ApoB-54.8, a truncated apolipoprotein found primarily in VLDL, is associated with a nonsense mutation in the apoB gene and hypobetalipoproteinemia

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Abstract A new, large kindred with hypobetalipoproteinemia and a previously undescribed truncated form of apolipoprotein B (apoB) has been identified. The asymptomatic, Caucasian male proband (CK, aged 37 years) has total plasma cholesterol, triglyceride, low density lipoprotein- (LDL) cholesterol, high density lipoprotein- (HDL) cholesterol, and apoB concentrations of 108, 131, 32, 50, and 16 mg/dl, respectively. Plasma samples of 11 family members spanning three generations, which had less than 5th percentile concentrations of LDL-cholesterol, contained three apoB bands detected on immunoblots: the normal apoB-100 and apoB-48 and an unusual band of apparent molecular mass of 299,356 ± 9580 daltons (~54% the molecular weight of apoB-100). Additional immunoblotting experiments using several different anti-apoB monoclonal antibodies showed that the carboxyl terminal of apoB-100 had been deleted somewhere between amino acid residues 2148–2488. A segment of genomic DNA from the proband was amplified by polymerase chain reaction (PCR) between nucleotides 7491–7791 of Exon 26 of the apoB gene. The DNA segment was cloned into pGEM3Zf(−) and sequenced. A C→T transition was found at nucleotide 7665, resulting in a premature stop codon at amino acid residue 2486 corresponding to apoB-54.8. These results were confirmed by direct sequencing of PCR products from three apoB-54.8 positive and three apoB-54.8 negative kindred members. Allele-specific oligonucleotides were used to identify other affected family members. Cosegregation of apoB-54.8 with the C→T transition occurred in all cases. Based on haplotypes constructed from restriction fragment length polymorphism, variable number of tandem repeats, and 5' insertion/deletion analyses and from the presence or absence of apoB-54.8, it was possible to assign a single allele of apoB to the mutation throughout the family. In contrast with other shorter truncations such as apoB-31, apoB-40, and apoB-46, which are found with particles in the HDL density range, and apoB-89 that is found primarily with LDL, apoB-54.8 was found primarily in very low density lipoproteins, much less in LDL, and was virtually absent in HDL. This suggests that the length of the truncation may significantly affect the metabolism of the associated lipoprotein particles. **—Wagner, R. D., E. S. Krul, J. Tang, K. G. Parhofer, K. Garlock, P. Talmud, and G. Schonfeld.** ApoB-54.8, a truncated apolipoprotein found primarily in VLDL, is associated with a nonsense mutation in the apoB gene and hypobetalipoproteinemia. *J. Lipid Res.* 1991. **32:** 1001–1011.

Supplementary key words hypobetalipoproteinemia • apolipoprotein B • truncated apoB • genetic defects

Several mutations of apoB-100 have been reported in recent years. One mutation, directing an amino acid substitution at amino acid 3500, inhibits the binding of LDL to its receptor, and produces hypercholesterolemia that, in many patients, clinically greatly resembles the LDL-receptor-defective familial hypercholesterolemias (1–4). Other mutations, mostly base deletions, produce shifts in the reading frames of transcription and premature stop codons that direct the synthesis of truncated apoB molecules of varying lengths, due to deletions of carboxylterminal portions of the molecule, resulting in the secretion of lipoproteins containing unusual short forms of apoB detectable in plasma (5–12). Individuals producing these unusual truncated apoB molecules have hypocholesterolemia and hypobetalipoproteinemia. The underlying physiologic mechanisms causing the low LDL levels are unknown for most defects, but we have previously demonstrated that LDL particles containing apoB-89 are cleared from plasma at enhanced rates (13) due to their increased affinity to LDL-receptors (5), which results in low concentrations of apoB-89-containing lipoproteins in plasma. Thus, studies of individuals with truncated apoB variants

Abbreviations: apo, apolipoprotein; VLDL, very low density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; RFLP, restriction fragment length polymorphism; VNTR, variable number of tandem repeats; PAGE, polyacrylamide gel electrophoresis; EDTA, ethylenedinitrilotetraacetic acid; PPACK, D-phe-L-phe-L-arg chloromethylketone; DTPA, diethylenetriamine pentaacetic acid; GGE, non-denaturing gradient gel electrophoresis; PBS, phosphate-buffered saline; SSC, 0.15 M NaCl, 0.015 M sodium citrate; ASO, allele-specific oligonucleotide; FPLC, fast protein liquid chromatography; MAb, monoclonal antibody.

have provided important information on the molecular genetics and pathophysiology of lipid disorders and on the structure-function relationship of apoB-100.

One of the major activities of our laboratory has been the continuing identification and characterization of apoB defects in humans (5, 6, 13). In this report we describe a kindred whose affected members have hypobetalipoproteinemia in association with a truncation of apoB called apoB-54.8 according to the centile system. The molecular defect and the distribution of apoB-54.8 among the lipoproteins are described in this communication.

METHODS

Population screening for apoB mutants

The population screened included blood donors at the St. Louis Red Cross Blood Bank, volunteers at community screenings, and patients undergoing coronary catheterization at Barnes and Jewish Hospitals in St. Louis, MO. Screenees with ≤10th percentile total cholesterol were evaluated for fasting total cholesterol, total triglycerides, and HDL-cholesterol in the Washington University Lipid Research Center (LRC) Core Laboratory (14, 15) using automated enzymatic methods (RA-1000, Technicon Instruments, Tarrytown, NY). The laboratory meets the lipid standardization requirements of the U.S. Public Health Service Center for Disease Control, Atlanta, Ga. ApoB concentrations were determined by radioimmunoassay (16). Total plasma proteins were separated on SDS-polyacrylamide gels, and subjected to immunoblotting using a monoclonal anti-apoB antibody (see below). Individuals displaying truncated apoB bands were invited to return for confirmation of the original findings of a truncated apoB. The proband was identified in this way and his kindred were studied in more detail as outlined below. Between April 1989, and July 1990, 431 subjects were confirmed as having ≤10th percentile total cholesterol concentrations and, of these, 292 had <5th percentile values. These 431 plasmas were analyzed by immunoblotting and two unusual truncated apoB variants were identified in probands of two kindreds (apoB-76, to be reported later, and the present apoB-54.8). One kindred with two different truncated apoBs, apoB-40 and apoB-89, was found in a previous screening involving ~900 volunteers evaluated for total cholesterol at Barnes and Jewish Hospitals. Since there were approximately 90 individuals with <10th percentile total cholesterol concentrations in the second group, we find an approximate frequency of four truncations per 1050 genes (525 subjects with ≤10 percentile total cholesterol concentrations). These frequencies provide only a rough estimate of the prevalence of truncated apoBs among subjects with low total cholesterols, because the base populations represent self-selected volunteers and plasmas examined for specific reasons.

Blood collection and plasma analyses

Blood from fasted donors was drawn into tubes containing EDTA (1.0 mg/ml). Blood cells were separated from plasma by centrifugation, and the following protease inhibitors and antibiotic were added promptly to the plasma: 20 μM D-phe-L-phe-L-arg chloromethylketone (PPACK) and 20 μM D-phe-L-pro-L-arg chloromethylketone (Calbiochem, La Jolla, CA) and gentamicin sulfate (100 μg/ml plasma) (Sigma Chemical Co., St. Louis, MO). A small volume of plasma was stored at −70°C for immunoblotting. Blood cells were saved and stored at −20°C for subsequent isolation of DNA (see below).

Isolation and storage of lipoprotein preparations

VLDL plus IDL were isolated at d<1.019 g/ml, LDL between d 1.019 and 1.063 g/ml, and HDL at d>1.063 g/ml or d 1.063–1.219 g/ml (17). Lipoproteins were dialyzed at 4°C against 50 mM borate-buffered saline, 20 μM diethylenetriamine pentaacetic acid (DTPA), and 0.13% (wt/vol) ε-amino caproic acid (Sigma Chemical Co.), pH 7.4, sterile-filtered, and stored tightly capped under N_2 at 4°C in the dark. VLDL subfractions were prepared either by gradient density ultracentrifugation in a swinging bucket SW40 rotor (Beckman Instruments, Palo Alto, CA) (18, 19), or by immunoaffinity chromatography using monoclonal antibodies of defined specificities (20). Immunoaffinity columns were constructed and used as previously described (21). Protein contents of VLDL subfractions were determined by a modification of the Lowry method (22).

Electrophoresis and immunoblotting

LDL apoB was analyzed by electrophoresis of delipidated LDL on gradient 3% to 6% sodium dodecyl sulfate (SDS) polyacrylamide gels (5, 23). Some LDL preparations also were analyzed by nondenaturing gradient polyacrylamide gel electrophoresis (GGE) on 2% to 16% Pharmacia PAA gels (24).

Electrotransfer and Western blotting of proteins separated by SDS-polyacrylamide gel electrophoresis (PAGE) was carried out as described previously (5). MAbs to apoB have been described previously (20). MAb BSol4 was kindly provided by Dr. Ross Milne (25).

Apolipoproteins of VLDL subfractions prepared by immunoaffinity chromatography were analyzed by 3–20% SDS polyacrylamide gel electrophoresis (26), followed by Coomassie blue staining and scanning of gels by laser densitometry (LKB-Model 2202 Ultrascan, Bromma, Sweden). Areas under gel bands were quantified using a digitizer and the Sigma-Scan program (Jandel Scientific,

TABLE 1. Primer oligonucleotides for polymerase chain reactions and apoB-54.8 allele-specific oligonucleotides

Amplification Site (cDNA seq)	5' Oligonucleotide	3' Oligonucleotide	PCR Product Size (bp)	PCR Conditions
	RFLP, VNTR, and I/D Primers[a]			
7464 *Xba* I	7141–7161 GGAGACTATTCAGAAGCTAA	7850–7830 GAAGAGCCTGAAGACTGACT	709	92°C, 1 min 58°C, 5 min
10834 *Msp* I	10560–10571 GAACTATTGCTAGTGAGGCCA	11024–10991 CTAAGGATCCTGGCAATGTGTCAAGC	462	92°C, 1 min 58°C, 5 min
12540 *Eco* RI	12316–12344 CTGAGAGAAGTGTCTTCGAAG	12794–12773 CTCGAAAGGAAGTGTAATCAC	478	92°C, 1 min 58°C, 3 min
3' VNTR	3' untranslated region ATGGAAACGGAGAAATTATG	CCTTCTTCACTTGGCAAATAC	variable	92°C, 1 min 58°C, 3 min
5' Ins/Del	5' region CAGCTGGCGATGGGACCCGCCGA	ACCGGCCCTGGCGCCCGCCAGCA	variable	92°C, 1 min 64°C, 1.5 min
	ApoB-54.8 Region Primers[b]			
7665 B-54.8	*Xba* I: 7457–7486 GCTCTAGACCGTGAGGTGACT- CAGAGACTCAATGGTGA	*Bam* HI: 7790–7761 GGGGATCCCCACCAATCAGAA- ATGTAGGTGACAAGTGT	333	95°C, 30 sec 55°C, 30 sec 72°C, 3 min
	Allele-Specific Oligonucleotides[c]			
Sequence position 7665	Normal oligonucleotide 7674–7655 GAGTCTCTCGGAATTTGGC	Mutant oligonucleotide 7674–7655 GAGTCTCTCAGAATTTGGC		

[a]Sequences of primers and the conditions for polymerase chain reaction were obtained from Boerwinkle et al. (30).
[b]Sequences spanning the apoB nucleotides indicated for the apoB-54.8 region primers are shown in upper case letters. The underlined nucleotide sequences represent additional 5' nucleotides added to incorporate either the *Xba* I or *Bam* HI restriction enzyme recognition sites.
[c]The two oligonucleotides, spanning the apoB nucleotides indicated, varied only in the underlined nucleotide 7665.

Corte Madera, CA). Molecular weights of truncated apoB forms were derived from plots of molecular weight of standard proteins (myosin, β-galactosidase, thrombin fragments of apoB, and known truncated apoB forms) versus distance migrated on the gels.

DNA preparation

Leukocytes were recovered from freshly drawn 30-ml blood samples in EDTA anticoagulant using Histopaque-1077 (Sigma Chemical Co.) gradient centrifugation as per the instructions of the suppliers. Genomic DNA was purified by the method of Davis, Dibner, and Battey (27).

Oligonucleotides

A pair of oligonucleotides was synthesized (Protein Chemistry Core Facility at Washington University School of Medicine) for use as polymerase chain reaction (PCR) amplification primers to produce a fragment of the apoB gene containing the region of the apoB-54.8 mutation as determined by protein size estimations. Recognition sites for *Xba* I and *Bam* HI restriction enzymes were incorporated into the 5' ends of the 5' and 3' oligonucleotides, respectively (**Table 1**). The apoB cDNA sequence numbering follows that of Knott et al. (28).

Oligonucleotide primers for PCR-mediated restriction fragment length polymorphism (RFLP) and variable number of tandem repeat (VNTR) analyses were synthesized from sequences reported by Boerwinkle et al. (29, 30, and Table 1).

Polymerase chain reaction

Five μg of genomic DNA and 0.5 pmol of each 5' and 3' primer were amplified in 100 μl reactions with recombinant *Taq* polymerase (Perkin Elmer Cetus, Norwalk, CT) in buffer supplied by the manufacturer (31). Thirty (apoB-54.8 and RFLP amplifications) and 26 (VNTR amplification) cycles of PCR were performed in a commercial temperature cycler (Coy Laboratory Products, Ann Arbor, MI) (Table 1).

Subcloning and sequencing

Amplified DNA from the proband CK, and a phenotypically normal relative GK, were purified with GeneClean (Bio 100, La Jolla, CA). The purified DNA was digested with 5 units each of *Xba* I and *Bam* HI and repurified with GeneClean. The DNA fragments were subcloned into a *Xba* I- and *Bam* HI-cut, dephosphorylated plasmid pGEM3Zf(−) preparation. The recombinant plasmids were sequenced using Sequenase II (United States Biochemical Corp., Cleveland, OH) with an SP6 promoter sequencing primer (Promega Corp., Madison, WI) and analyzed on 6% denaturing polyacrylamide gels (32).

TABLE 2. Clinical characteristics of CK kindred

ID	Sex	Age	BMI	TChol	TG	LDL-C	HDL-C	ApoB	ApoE Form
		yr	*kg/m²*						
Affected members									
IV-33	F	27	40.5	113	56	50	52	22	4/4
IV-32	F	28	19.5	122	64	41	68	29	4/4
IV-31	F	29	42.4	105	55	32	62	23	3/4
III-36	M	37	27.8	108	131	32	50	16	3/3
III-17	M	40	24.2	146	60	61	73	32	3/3
III-34	F	47	38.6	161	68	51	96	20	ND
III-32	M	49	29.9	108	74	38	55	15	3/3
III-15	M	54	25.4	98	41	41	49	21	ND
II-14	M	68	21.0	144	85	74	53	33	3/3
II-12	F	71	31.6	123	71	56	53	26	ND
II-5	M	82	23.7	121	57	67	43	43	3/3
Mean ± SD		48 ± 19*	29.5 ± 2.4	122.6 ± 19.8##	69.3 ± 23.5#	49.4 ± 14.0##	59.5 ± 14.9	25.5 ± 8.3###	
Unaffected members									
IV-38	M	11	19.1	126	80	69	41	57	3/3
IV-37	M	16	30.9	182	193	95	48	60	3/4
IV-11	M	19	29.0	146	84	84	45	75	3/3
IV-10	F	21	34.1	243	294	134	60	103	ND
IV-9	M	23	25.9	148	64	96	39	89	3/3
IV-7	M	24	33.3	207	186	128	42	63	3/3
IV-8	F	25	25.4	168	63	111	44	60	ND
IV-30	M	25	27.8	125	69	47	64	25	2/3
IV-28	M	30	33.6	156	367	55	28	59	3/3
III-37	F	33	24.3	175	163	93	49	64	3/4
III-7	F	46	23.7	234	84	138	79	106	3/3
III-6	M	47	38.2	222	227	125	52	95	3/3
III-33	F	49	19.8	174	88	87	69	50	2/3
III-35	M	51	29.3	238	134	169	42	72	3/4
II-13	M	73	34.0	239	393	126	34	103	3/3
Mean ± SD		33 ± 17	28.6 ± 5.6	185.5 ± 42.0	165.9 ± 110.7	103.8 ± 33.4	49.1 ± 13.7	72.1 ± 23.0	

Affected members of the kindred were heterozygous for apoB-54.8/apoB-100 as determined by Western blotting of plasma as described in Methods. Unaffected members of the kindred all were homozygous for apoB-100/apoB-100. Plasma lipid profiles were determined by using the protocols of the Lipid Research Clinics and apoB was determined by established radioimmunoassay. All values are mg/dl. ApoE phenotypes were determined by a restriction isotyping method (35). BMI, body mass index; TChol, total plasma cholesterol; LDL-C, LDL cholesterol; HDL-C, HDL cholesterol; apoB, plasma apolipoprotein B; apoE form, apolipoprotein E phenotype; SD, standard deviation. *, $P < 0.05$ by unpaired *t*-test compared to unaffected members; *, **, ***, $P < 0.005$, $P < 0.0005$, and $P < 0.0001$ by Mann-Whitney Two Sample Test compared to unaffected members, respectively.

The sequence was further confirmed by direct sequencing of amplified DNA from three phenotypically positive and three phenotypically negative kindred members using the 5' PCR primer (B54.8-1) as the sequencing primer (Table 1).

Allele-specific oligonucleotide analyses

A pair of allele-specific oligonucleotides (ASO) was synthesized with the sequences shown in Table 1. The oligonucleotides were 5' end-labeled with [^{32}P]ATP (ICN, Biomedicals, Inc., Costa Mesa, CA) using T4 polynucleotide kinase (Boehringer Mannheim, Indianapolis, IN) (33). Twenty μl of each 100 μl PCR product was denatured by the addition of 0.4 N NaOH to a final concentration of 0.2 N NaOH. The mixture was heated to 95°C for 5 min and quenched with 400 μl of ice-cold 15× SSC (SSC: 0.15 M NaCl, 0.015 M sodium citrate, pH 7.6). A 200-μl aliquot of each denatured PCR product was applied to a GeneScreen membrane (DuPont NEN, Boston, MA) in a slot-blot apparatus. The remaining 200-μl aliquots were blotted on a second membrane. The membranes were baked in a vacuum oven at 80°C for 2 h and placed into heat-sealable bags with 5 ml of 6× SSC, 0.1% SDS, 10× Denhardt's solution (0.2% ficoll, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin, and 250 μg/ml denatured salmon sperm DNA) and prehybridized 18 h at 42°C. The membranes were hybridized with the normal allele-specific or the mutant allele-specific probes for 24 h at 42°C. The membranes were washed twice with 6× SSC, 0.1% SDS for 5 min at room temperature and once for 5 min at 55°C. The membranes were air dried and subjected to autoradiography with Kodak XAR-5 film for 2 h at −70°C using intensifying screens.

RFLP, VNTR and insertion/deletion analyses

Oligonucleotide pairs were used as PCR primers to amplify the fragments of the subjects' DNA containing the *Xba* I, *Msp* I, and *Eco* RI RFLP sites of the apoB gene. A pair of oligonucleotides was used to amplify the 5' region of the apoB gene containing the insertion/dele-

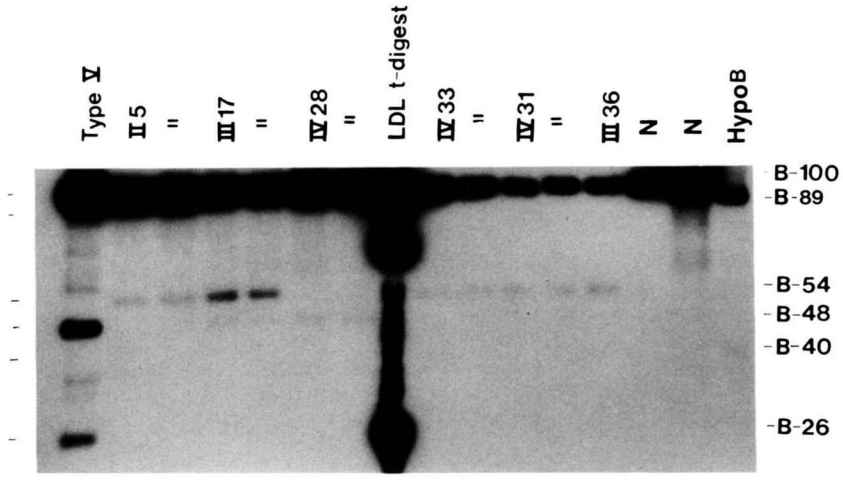

Fig. 1. Immunoblots of whole plasma. Ten μl of plasma was delipidated, subjected to electrophoresis on a 3–6% SDS-gradient polyacrylamide gel, and electrophoretically transferred to nitrocellulose. ApoB bands were visualized by immunoblotting and autoradiography, using monoclonal antibody C1.4. Type V, plasma from patient with Type V hyperlipoproteinemia whose major apoB bands were apoB-100 and apoB-48; hypobeta, plasma from a patient with compound heterozygous hypobetalipoproteinemia whose plasma contained apoB-89 and apoB-40 (5); N, plasmas of two normolipidemic controls; LDL-t-digest, thrombin-digested LDL. The Roman/Arabic numerals refer to subjects from the CK kindred (see Table 2). Ditto marks designate the same plasmas as the adjoining lefthand lanes except that they were collected in the absence of protease inhibitors. This film was deliberately overexposed for apoB-100 in order to show the apoB-54.8 bands. The various forms of apoB are indicated on the right side of the figure. ApoB-26 refers to a fragment generated by thrombin cleavage of apoB (see LDL-t-digest lane).

tion (I/D) polymorphism (34). An additional oligonucleotide pair was used to amplify the 3' region of the apoB gene containing the VNTR sequences at the 3' end of exon 29. The sequences of the PCR oligonucleotide primers and the specific amplification conditions are listed in Table 1.

DNA amplifications were made in 100-μl final volumes for each polymorphism. The template DNA was 0.5 μg of each subject's genomic DNA and 0.01 μg of each primer was used. Ten units of each restriction enzyme (Boehringer Mannheim) was used for the RFLP analyses. The restriction digests were analyzed by electrophoresis on 2% agarose gels run at 70 volts for 4 h in TBE buffer (0.089 M Tris, pH 8, 0.089 M boric acid, 2 mM EDTA). The amplification products of the apoB gene regions containing the I/D polymorphism were analyzed on 7% horizontal agarose gels consisting of 5% (wt/vol) NuSieve GTG agarose (FMC BioProducts, Rockland, ME) and 2% agarose (Bethesda Research Laboratories, Gaithersburg, MD) run at 70 volts for 6 h. The ethidium bromide-stained bands were visualized and photographed on an ultraviolet transilluminator.

ApoE phenotyping

ApoE phenotyping was performed according to the restriction isotyping method of Hixson and Vernier (35).

Pedigree analysis

The phase of inheritance of the polymorphic markers and the apoB-54.8 phenotype were traced through the CK family by conventional segregation analysis.

RESULTS

Proband and his kindred

The proband is an asymptomatic 37 year old male Caucasian with total plasma cholesterol, LDL-cholesterol, and apoB concentrations of 108 mg/dl, 32 mg/dl, and 16 mg/dl, respectively (III-36, **Table 2**). These values were confirmed on two other occasions over several months. His medical history, physical examination, plasma glucose, and tests of liver, kidney, and thyroid function as well as blood cell counts and urinalysis were normal. On immunoblotting of this plasma (collected either in the presence or absence or protease inhibitors) apoB-100 was present as the predominant form of apoB (**Fig. 1**). In addition, an unusual band of a calculated molecular mass of 299,356 $\pm$ 9580 daltons on the SDS-polyacrylamide gel was found that was not seen on any of the apoB immunoblots performed on >1500 individual plasma samples analyzed over the last 30 months in this laboratory. This apoB species corresponded to 54.4% of the molecular mass of apoB-100 (550,000 daltons) and was also detected on two other proband plasma samples, analyzed on separate occasions. Of the 26 other members of the kindred tested to date (Table 2), 13 had <5th percentile (age, race and sex specific) LDL-cholesterol concentrations (37) and low plasma apoB concentrations. Eleven of the 13 subjects also had identifiable apoB-100 and apoB-54 in their plasmas (Table 2), the latter representing 5–10% of total apoB as estimated from the immunoblots. In the plasma of the two other subjects (brothers IV-28 and IV-30), only apoB-100 was detected in three separate samples taken

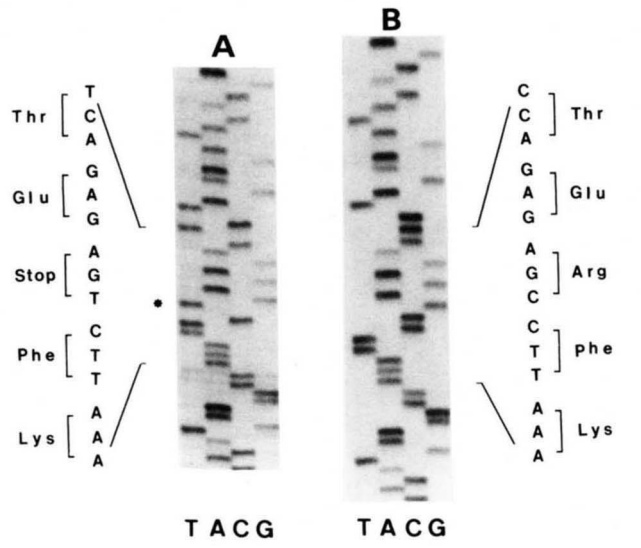

Fig. 2. Nucleotide sequence analysis of the apoB-54.8 mutant clone (A) and normal clone (B). DNA sequencing gels are shown for PCR products subcloned into pGEM 3Zf(−) vectors. The C→T transition at position 7665 is indicated by the star and is responsible for the introduction of the stop codon. The second C→T difference between the two alleles, at position 7673 (36) is responsible for the *Xba* I RFLP (the start of the restriction site is the last nucleotide reading from bottom upwards).

from each brother. These subjects are the offspring of a father with an apoB-54/apoB-100 pattern (III-32) and a mother with an apoB-100/apoB-100 pattern (III-33) whose LDL-cholesterol concentration is <10th percentile.

ApoB-54 was discovered by immunoblotting of whole plasma with anti-apoB MAb C1.4 whose specificity is directed against an epitope near the amino terminal region of apoB, suggesting that apoB-54 resulted from a carboxylterminal truncation of the apolipoprotein. To confirm this, a series of immunoblots was performed using a panel of eight anti-apoB MAbs with known apoB-100 regional specificities. MAbs with epitope specificities proximal to amino acid 2148 bound to the truncated apoB, whereas MAbs with epitope specificities distal to this residue were not reactive. This analysis suggested that the truncation indeed was in the carboxylterminal region and was located between amino acids 2148 and 2488 bordered by epitopes D7.2 (20) on the amino terminal end and epitope BSol4 (25) on the carboxyl end. Based on the estimated size of the truncated apoB by SDS-PAGE, the site of truncation was predicted to be near amino acid 2449.

Mutation

The appropriate region of the apoB-100 gene was amplified by PCR using leukocyte DNA and the primers listed in Table 1. The PCR products were sequenced directly by double-stranded sequencing or after ligation and subcloning in the pGEM 3Zf(−) vector system (**Fig. 2**). Both sequencing methods detected a nonsense mutation involving a C→T transition of nucleotide 7665 resulting in the conversion of an arginine codon in amino acid position 2486 to a stop codon. (The discrepancy between the nucleotide numbering system and the amino acid positions of the mature protein is due to the inclusion of 209 nucleotides comprising a 128 nucleotide segment of the 5' untranslated region and an 81 nucleotide segment comprising the 27 amino acid leader sequence in the report of the original sequence (38)). The stop codon was absent in alleles from phenotypically normal family members. Based on the centile system, the defect was named apoB-54.8.

Next we assessed whether the hypobetalipoproteinemia cosegregated with the presence of apoB-54.8 in other members of the kindred using allele-specific oligonucleotides (ASO) to probe PCR products prepared from DNA of relatives (Table 1 and **Fig. 3**). All subjects heterozygous for apoB-100 and apoB-54.8 had hypobetalipoproteinemia

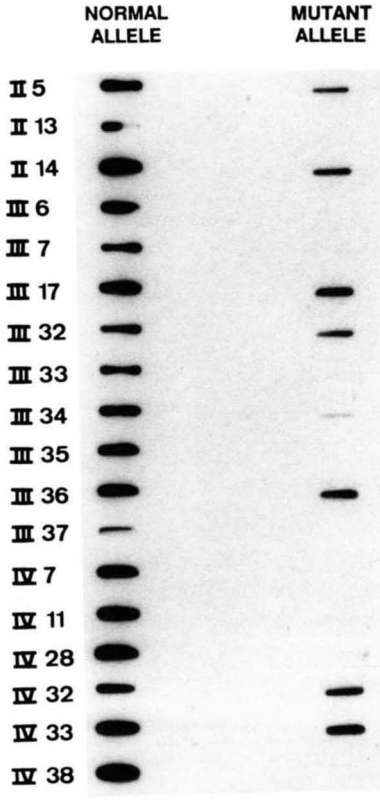

Fig. 3. Identification of members of the CK kindred homozygous for apoB-100 and heterozygous for apoB-100/apoB-54.8. The 5'-end ^{32}P-labeled allele-specific oligonucleotides (Table 1) were hybridized to PCR products of the relevant region of the apoB gene. PCR products were obtained using the oligonucleotides and conditions described in Table 1. Subjects are identified by Roman/Arabic numerals used in Table 2. Subjects whose DNA reacted with only the normal allele are homozygous for apoB-100; those whose DNA reacts with both alleles are apoB-100/apoB-54.8 heterozygotes.

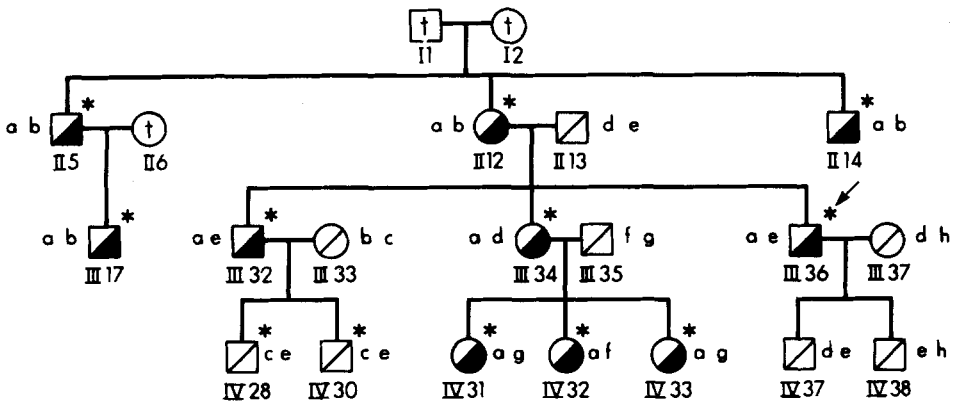

Fig. 4. The pedigree of the CK kindred showing only those members whose apoB haplotypes were determined as described in Methods. ⊘, ◪, Homozygous for apoB-100; ◔, ◪, heterozygotes were ≤5th percentile for age, race and sex; †, deceased. Haplotypes (a–h) are assigned according to the presence or absence of *Xba* I (X), *Msp* I (M), *Eco* RI (R) cut sites, the numbers of VNTRs, the insertion or deletion of a repeat sequence in the signal peptide coding region, and the presence or absence of apoB-54.8 as shown at right (haplotypes are designated with a 1 for the common allele and a 2 for the rare allele (39)).

Haplotype	*Xba* I	*Msp* I	*Eco* RI	VNTR	5'I/D	ApoB Phenotype
a	X2	M1	R1	3'β37	D	54.8
b	X2	M1	R1	3'β37	I	100
c	X2	M2	R1	3'β37	D	100
d	X1	M1	R1	3'β35	D	100
e	X1	M1	R1	3'β35	I	100
f	X1	M1	R1	3'β47	I	100
g	X1	M1	R2	3'β34	I	100
h	X2	M2	R1	3'β37	I	100

(Table 2 and Fig. 3) and the cosegregation of apoB-54.8 with the C→T transition could be shown in all cases.

Haplotypes of various apoB alleles present in the kindred were constructed, based on RFLP, VNTR, and insertion/deletion analyses, and the presence of apoB-54.8. Eight distinct haplotypes were found. The haplotypes segregated in the kindred in accord with the information obtained from the relatives as to their relationships with each other (**Fig. 4**). Neither subject IV-28 nor subject IV-30, who demonstrated <5th percentile for LDL-cholesterol concentrations, had the haplotype associated with apoB-54.8, nor did either subject's DNA hybridize to the mutant ASO. This is in accord with the absence of apoB-54.8 from their plasmas.

Characterization of lipoproteins

Distribution of apoB-54.8 among the major classes of lipoproteins was assessed on 3–6% SDS polyacrylamide gels stained by Coomassie Blue; identities of bands were confirmed by sizing and immunoblotting (**Fig. 5**). ApoB-54.8 appeared to be more abundant in d<1.019 g/ml (apoB-54.8/apoB-100 ratio ≈1:6), less was present at d 1.019–1.063 g/ml (apoB-54.8/apoB-100 ratio ≈1:18), and virtually none was seen at d>1.063 g/ml (including the HDL and non-lipoprotein fractions). The lipid compositions of these density classes were not unusual, and apoE and apoCs were present in VLDL in expected proportions (data not shown).

Lipoprotein size distribution were examined by gel permeation chromatography on FPLC (40) and by GGE. Elution profiles of whole plasma on FPLC resembled those of normal plasma except that LDL were present in lower amounts (data not shown). The LDL (d 1.019–1.063 g/ml fractions) isolated from plasma were examined by GGE (data not shown). A single class of LDL of normal diameter was found, probably reflecting the predominant presence of apoB-100-containing LDL particles in this density fraction.

In order to further assess whether apoB-containing particles of unusual size were present in plasma, whole plasma was subjected to GGE, followed by immunoblotting with ^{125}I-MAb C1.4 (**Fig. 6**). Again, only a single population of LDL of normal size was found. No smaller apoB-containing particles were present (in contrast with the apoB-40-containing particles seen in apoB-89/apoB-40 compound heterozygotes (5)). No particles migrated between VLDL and LDL (not shown). VLDL subfractions cannot be analyzed by these gels (or by FPLC) because VLDL are excluded by both methodologies due to their size.

Next we assessed whether the apoB-100, apoB-54.8, and apoB-48 forms of apoB were associated to the same extent with all the particles isolated in the d<1.019 g/ml density range. After subfractionating the d<1.019 g/ml particles by gradient ultracentrifugation into three subfractions, the relative proportions of apoB-100, apoB-54.8, and apoB-48 were assessed by SDS-gradient polyacrylamide gel electrophoresis. The proportions differed in the three subfractions (**Table 3**), with the largest $VLDL_1$ particles having relatively higher proportions of apoB-54.8 compared to the smaller $VLDL_2$ and $VLDL_3$ particles. The d<1.019 g/ml lipoproteins also were subfractionated by immunoaffinity chromatography on a column containing anti-apoB MAb 29EE2.1 (amino acids 2658–3287)

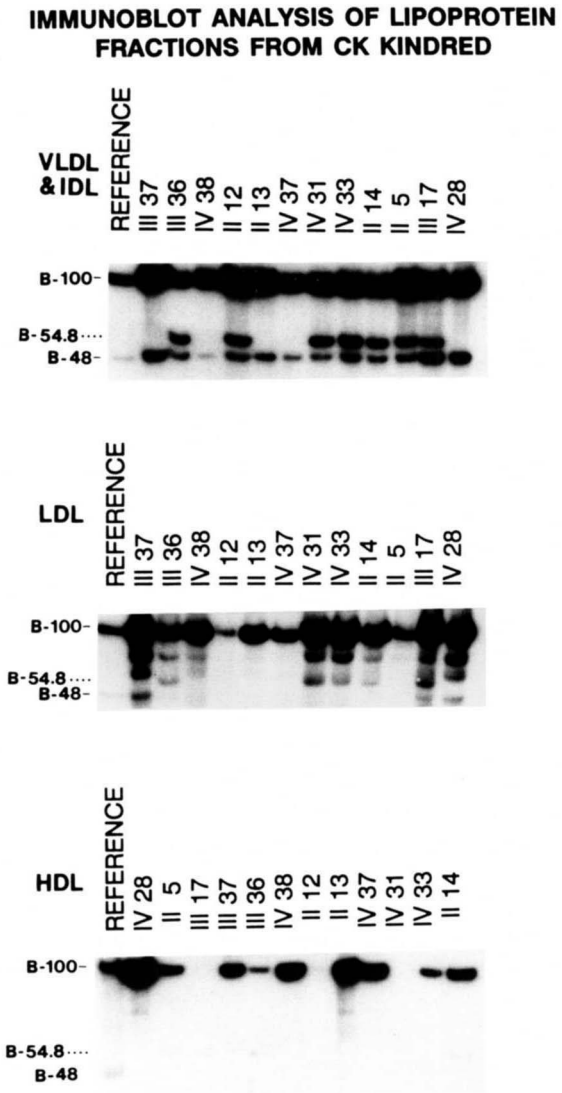

Fig. 5. Immunoblot analyses of VLDL and IDL (d < 1.019 g/ml), LDL (1.019–1.063 g/ml), and HDL (d > 1.066 g/ml) isolated from members of the CK kindred. Lipoproteins were delipidated, electrophoresed on 3–6% SDS-gradient polyacrylamide gels, electrophoretically transferred to nitrocellulose sheets, and immunoblotted with anti-apoB MAb C1.4. Members of the CK kindred are identified by Roman/Arabic numbers (see Table 2). The migrations of various forms of apoB used as size markers are indicated on the left side of the gels.

(**Fig. 7**). The pooled unbound fraction contained ~35% and the bound fraction ~65% of the recovered VLDL protein. Recovery of protein was approximately 70% in two separate experiments. As expected from the epitope specificity of the MAb and its lack of interaction with apoB-54.8, apoB-100 was bound to the column, whereas apoB-54.8 was not bound to any significant degree (Fig. 7). Not all apoB-100 in the VLDL preparations was retained by the affinity columns however, in agreement with earlier results (41). These results indicate, then, that in this kindred at least two populations of VLDL are present: apoB-100-containing VLDL, apoB-100 and apoB-54.8-containing VLDL, or separate apoB-54.8-containing VLDL.

DISCUSSION

The genetic, biochemical, and physiologic bases of the hypobetalipoproteinemias appear to be diverse and in most cases not understood. The relatively well-understood hypobetalipoproteinemias are those associated with the truncated forms of apoB-100. Although no formal surveys are available, heterozygotes with truncated forms of apoB, as opposed to normal apoB-100, probably comprise <1% of subjects with ≤10th percentile LDL-cholesterol plasma concentrations. Despite their rarity, patients with truncated forms of apoB have provided and continue to provide valuable information about the structure–function relationships of apoB and lipoprotein metabolism.

The proband's plasma LDL-cholesterol and apoB levels were well below 5th percentile for his age, race, and sex, and his plasma contained in addition to apoB-100 and apoB-48, the natural forms of apoB, an unusual form, apoB-54.8. Eleven other members of the kindred in three generations had similar low concentrations of LDL-cholesterol and apoB-54.8 was present. On immunoblotting of whole plasma, apoB-54.8 appeared to represent ~5–10% of total apoB. None of the affected individuals complained of visual, neurologic, or gastrointestinal symptoms and the proband was normal on detailed examination.

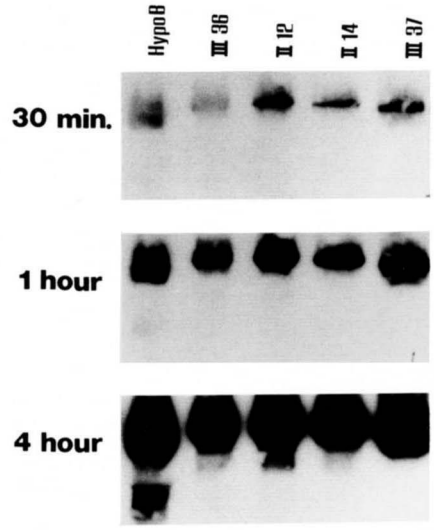

Fig. 6. Immunoblots of whole plasma electrophoresed in nondenaturing 2–16% GGE. Each lane identifies a member of the kindred except for the lane labeled hypobeta which contains plasma from a hypobetalipoproteinemic apoB-89/apoB-40 heterozygote. The times denote the duration of exposure to the blots to the X-ray films. The more rapid migration of apoB-89 containing LDL is evident, particularly at 30 min and 1 h. The presence of a second population of apoB-containing lipoproteins in the hypobeta patient becomes increasingly obvious with increasing duration of exposure. These are apoB-40-containing lipoproteins that float at d 1.063–1.21 g/ml (5). Second populations of LDL-like or HDL-like particles are not evident in the plasmas of either the apoB-100/apoB-54.8 heterozygous or the apoB-100/apoB-100 homozygous members of the kindred.

TABLE 3. Relative proportions of apolipoproteins B-100, B-54.8, and B-48 in density subfractions of VLDL

Subject	Subfractions	Area Ratios		
		B-48/B-100	B-54.8/B-100	B-54.8/B-48
III-36	$VLDL_1$	0.09	0.23	2.47
III-36	$VLDL_2$	0.12	0.22	1.8
III-36	$VLDL_3$	0.08	0.11	1.4
IV-37	$VLDL_1$	0.04		
IV-37	$VLDL_2$	0.08		
IV-37	$VLDL_3$	0.09		

Results represent area ratios of scans of Coomassie-blue stained SDS polyacrylamide gradient gels. VLDL subfractions were obtained by density gradient ultracentrifugation as described in Methods. Fifty μg of lipoprotein protein from each subfraction was applied to 3–10% SDS-PAGE gels. After staining with Coomassie blue, the protein bands corresponding to apoB-100, apoB-54.8, and apoB-48 were scanned by laser densitometry. Areas under the protein peaks were quantified as described in Methods. Subject III-36 is the proband heterozygous for apoB-100/apoB-54.8. Subject IV-37 is a normolipidemic relative homozygous for apoB-100.

Two members of the kindred, brothers IV-28 and IV-30, had less than 5th percentile values for LDL cholesterol concentrations but did not have apoB-54.8. Their father (III-32) had apoB-54.8 and a hypobetalipoproteinemic phenotype, but their mother (III-33), although homozygous for apoB-100, had <10th percentile values for LDL cholesterol concentration, suggesting the low LDL cholesterol concentration in subjects IV-28 and IV-30 may have been an independent trait inherited from their mother. The cause of her low LDL levels and theirs has not yet been determined. All other members of the CK kindred tested thus far that are homozygous for apoB-100 were normolipidemic.

ApoB-54.8 results from a nonsense mutation consisting of a C→T transition at nucleotide 7665 leading to a premature stop codon at residue 2486. This C→T transition was anticipated by Collins et al. (42). The mutation was confirmed in other members of the kindred by direct sequencing of PCR amplified products of genomic DNA and by sequencing of subcloned PCR amplified products of the appropriate region of the apoB gene. ASOs specific for the mutant allele detected each of the ten affected relatives with apoB-54.8 and none who were apoB-100/apoB-100 homozygotes including two relatives with apoB-100/apoB-100 phenotypes who had <5th percentile LDL-cholesterol concentrations. Although definitive proof of the role of this mutation in causing the production of apoB-54.8 awaits reproduction of the defect in an in vitro expression system, the available data strongly suggest that this mutation results in production of apoB-54.8. All affected individuals were apoB-100/apoB-54.8 heterozygotes. Based on haplotypes constructed from RFLP, VNTR, and 5' insertion/deletion analyses and from the presence or absence of the apoB-54.8 gene defect, it was possible to assign a single allele of apoB to the gene defect throughout the family and to establish the genotype of the parents of the proband (Fig. 4).

Based on the proportion of apoB-54.8 to apoB-100 in the d<1.019 g/ml lipoproteins and in LDL (≈1:6 and ≈1:18, respectively) it is likely that the great majority of apoB-54.8 was in the d<1.019 g/ml fraction. Thus, apoB-54.8 is of sufficient length to permit the secretion of VLDL-size particles either from the liver, intestine, or both. We were unable to distinctly separate apoB-100-containing d<1.019 g/ml particles from particles containing apoB-54.8 or apoB-48 by density gradient ultracentrifugation (Table 3) or immunoaffinity chromatography (Fig. 7). Separate subpopulations of VLDL also were not detectable on acrylamide/agarose gel electrophoresis (data not shown). Nevertheless, it was possible to demonstrate that the relative proportions of apoB-100, apoB-54.8, and apoB-48 differed both in the three density classes of VLDL and the two immunoaffinity fractions. These findings are compatible with the concept that each of the three forms of apoB is associated with a separate set of particles, which is in accord with findings in other cases of apoB truncation (5, 13, 43). However, this remains to be demonstrated. Similarly, it was not possible to identify distinct populations of LDL particles by immunoblotting

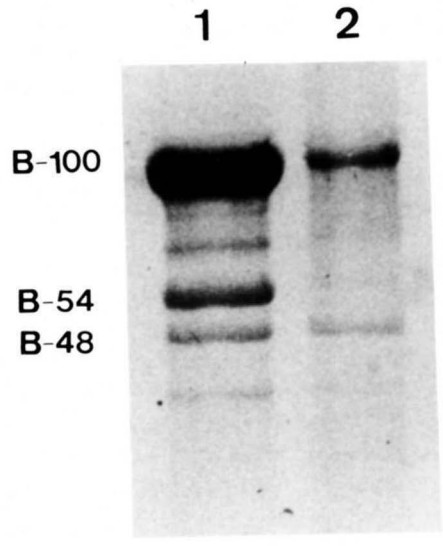

Fig. 7. Coomassie-blue stained 3–20% SDS-PAGE gel of d<1.019 g/ml subfractions from subject III-32 separated by immunoaffinity chromatography on a column prepared with monoclonal antibody 29EE2.1. Five hundred μg of d<1.019 g/ml protein was applied to the immunoaffinity column. The column was rinsed and the protein specifically bound was eluted as described in Methods. Lane 1, unretained fraction; lane 2, retained fraction. Twenty μg protein of each fraction was applied to the gel. Only the top portion of the gel is shown. Ratios of apoB-54.8/apoB-100 for the d<1.019 g/ml unretained and retained fraction were determined after densitometric scanning of the apoB-54.8 and apoB-100 bands and were 0.19 and 0.03, respectively. The band migrating like apoB-48 in the retained fraction most likely represents a proteolytic product of apoB-100 and not actual apoB-48.

of whole plasma on nondenaturing gels (Fig. 6). Perhaps this is because, *a*) in fact, none were present; *b*) immunoblotting was too insensitive to detect a second, minor LDL population; *c*) apoB-54.8- and apoB-100-containing LDL populations were too similar in diameter and charge to be resolved by GGE, and/or; *d*) that apoB-54.8 and apoB-100 may be present on the same particles.

The reasons for the low plasma concentrations of apoB-54.8-containing lipoproteins are not known. Rates of biosynthesis of apoB-54.8 may differ from that of apoB-100 or the metabolic fates of apoB-54.8 lipoproteins (e.g., lipoprotein lipase or hepatic triglyceride lipase-catalyzed processing, lipid exchange reactions or receptor-mediated clearances) also could differ from normal lipoproteins. The metabolism of the various truncations is most likely affected in characteristic ways depending on the specific apoB variant. For example, non-LDL-receptor-mediated clearance is likely to occur for apoB-54.8-LDL, which is probably not recognized by the LDL-receptor because the putative receptor recognition site has been deleted. Hence, apoB-54.8-lipoproteins must be cleared from plasma by other mechanisms; on the other hand, apoB-89-containing LDL, which interacts with the LDL-receptor with enhanced affinity (5), is cleared from plasma with unusual rapidity (13). Clearly, much remains to be learned about the compositions, structures, and metabolism of the various truncated apoB-containing lipoproteins. It may be anticipated that this information will enhance understanding of the several important functions of apoB in lipoprotein metabolism and atherogenesis.

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