The molecular basis of truncated forms of apolipoprotein B in a kindred with compound heterozygous hypobetalipoproteinemia

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Abstract Krul et al. (1) have identified two truncated species of apolipoprotein B-100 in a kindred with familial hypobetalipoproteinemia. Five family members were identified who produce either one or both of two truncated apolipoprotein B-100 proteins estimated to be 40% and 90% the amino-terminal end of apolipoprotein B-100. Low density lipoprotein with the apolipoprotein B-90 binds more strongly to the low density lipoprotein-receptor on cultured fibroblasts. In this present study, we have identified the DNA mutations leading to these truncated apolipoprotein B-100 variants in this kindred. Sequencing of amplified DNA from the proband revealed that deletions of one or two nucleotide bases produced frameshift mutations and generated premature stop codons in both cases. Apolipoprotein B-40 (Val_{1829}→Cys-TERM) is the result of a dinucleotide (TG) deletion in exon 26 that generates a stop codon at position 1830 and produces a protein with a predicted molecular mass of 207.14 kDa. The other truncated apolipoprotein B (Glu_{4034}→Arg-Gln-Leu-Leu-Ala-Cys-TERM) is due to a single nucleotide (G) deletion in exon 29. This results in a protein with 4039 amino acids and a predicted molecular mass of 457.6 kDa that is now designated apolipoprotein B-89. Mechanisms by which the removal of the last 497 amino acids might increase the binding of the apoB-89 to the LDL-receptor are discussed. —**Talmud, P., L. King-Underwood, E. Krul, G. Schonfeld, and S. Humphries.** The molecular basis of truncated forms of apolipoprotein B in a kindred with compound heterozygous hypobetalipoproteinemia. *J. Lipid Res.* 1989. **30:** 1773–1779.

Supplementary key words hypobetalipoproteinemia • polymerase chain reaction • sequencing • stop codon

Hypobetalipoproteinemia (HBL) is a disorder characterized by low levels of apoB and low density lipoprotein (LDL) levels below the fifth percentile (2). To date all the molecular and genetic evidence indicates that the defect leading to HBL is in the apoB gene itself. Since each LDL particle has one molecule of apoB-100, the predominant protein and ligand for the LDL-receptor, mutations leading to defects in the apoB gene would express themselves in a co-dominant manner. This is the mode of inheritance of HBL.

Recently a kindred displaying compound heterozygous HBL was reported by Krul et al. (1). In this kindred HBL is caused by the co-inheritance of two truncated forms of apoB-100 designated apoB-40 and apoB-90, being 40% and 90% of the normal length of apoB-100. In vitro studies revealed that LDL containing the apoB-90 variant binds more strongly than apoB-100 to the LDL-receptor on cultured fibroblasts (1) and is the first example of a mutation in the apoB gene causing increased binding to the LDL-receptor. We have identified the molecular basis of the apoB-40 and apoB-90 variants and investigated the structural changes in the apoB-90 to gain insight into domains of the protein that may be functionally important for binding to the LDL-receptor.

METHODS

The SH kindred

Clinical information, apolipoprotein and lipoprotein levels of family members have been previously reported (1). The pedigree (**Fig. 1**) consists of eleven siblings, five of whom were diagnosed as having HBL, with low apoB concentrations and LDL cholesterol levels below the fifth percentile. The use of SDS-PAGE and immunoblots probed with four monoclonal antibodies to defined epitopes of apoB made it possible to identify truncated apoB species as apoB-40 and apoB-90. Three siblings, including the proband (SH), have inherited both mutant alleles

Abbreviations: HBL, hypobetalipoproteinemia; LDL, low density lipoprotein; ASO, allele-specific oligonucleotide; PCR, polymerase chain reaction.

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and display greatly reduced apoB levels. One brother has inherited apoB-90/apoB-100 and another brother apoB-40/apoB-100. All family members were free of neurological symptoms.

DNA preparation

Blood from available family members was collected into tubes with EDTA. DNA was extracted from packed white blood cells by the Triton X-100 method (3).

Oligonucleotides

Oligonucleotides were synthesized on a Pharmacia Gene Assembler using the phosphoramidite procedure. Purification was performed using G-25 Sephadex columns (Pharmacia, Sweden) and resolubilization in water. On the basis of the protein size estimations (1) we synthesized the following oligonucleotides to span the region where mutations would generate apoB-40 and apoB-90. Recognition sequences for SmaI and EcoRI were added to the 5' end of the 5' and 3' oligonucleotides, respectively (lower case letters). Nucleotide and amino acid numbering was according to Knott et al. (4).

B40-1 5490-5520 5' ggcccggg CTACAGCCCTATTCTCTGGTAACTACTTTA 3'

B40-2 6271-6241 3' CTACGAAATCTCTACTCTCTACGGCAACTCCttaaggg 5'

B90-1 12216-12236 5' ggcccgggCCTGAGCAGACCATTGAGATTCCCTCCATT 3'

B90-2 12479-12449 3' GACGTCTTGTTACGACTCACCCAAATAGTTCttaaggg 5'

Polymerase chain reaction

Five μg of genomic DNA was amplified (5) for 30 rounds of amplification using 1 U Taq polymerase (Perkin Elmer-Cetus) per sample in the buffer recommended by the manufacturers and 1 μg of each synthetic oligonucleotide as a primer in a total volume of 100 μl. Amplification was performed using a Cambio Intelligent Heating Block (Cambio). After an initial denaturing period of 5 min at 95°C, the following cycles were used: 0.5 min at 95°C, 0.5 min at 55°C, and 3 min at 72°C.

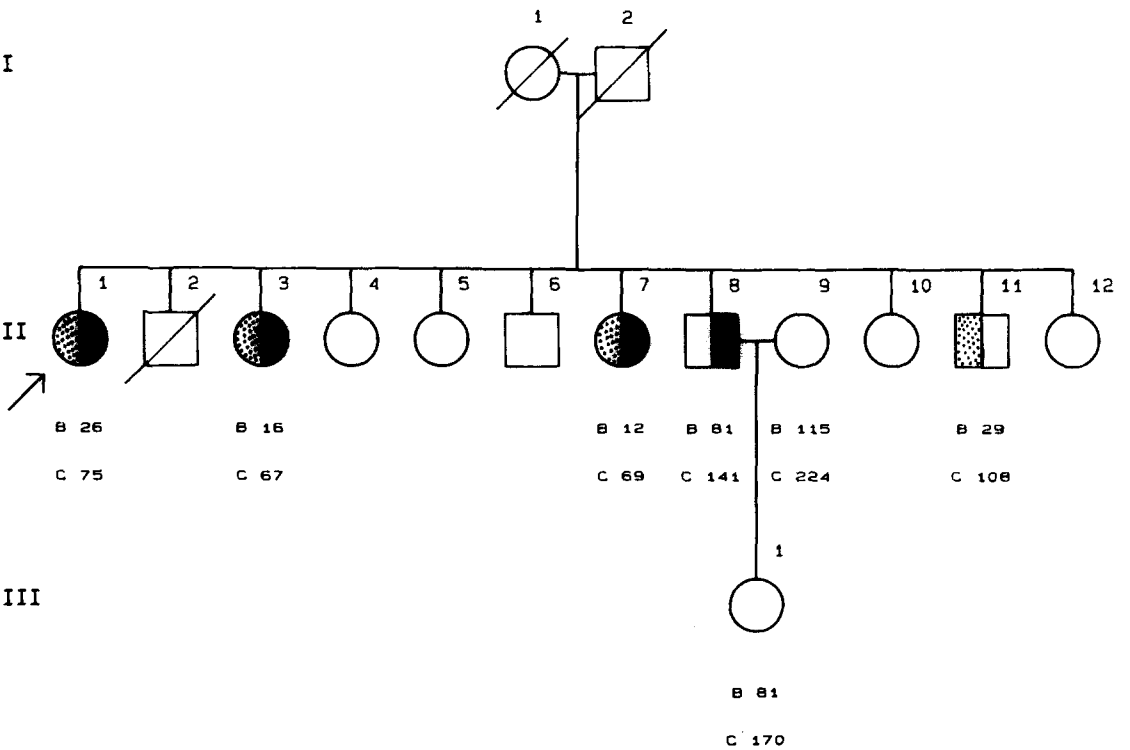

Fig. 1. Pedigree of the SH kindred. The proband is identified by an arrow. Total cholesterol (mg/dl) and apoB (mg/dl) levels as reported in our previous publication (1) are given below each family member sampled; (▨) apoB-40; (■) apoB-89.

Subcloning and sequencing

Amplified DNA from the proband SH, was purifed using GeneClean (Stratic Scientific, London). DNA was then digested with EcoRI/SmaI (Bethesda Research Labs) making use of the restriction enzyme sites introduced into the 5' end of the oligonucleotides. The DNA was further purified using GeneClean and cloned into EcoRI/SmaI-digested M13 mp11 (Amersham) using standard techniques (6). Recombinant clones were picked and purified. Ten subclones from each amplification were sequenced using the Sequenase Kit (U.S. Biochemical Corp.) and analyzed on 8% denaturing polyacrylamide gels. Ten subclones were sequenced running all the As, Cs, Gs, and then Ts from the clones. This provided a rapid method of scanning the autoradiograph for mutations. As we reported previously (7), the PCR generates a number of errors. In no case were the same errors seen in more than one clone.

Oligonucleotide melting

Two pairs of allele-specific oligonucleotides (ASO) were synthesized and purified as before. S70 and S72 were used in oligonucleotide melt experiments to detect the apoB-40 mutations. S71 and S73 were used to detect the apoB-90 mutation.

S70 5' GACACTGTTGCTA 3' 5688-5700

S72 5' CAGACACTTGCTA 3' 5686-5698

S71 5' GAAGAAAGGCAGCT 3' 12303-12316

S73 5' GAAGAAGAGGCAG 3' 12303-12315

ASOs were phosphorylated at their 5' ends with [γ^{32}P]ATP (Amersham) and T4 nucleotide kinase (BRL). The specific activity of the probes was approximately 0.1 μCi/pmol. Two μl of the final 100 μl PCR reaction was diluted in 200 μl 15 × SSC (SSC: NaCl 0.15 M, trisodium citrate 0.015 M, pH 7.6), denatured by heating at 95°C for 7 min, and applied to Hybond-N filters (Amersham) in duplicate using a slot-blotting apparatus (Schleicher and Schuell, FRG). DNA was bonded to the filter by 3 min of UV irradiation (Chromato-uve TM20, UVP transilluminator). Filters were prehybridized in 4 ml 5 × SSPE for 30 min at 40°C (SSPE: 0.15 M NaCl, 10 mM NaH_2Po_4, 1 mM EDTA, pH 7.4). Five pmol probe was added and hybridized at 28°C overnight. Filters were washed in 5 × SSPE 0.1% SDS for 10 min. Filters hybridized with ASOs S70 and S72 were washed at 36°C, S71 and S73 at 40°C, and exposed to X-ray film (Konica) at −70°C for 3 h.

RESULTS

As previously reported, the proband and four other family members of the SH kindred were diagnosed as having HBL, with low apoB concentrations and total and LDL cholesterol levels below the fifth percentile (1); the

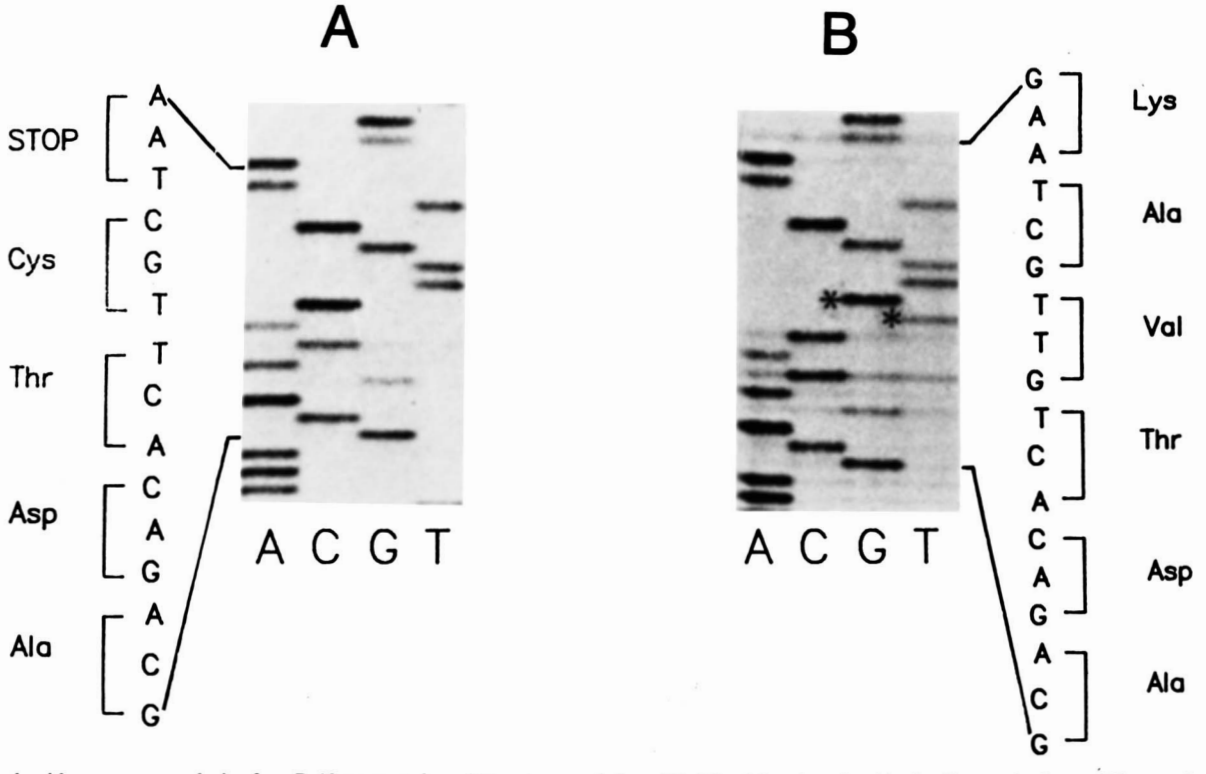

Fig. 2. Nucleotide sequence analysis of apoB-40 mutant clone (A) and normal clone (B). The deleted nucleotides leading to the frameshift mutation and creating a stop codon are marked with an asterisk.

pedigree is shown in Fig. 1. Results from SDS-PAGE and immunoblots revealed two truncated apoB species in the proband and two other siblings. These species were sized as apoB-40 and apoB-90. One affected brother was apoB-90/apoB-100 and another affected brother was apoB-40/apoB-100.

In order to determine the mutation causing the apoB-40, we used the polymerase chain reaction (PCR) (5) to amplify a 781 bp fragment from DNA of the proband SH, from nucleotide 5490 to 6271. Amplified DNA was digested with SmaI/EcoRI (sites that had been introduced into the 5' end of the oligonucleotides) and cloned into M13 mp11. In six out of the ten clones sequenced, a dinucleotide, TG, deletion was detected at nucleotides 5693 and 5694. The GTT codon at $Valine_{1829}$ becomes GTC, coding for a CYS residue followed by a stop codon TAA (**Fig. 2**). The truncated protein is 1829 amino acids in length producing a protein 40.3% the size of apoB-100.

From the size of the apoB-90, we predicted that the mutation would be in exon 29. We amplified 263 bp from the start of the exon at nucleotide 12216 to nucleotide 12479 using the same subcloning procedure as for apoB-40. Of the ten subclones sequenced, five had a single G deletion at nucleotide 12309, the first base of Glu residue 4034. This single base deletion causes a frameshift mutation generating amino acids Arg, Gln, Leu, Leu, Ala, Cys before resulting in a stop codon and termination of the protein. The resultant protein product is 4039 amino acids long producing an apoB-89.04 (**Fig. 3**).

Using the PCR in conjunction with ASOs we were able to demonstrate the inheritance of apoB-40 and apoB-89 in other family members. The PCR product from available family members was applied to a slot-blotting apparatus in duplicate. One filter was hybridized with the labeled ASO specific for the normal allele and the duplicate filter with the ASO for the mutant sequence. The results of these experiments are shown in **Fig. 4, A and B**. Using four RFLPs of the apoB gene and the 3' variable number of tandem repeats (VNTRs), we have previously shown the inheritance of the four parental apoB alleles in this kindred (1). These results were confirmed using ASOs.

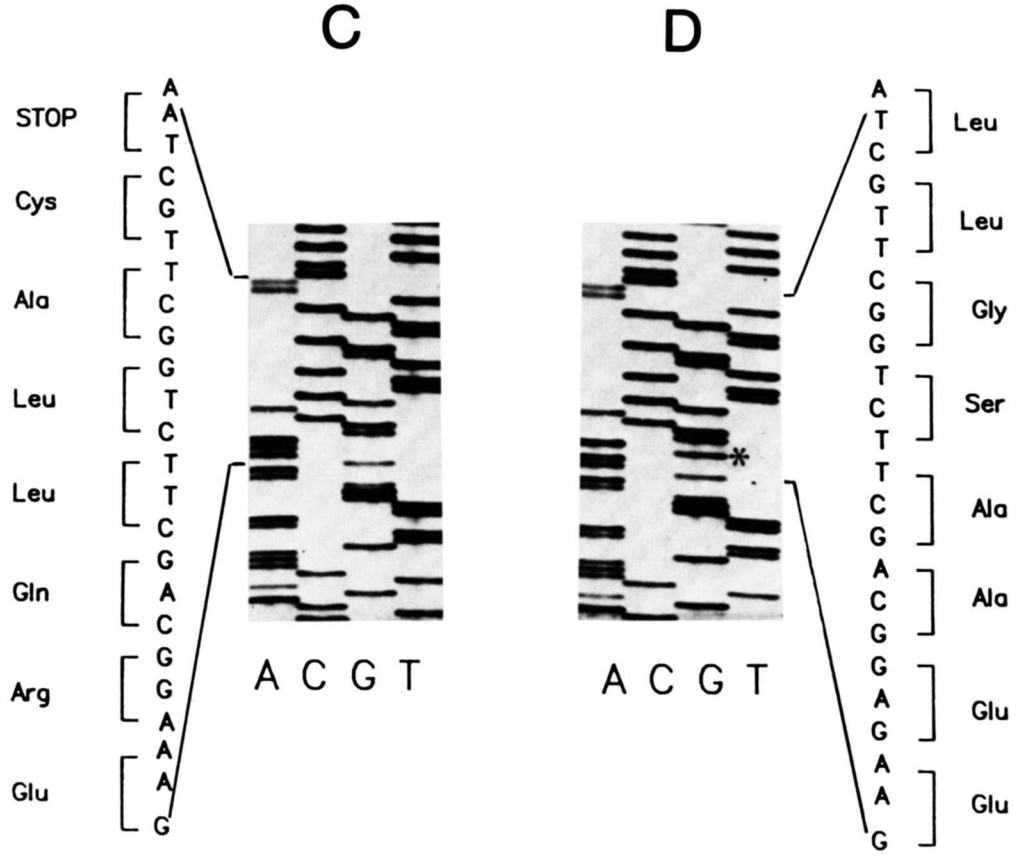

Fig. 3. Nucleotide sequence analysis of apoB-89 mutant clone (C) and normal clone (D). The deleted base is marked with an asterisk.

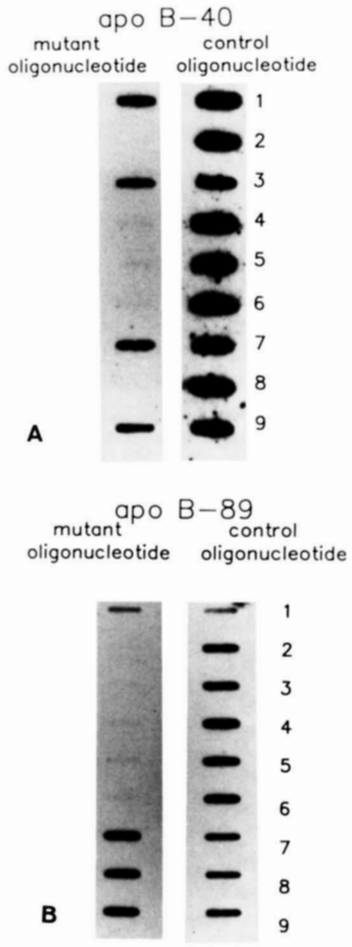

Fig. 4. A: Autoradiograph of the slot-blot of DNA from available family members amplified with the apoB-40 oligonucleotides and probed with ASOs S70 (complementary to the control sequence) and S72 (complementary to the apoB-40 mutant sequence), after a 10-min wash at 36°C. (1, II-1; 2, unrelated control; 3, II-11; 4, II-9; 5, unrelated control; 6, III-1; 7, II-7; 8, II-8; 9, II-3). B: Autoradiograph of the slot-blot of DNA from available family members amplified with the apoB-89 oligonucleotides and probed with ASOs S71 (complementary to the apoB-89 mutant sequence) and S73 (complementary to the control sequence), after a 10-min wash at 40°C. Numbering as above.

DISCUSSION

The SH kindred is a family displaying compound heterozygous HBL. In the proband we have identified a dinucleotide deletion causing a frameshift mutation and stop codon producing an apoB-40 (Val_{1829}→Cys TERM). The site of the apoB-40 mutation accurately matches the molecular weight estimates of apoB-40 derived from SDS-PAGE and molecular weight markers. In the other allele of apoB, a single G deletion results in a frameshift mutation altering the next six amino acids, finally ending in a stop codon and producing a truncated apoB which we designated apoB-89 (Glu_{4034}→Arg-Gln-Leu-Leu-Ala-Cys-TERM). The use of oligo-melting techniques and ASOs revealed that the whole spectrum of inheritance of these two mutant alleles exist in this kindred. Thus, there are siblings who are compound heterozygotes inheriting both mutant alleles, siblings who have inherited either apoB-89 or apoB-40 mutant alleles together with an apoB-100 allele, and those who have inherited normal apoB-100 alleles from both parents.

To date, six different mutations in the apoB gene leading to HBL have been defined with no two unrelated patients having the same defect. **Fig. 5** is a compilation of known apoB mutations leading to HBL, including the two reported here. ApoB-39 (8) and apoB-37 (9) both result from nucleotide deletions. ApoB-37 is a truncated protein identified in a kindred with compound heterozygous HBL; the mutation in the other allele, leading to low levels of apoB-100, is yet to be identified. ApoB-28 is the result of a C–T substitution mutating ARG_{1306} to a stop codon (8). Huang et al. (10) found that exon 21 was deleted in an HBL patient by alu-alu recombination. Three of the five HBL mutations are clustered in exon 26: apoB-37, apoB-39, and apoB-40. Within this exon lies the putative receptor binding domain for the LDL receptor, between residues 3147–3157 and 3359–3367 (4). All these truncations, including apoB-28, terminate before this

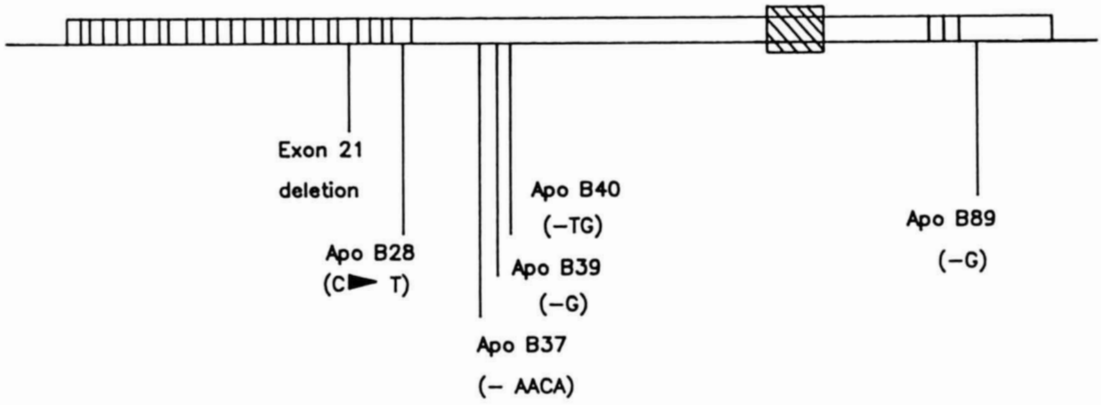

Fig. 5. Schematic map of apoB cDNA identifying reported mutations of apoB that lead to HBL. Vertical lines on the cDNA map represent introns; the hatched box depicts the proposed LDL-receptor binding domain.

region. Exon 26 is 7.5 kb long, the longest reported exon in a mammalian gene (11). This exon is more than half the size of the cDNA and this may account for the fact that three of the five mutations leading to HBL occur within it.

The metabolism of these truncated apoB proteins provides insight into the functions of certain domains of the protein. ApoB-28 (8) was not detected in the plasma of the affected patient, suggesting that the protein is rapidly degraded or that the apoB-28 is smaller than a critical size needed for lipoprotein assembly. ApoB-37 (9) is found primarily in the very low density lipoprotein (VLDL) and high density lipoprotein (HDL) density range fractions. Both apoB-39 (8) and apoB-40 (1) were detected in low amounts in the LDL fraction of plasma of affected patients. Since the receptor binding domain is absent in these apoB species, one would expect higher levels of LDL containing these truncated forms of apoB. These low levels suggest that either the resultant lipoproteins are unstable in the plasma and are degraded, or it is possible that they are not transported from the liver and, therefore, never reach the plasma.

The mutation generating apoB-89 is in exon 29. It has been suggested that exon 29 may contain secondary binding domains of apoB that contribute to LDL-receptor binding. Forgez et al. (12) raised a monoclonal antibody (TP1) to a synthetic peptide corresponding to basic residues 4007–4019 and showed competitive binding to the LDL-receptor on U937 cells. Furthermore in the pig, sequence analysis of the apoB gene from four different alleles associated with different cholesterol levels showed no sequence differences in the proposed receptor-binding domain, which suggests that the molecular changes leading to these phenotypic differences lie outside this domain (13). Amino acids within the 4100–4536 terminal region [domain V of Yang et al. (14)] are trypsin nonreleasable and firmly embedded in the lipoprotein molecule (M. Rosseneu, personal communication); this domain is absent in apoB-89. We speculate that the deletion of all or part of this region might affect tertiary structure and availability of both primary and secondary binding sites of apoB to the LDL-receptor. Using the Chou-Fasman (15) algorithm, there was no difference in the predicted degree of α-helix, hydrophobicity, or hydrophobic moment between apoB-89 or apoB-100.

The results from the binding studies on culture fibroblasts (1) suggest that the reduced plasma levels of apoB-89 may be due to increased binding of apoB-89 to the LDL-receptor. However, at this stage we cannot exclude the possibility that these low levels may also reflect reduced synthesis of the truncated apoB protein. In vivo turnover studies on individuals with this mutation, using specifically labeled apoB-89, will be necessary to clarify this. We have thus found a functional difference between apoB-89 and apoB-100 which partially explains the low levels of LDL seen in the individuals with apoB-89.

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