed ribbon surfaces indicate the absence of hydrophobic residues. The red dotted circle highlights the local hydrophobic core involving Leu343. The homology model is calculated based on the crystal structure of lipovitellin using MODELLER. The graphics representation is achieved by MOLMOL.

The APOE genotype accounts for ~10% of the variation in plasma LDL cholesterol in the general population. However, recently it has been reported that the APOE genotype accounts for 15–60% of this variation in FHBL heterozygotes (57). ApoE4, apoE3, and apoE2 are inherited with allele frequencies of 0.13, 0.81, and 0.06, respectively, in a Western Australian population (58). Thus, the apoE2/3 isoform, which is associated with low plasma LDL cholesterol, is over-represented in the LV kindred. As expected, the apoE2/3 genotype was present in those unaffected family members with the lowest plasma LDL cholesterol and apoB concentrations. We speculate that the apoE2/3 genotype would have a lesser absolute impact on decreasing plasma LDL cholesterol and apoB levels in LV heterozygotes compared with unaffected family members.

The L343V mutation found in the FHBL kindred defines a local domain that would appear to be critical for the efficient secretion of apoB-containing lipoproteins. Both Leu343 and Arg463 residues are located within the predicted α-helical domain of apoB, which contains sequence elements shown to be important for proper folding of apoB, for the physical interaction between MTP and apoB, and for lipoprotein assembly. Transfection studies have suggested that apoB segments containing sequences lacking the βα1 domain were unable to be secreted (13) or else secreted poorly (14). Mutational analysis has identified critical disulfide linkages within the βα1 domain that are essential for efficient secretion of apoB and apoB-containing lipoproteins (15, 16, 18). The current work on the L343V mutation, together with the previous R463W studies (35), provides additional evidence for the functional importance of the βα1 domain. The Leu343 and Arg463 residues are conserved in apoB among all species examined, including human, mouse, pig, and rat. The current work has demonstrated decreased secretion efficiency for apoB-100, B-48, and B-17 that contained L343V mutation (Figs. 2 and 5). Because apoB-17 has only limited capacity to assemble lipids and can be secreted as a lipid-poor protein, our results suggest that the α-helix domain where Leu343 and Arg463 reside must be critical in apoB folding. It is rather striking to consider that a single residue in the α-helical domain could have such a profound effect on apoB function. As stated above, the only other phenotype that has been associated with naturally occurring missense mutations in APOB is familial defective apoB-100 (55). Of interest, substitution of Arg3500 with Lys3500 in apoB-100 was as defective as the R3500Q mutation, indicating that it is the presence of Arg3500 and not a positive charge alone that is essential for normal LDL receptor binding (60).

The current data indicate that although both R463W and L343V mutations resulted in impaired apoB-100 secretion, they exerted different effects on the ability of apoB-100 to assemble VLDL1 (Fig. 3). Thus, whereas B100LV markedly lost its ability to form VLDL1, B100RW retained this function. Our biochemical and biophysical analyses of the apoB domain constructs showed that R463W and L343V mutations probably induced different structural perturbations in the native folding of the α-helical domain. The lipovitellin-based model of apoB17 predicts that Leu343 is located in the third α-helix of the α-helical domain (Fig. 7A). The increased trypsin accessibility at residue Arg329 suggests that L343V mutation renders folding of the first two α-helices unstable. The homology model of apoB17 predicts that the α-helical domain is composed of two layers of α-helices, held together by a continuous hydrophobic interface (42). The building blocks of this domain are pairs of double helices that interact with each other through hydrophobic residues (Fig. 7, A and B). The inter-helix pair tethering through
interaction of hydrophobic side chains provides further structural integrity of the entire \(\alpha\)-helical domain (Fig. 7C). Leu\(^{343}\), together with other hydrophobic residues (Leu\(^{299}\), Leu\(^{324}\), and Leu\(^{339}\)), may contribute to the tethering of the first and second helix pairs (Fig. 7B). Shortening the side chain of Leu\(^{343}\) presumably will weaken the tethering and lead to loosening of the folding of helice one and two (the first helical pair). In the structure of lipovitellin, the second \(\alpha\)-helix interacts with an extended amphipathic \(\beta\)-sheet, termed A-sheet (10). It is possible that the L343V mutation may also indirectly alter the tertiary folding of the A-sheet in apoB. It should be noted that at higher trypsin concentrations (1:10 and 1:100; w/w), cleavage at the C terminus of the first two \(\alpha\)-helices occurred in apoB17wt (45). Thus, the first two helices are not as tightly folded as the rest of the \(\alpha\)-helical domain. In L343V, this cleavage completed within 2 h at trypsin concentrations as low as 1:1,000 (w/w) (Fig. 6B), suggesting that L343V mutation has increased the degree of flexibility in this region.

In comparison to L343V, the structural perturbation of R463W is of longer range and more significant (Fig. 7A). The inability to form dimer between B6.4-13RW and B13-17 suggests that this mutation either abolished the interaction between these two domains or altered the structure of the heterodimer, so that it could not be cross-linked under the same condition. A weakened interaction between the \(\alpha\)-helical and the C-sheet domains is also implicated in limited proteolysis and chemical denaturation experiments. The remnant of the C-sheet domain disappeared faster in the proteolysis of B6.4-17RW than that in the digestions of either B6.4-17wt or B6.4-17LV (Fig. 6B, black arrow). A reduction in the unfolding cooperativity of the first transition in the denaturation curve of B6.4-17RW can also be explained by this weakened interaction. The apparent two-transitional unfolding of B6.4-17 is because of the existence of two relatively independent folding units in the \(\alpha\)-helical domain. It has been suggested that the N-terminal half of the \(\alpha\)-helical domain (B6.4-10) behaves like a molten globule and can actively interact with phospholipids, whereas its C-terminal half (B9-13) has a rather cooperative unfolding, and it is mainly responsible for the interaction with the C-sheet domain (61, 62). The first unfolding transition arises from the C-terminal half of the \(\alpha\)-helical domain, and the second apparent transition is primarily contributed by the gradual unfolding of the N-terminal half of the \(\alpha\)-helical domain (61). Therefore, an increased stability of B6.4-17RW in the second transition probably indicates the formation or stabilization of a local hydrophobic core involving Trp\(^{63}\). This hypothesis is also supported by the absence of changes in the second transition in B6.4-17RA, although this mutant displays a decrease in cooperativity in the first transition and an impaired cross-linking with the C-sheet domain.\(^7\)

Previous cell culture studies with a variety of truncated apoB forms (ranging from apoB-15 to apoB-94) showed that most of the C-terminally truncated apoB forms were secreted as efficiently as normal apoB-100 or apoB-48 (39, 63). Thus, it is rather unusual that substitution of Leu\(^{343}\) with Val, both being hydrophobic and differing merely a methylene group that should have a much less severe impact than C-terminal truncations of apoB (27, 28, 30), can cause FHBL. In addition to decreased secretion, increased catabolism of apoB-containing lipoproteins has been implicated in causing the FHBL lipid phenotype (64–68). The secretion defect suggested by the current studies needs to be further confirmed by in vivo human lipoprotein turnover studies using the L343V FHBL subjects and ultimately in knock-in mice that harbor the L343V mutation in APOB.

Hepatic steatosis has been reported in FHBL kindreds (69–71). The mildly elevated mean serum liver enzyme concentrations in the L343V heterozygotes could represent a subclinical phenotype, consistent with previous observations in R463W FHBL subjects (35). Sensitive noninvasive imaging studies of the liver in mutation carriers would be of interest. When compared with unaffected family members, LV heterozygotes had significantly reduced serum \(\alpha\)-tocopherol concentrations that were positively correlated with plasma cholesterol and apoB levels. Vitamin E supplementation of FHBL heterozygotes has been recommended (72); however, recent studies of the effect of truncated apoB variants on vitamin E metabolism and oxidative stress refute this advice (73).

In conclusion, we investigated and contrasted two nonsynonymous nontruncating APOB gene mutations in FHBL, namely L343V and R463W, which appear to impair the secretion of apoB and apoB-containing lipoproteins. The structural perturbation by the L343V and R463W mutations pinpoints the essential role of the \(\alpha\)-helical domain during apoB folding. FHBL resulting from single amino acid substitution is rare. The identification of L343V and R463W shows that proper folding of the \(\alpha\)-helical domain within the N terminus of apoB is important for efficient secretion of apoB-containing lipoproteins.

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