Characterization of an Abnormal Species of Apolipoprotein B, Apolipoprotein B-37, Associated with Familial Hypobetalipoproteinemia

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Abstract

Steinberg and colleagues have previously described a unique kindred with normotriglyceridemic hypobetalipoproteinemia (1979. *J. Clin. Invest.* 64:292–301). In a reexamination of this kindred, we found an abnormal apolipoprotein (apo) B species, apo B-37 (203,000 mol wt), in the plasma lipoproteins of multiple members of the kindred. In affected individuals apo B-37 was found in very low density lipoproteins, along with the normal apo B species, apo B-100 and apo B-48. High density lipoproteins (HDL) also contained apo B-37, but no other apo B species. The first 13 amino-terminal amino acids of apo B-37 were identical to those of normal apo B-100. We utilized a panel of 18 different apo B-specific monoclonal antibodies and polyclonal antisera specific for apo B-37 and the thrombin cleavage products of apo B-100 to map apo B-37 in relation to apo B-100, apo B-48, and the thrombin cleavage products of apo B-100. The results of those immunochemical studies indicated that apo B-37 contains only amino-terminal domains of apo B-100.

In affected individuals, the majority of apo B-37 in plasma was contained in the HDL density fraction. Within that fraction apo B-37 was found on discrete lipoprotein particles, termed Lp-B37, that had properties distinct from normal HDL particles containing apo A-I.

This report documents for the first time the existence of an abnormal apo B species in humans. Further study of apo B-37 and lipoprotein particles containing apo B-37 should lead to an improved understanding of apo B structure and function.

Introduction

In 1980, Kane and co-workers (1) demonstrated the existence of two species of apolipoprotein (apo)¹ B in human plasma lipoproteins. The larger apo B species, apo B-100 (550,000 mol wt), is made by the liver (2) and is found in very low density lipoproteins (VLDL) and low density lipoproteins (LDL). In humans, the smaller apo B species, apo B-48 (264,000 mol wt), is made only by the intestinal mucosal cells (3), and is found in plasma chylomicrons and chylomicron remnants (1). Apo B-100 contains the LDL receptor-binding domain(s), and is the ligand responsible for uptake of LDL by cells (4–6). Apo B-48 is not believed to contain an LDL receptor-binding domain, but definitive evidence on this point is lacking. Apo B-48-containing particles, which are triglyceride-rich, also contain apo E (7), and

are thought to be removed from plasma by specific receptors that recognize apo E (8). This process appears to be independent of apo B-48 (8).

Recently, our laboratory demonstrated that a specific monoclonal antibody, MB19, detects a common form of genetic polymorphism in apo B-100 (9). This polymorphism was invariably expressed in parallel in apo B-100 and apo B-48, providing the best evidence to date that the two normally occurring apo B species are products of a single gene (10). The molecular basis whereby a single apo B gene yields two different forms of apo B is incompletely understood. Recently, Law and co-workers (11) presented evidence that intestinal cells have two apo B messenger RNAs, one large message capable of encoding for apo B-100, and a smaller message for apo B-48. However, Glickman et al. (3) have found only the large message in human intestinal mucosal cells. The mechanism underlying the formation of the two normally occurring apo B species requires further study.

There have been many reports of patients who make little or no apo B-100 or B-48 (12, 13). There have also been several reports of human subjects who synthesize B-48, but not B-100 (14–16). However, no one has previously reported an abnormal apo B species (i.e., an apo B with an abnormal molecular weight), either in humans or experimental animals.

In 1979 Steinberg and colleagues (17) at La Jolla identified an unusual family, the H.J.B. kindred, with hypobetalipoproteinemia. Affected members of this unique kindred had severe hypobetalipoproteinemia, yet were normotriglyceridemic and clinically well. Studies performed at that time to elucidate the mechanism of the hypobetalipoproteinemia indicated that LDL from the proband had normal uptake in cultured fibroblasts, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of his LDL was said to show normal apo B. Because it was subsequently learned that normal plasma contains two apo B species, as noted above, we recently undertook an extensive reevaluation of this family.

In this report, we document the existence of an abnormal apo B species, apo B-37 (203,000 mol wt), present in the lipoproteins of multiple members of this kindred with hypobetalipoproteinemia. We demonstrate that apo B-37 is not simply an artifact produced during isolation of the lipoprotein fractions, but a distinct apo B species that contains only the amino-terminal domains of apo B-100. We also document the presence of a novel lipoprotein found in the HDL density region, termed Lp-B37, in which apo B-37 is the major apoprotein. In a following article (18), we demonstrate that apo B-37 is the sole product of one of the two apo B alleles, and we trace the inheritance of this abnormal allele over three generations.

Methods

Preparation of plasma samples. Fresh plasma samples were obtained from H.J.B., the proband, members of the H.J.B. kindred, and selected normal control subjects. Nonfasting samples were obtained to increase

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1. Abbreviations used in this paper: apo, apolipoprotein.

the yield of chylomicrons and VLDL. From H.J.B., 250 ml of plasma was obtained by plasmapheresis. From the other subjects, 25–80 ml of blood was collected into tubes containing EDTA (1.5 mg/ml blood), and the plasma was immediately isolated by centrifugation at 4°C. One aliquot was immediately set aside for measurement of lipoprotein and apoprotein concentrations. To the remainder of the plasma, we added multiple proteolytic inhibitors and antimicrobial agents, similar to the group of inhibitors reported by Cardin et al. (19). The inhibitors (and their final concentrations) were as follows: aprotinin (100 kallikrein inhibitor units/ml), polybrene (25 µg/ml), lima bean trypsin inhibitor (20 µg/ml), soybean trypsin inhibitor (20 µg/ml), benzamidine (2.0 mM), D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK, 1.0 µM), and glutathione (0.02%). On one occasion, blood from H.J.B. was collected directly into syringes containing the inhibitors. On several occasions, we isolated plasma from H.J.B. in the absence of proteolytic inhibitors. These studies were approved by the Human Use Committee of the University of California, San Diego.

Lipoprotein isolation and characterization. VLDL ($d < 1.006$ g/ml), IDL ($d = 1.006$ – 1.025 g/ml), LDL ($d = 1.025$ – 1.063 g/ml), HDL ($d = 1.063$ – 1.21 g/ml), and lipoprotein-depleted plasma ($d > 1.21$ g/ml) were isolated under strictly sterile conditions using standard ultracentrifugation techniques (20). Protein concentration of the lipoprotein fractions was determined by a modification of the Lowry technique, using a bovine serum albumin (BSA) standard (21).

Lipoprotein electrophoresis of fresh plasma and isolated lipoprotein fractions was performed using 1% agarose gels (no. AC470100, Fisher Scientific Co., Pittsburgh, PA) and the Corning electrophoresis system (Palo Alto, CA), according to manufacturer's instructions. Agarose gels were either stained with fat red 7B (Sigma Chemical Co.) or blotted onto a nitrocellulose membrane for Western blot analysis. We found that nearly all of the lipoproteins in the agarose gel could be transferred to the nitrocellulose sheet simply by incubating a moist sheet of nitrocellulose membrane on the gel for 18 h at 4°C.

SDS-PAGE. Apoproteins contained in the different lipoprotein fractions were separated by 3–12% or 3–15% polyacrylamide slab gels containing 0.1% SDS. The sterilely prepared lipoprotein fractions were first delipidated in 3:1 ether/ethanol containing 20 µM butylated hydroxytoluene (1). The delipidated lipoprotein preparations were dissolved in a sample buffer containing 10 mM Tris-HCl, 30% glycerol, 2% SDS, and unless otherwise indicated, 0.2% 2-mercaptoethanol. Samples were brought to 100°C for 5 min before being loaded onto the gels. After electrophoresis gels were stained with 0.1% Coomassie Blue or blotted onto nitrocellulose membranes for Western blot analysis, as previously described (10, 22).

Determination of the molecular weight of the abnormal apo B species. To determine the molecular weight of the abnormal apo B species in H.J.B. lipoproteins, 10–30 µg of delipidated H.J.B. VLDL and H.J.B. HDL were applied to lanes of a 3% polyacrylamide gel. Control lanes contained: (a) delipidated LDL isolated from a normal individual in the absence of proteolytic inhibitors; (b) delipidated thrombin-treated LDL; and (c) delipidated chylomicrons isolated from a normal subject. To calibrate the gels, we used the following published values for the molecular weights of the various proteins: B-100, 550,000; B-74, 407,000; B-26, 144,000; B-48, 264,000; T1, 385,000; T2, 170,000; T3, 238,000; and T4, 145,000 (1, 19).

The molecular weight of the abnormal apo B species in H.J.B. lipoproteins was determined by regression analysis and found to be 203,000. Using the centile system of nomenclature suggested by Kane and colleagues (1), the abnormal apo B species was designated "apo B-37."

Purification and amino-terminal sequencing of apo B-37. Because apo B-37 was the only apo B species found in the HDL of H.J.B. (see Results), we purified apo B-37 from HDL without concern of contamination by apo B-100 or apo B-48, or their proteolytic breakdown products. 3–5 mg of H.J.B. HDL was delipidated and applied to a 3–15% polyacrylamide slab gel containing 0.1% SDS. After electrophoresis, apo B-37 was visualized upon precipitation of the SDS in the gel by incubating the gel in 0.2 M KCl at 4°C. The apo B-37 band was sliced from the

gel, and electroeluted as previously described (23). Apo B-37 purified in this fashion migrated as a single band on analytical SDS-PAGE and had exactly the same mobility as the apo B-37 contained in H.J.B. HDL. The amino-terminal amino acid sequence of the purified apo B-37 protein was determined on a gas-phase model 470 sequencer (Applied Biosystems, Inc., Foster City, CA).

Antibodies. 18 monoclonal antibodies specific for apo B were used in this study. Antibodies B1, B2, B3, B11, B16, B18, B19, B20, and B24 have been previously characterized (9, 10, 22, 24). Here, to avoid possible confusion with various apo B species, they are referred to as MB1, MB2, etc. MB47, an antibody that binds to an epitope on or near the LDL receptor-binding domain of apo B-100, has previously been described in detail by our laboratory (25). Antibodies MB43, MB44, MB6, MB8, MB7, MB10, and OXB1 have not been previously described but each has been shown to be specific for apo B, using previously established techniques (24–26). Antibody 2D8 (5, 6) was kindly provided by Drs. Ross Milne and Yves Marcel. Ascites fluid or culture supernatant fluids were used as a source of antibody.

We generated a rabbit antisera against LDL-apo B-100, as previously described (25, 26). We also generated rabbit antisera against the abnormal apo B species, apo B-37, using purified apo B-37 isolated from preparative SDS-polyacrylamide gels (see below) as the immunogen. This antiserum was purified over a protein A- or LDL-Sepharose 4B column, as previously described (6, 24). Rabbit antisera specific for the apo B-100 thrombin cleavage fragments T4, T3, and T2 were kindly provided by Dr. Thomas Innerarity of the Gladstone Foundation Laboratories, San Francisco, CA.

To identify apo A-I in the Western blots we utilized a monoclonal antibody specific for apo A-I, designated A-I-10. This antibody was generated and characterized according to previously published techniques (27).

Characterization of antibodies. Western blot analysis was used to assess the ability of each of the 18 apo B-specific monoclonal antibodies to bind to apo B-100 and apo B-48 of chylomicrons isolated from the plasma of a normal subject. Western blots were also used to assess the binding of the thrombin fragment-specific antisera to apo B-37 and to assess the binding of the apo B-37-specific antisera to the thrombin cleavage products of apo B-100. Thrombin-treated LDL, and the individual thrombin fragments, which were purified from preparative SDS-polyacrylamide gels, were provided by Dr. Thomas Innerarity.

Monoclonal antibody binding to the thrombin fragments of apo B-100 was determined by solid-phase radioimmunoassay (RIA) (9, 22, 25). Briefly, the individual thrombin fragments of apo B-100 were used to coat flexible 96-well microtiter plates. Remaining binding sites on the plastic plates were blocked with a buffer containing 3% BSA. A fixed concentration of monoclonal antibody was then added to the plates for 16 h at 4°C. Binding of antibodies to the thrombin fragments was then quantitated by a final incubation with ¹²⁵I-labeled goat anti-mouse Ig. Binding of selected monoclonal antibodies to the thrombin cleavage fragments of apo B-100 was confirmed by Western blot analysis.

Competitive radioimmunoassay of apo B. The apo B concentration in whole plasma or in isolated lipoprotein fractions was measured by solid-phase, competitive RIA using antibody MB3 or antibody MB47, exactly as previously described (9, 25). This assay uses 96-well microtiter plates coated with normal LDL and utilizes normal LDL for the standard curve. Antibody MB3 binds apo B-100, B-48, and B-37, whereas MB47 binds only apo B-100 (see results below). Thus, an assay utilizing antibody MB3 measures "total apo B" present, whereas antibody MB47 measures only apo B-100 present. To determine the density distribution of total apo B and apo B-100 in H.J.B. plasma, separate RIAs were performed with MB3 and MB47 on whole plasma and on the top and bottom fractions of aliquots of whole plasma that was ultracentrifugally separated at the following densities: $d = 1.006$, 1.019, 1.063, and 1.21 g/ml. 4 ml of plasma was adjusted to the appropriate density with NaBr, and then spun in polyallomer tubes for 48 h in a 50.3 Ti rotor. The tubes were sliced, and the top and bottom fractions were recovered and dialyzed against phosphate-buffered saline (PBS). Total apo B and apo B-100 were measured on each fraction with RIAs using antibodies MB3 and

MB47, respectively. Recovery of apo B in the top and bottom fractions averaged 85%. Identical studies were performed in parallel with plasma pooled from normolipidemic control subjects.

Characterization of HDL isolated from H.J.B. To determine the size of the lipoprotein particles in the HDL density fraction, we performed gradient PAGE under nondenaturing conditions using commercially available gradient polyacrylamide gels (PAA 4/30 gels, Pharmacia Fine Chemicals, Piscataway, NJ), as described (27, 28). High molecular weight markers (Pharmacia Fine Chemicals) were included on one lane of each gel. Gels were stained using a silver stain kit (National Diagnostics, Summerville, NJ) according to manufacturer's instructions. In some cases these gels were electrophoretically blotted onto nitrocellulose membranes for Western blot analysis. Transfer of the size-separated lipoproteins was performed at 4°C at 150 V for 18 h, as previously described (22).

To determine the peak density of particles containing apo A-I and apo B-37 in the HDL density range, we used discontinuous salt gradient ultracentrifugation. This technique is similar to that previously described by Teng and co-workers (29). HDL preparations from three subjects with apo B-37 (H.J.B. and subjects 2 and 18 [see following article, Fig. 1]) and HDL preparations from two control subjects were analyzed in this manner in two separate experiments. 5 mg of the HDL fractions was adjusted to 2 ml with NaBr density solution to yield a density of 1.21 g/ml, then layered on the bottom of a SW40 polyallomer tube using an Auto-Densiflow gradient loader (Hakke-Buchler Instruments, Inc., Saddle Brook, NJ). We then overlaid 2.25 ml of a $d = 1.168$ g/ml solution, 2.25 ml of a $d = 1.126$ g/ml solution, 2.25 ml of a $d = 1.085$ g/ml solution, 2.25 ml of a $d = 1.070$ g/ml solution, and a 1.25 ml of a $d = 1.045$ g/ml solution. All solutions were at 4°C. The tubes were then spun at 38,000 rpm for 40 h at 10°C in a SW40 rotor. Using the Auto-Densiflow device, gradients were unloaded into 13 separate fractions. The density of each fraction was measured with a densitometer (DMA 45 Digital Density Meter, Anton Parr, Graz, Austria). Preliminary studies demonstrated that the salt gradients were highly reproducible from one experiment to another. After dialysis of each of the fractions against PBS, the apo B content of each fraction was measured by competitive RIA using monoclonal antibody MB3. In one of the experiments, antibody MB19 was also used. Apo A-I content of each fraction was determined in the laboratory of Dr. Richard Smith using apo A-I-specific monoclonal antibodies (27) and an immunoenzymometric assay.

Analytical ultracentrifugation studies on H.J.B. plasma were performed by Dr. Ronald Krauss of the Donner Laboratory, Berkeley, CA, according to published techniques (30).

Results

Human subjects and plasma samples. In this article, we characterize an abnormal apo B species in the lipoproteins of H.J.B., the proband of a kindred with familial hypobetalipoproteinemia, as well as in the lipoproteins of several other affected family members. Every subject whose lipoproteins contained the abnormal apo B species had hypobetalipoproteinemia. Lipoprotein concentrations of each family member, a family tree, and a genetic analysis of the family is found in the following article (18). A clinical description of H.J.B., plasma lipoprotein concentrations of several of his immediate family, as well as metabolic studies on H.J.B. were previously reported by Steinberg et al. (17).

Apo B species in H.J.B. lipoproteins. By analytical SDS-PAGE, and by Western blot techniques (see below), an abnormal apo B species was found in all H.J.B. lipoprotein fractions: chylomicrons, VLDL, IDL, LDL, and HDL. The molecular weight of this abnormal band was determined from carefully calibrated gels (as outlined in the Methods) and found to be 203,000. Using the nomenclature for apo B introduced by Kane et al. (1), the abnormal species was designated apo B-37. Apo B-37 was confined to the lipoprotein fraction of plasma. In RIAs using monoclonal antibodies MB3 or MB47, or an RIA using a polyclonal antiserum against LDL apo B-100, no apo B was detected in the $d = 1.21$ g/ml bottom fraction of H.J.B. plasma.

In addition to apo B-37, the VLDL, IDL, and LDL of H.J.B. contained apo B-100 and B-48. Apo B-100 and apo B-48 of H.J.B. lipoproteins had the same molecular weight (i.e., the same mobility of SDS-PAGE) as apo B-100 and apo B-48 of normal subjects. Apo B-37 migrated as a distinct band below apo B-48 (Fig. 1, A and B). Apo B-37 was the only apo B species in H.J.B. HDL. Apo B-37 was also found in eight other members of this kindred spanning three generations. A stained gel demonstrating apo B-37 in the VLDL of subjects 2 and 18 (a brother and nephew of H.J.B.; see following article) is shown in Fig. 2. Apo B-37 was never observed in the lipoproteins of normal control subjects, even when the lipoproteins were isolated in the absence of inhibitors and in a nonsterile manner.

Figure 1. Abnormal apo B species, apo B-37, in H.J.B. lipoproteins. (A) A 3–15% SDS-polyacrylamide slab gel stained with Coomassie Blue. 50 μ g of delipidated lipoprotein was loaded onto each lane. (Lane 1) Chylomicrons isolated from a normal subject; (lane 2) H.J.B. VLDL; (lane 3) HDL from a normal subject; (lane 4) H.J.B. HDL. The “apo B-90” band in H.J.B. VLDL was present in variable amounts in different preparations of H.J.B. VLDL. It was not observed in the VLDL of other members of the H.J.B. kindred whose lipoproteins contained apo B-37. We presumed it to be a minor breakdown product of apo B-100. (B) A separate 3–15% polyacrylamide slab gel stained with Coomassie Blue. (Lane 1) Normal HDL; (lane 2) H.J.B. HDL; (lane 3) normal LDL isolated in the absence of proteolytic inhibitors; (lane 4) normal chylomicrons. The H.J.B. HDL shown in B was isolated from a separate sample of H.J.B. plasma.

Figure 2. Demonstration of apo B-37 in VLDL of two members of the H.J.B. kindred. The apoproteins of delipidated VLDL preparations were separated on 3–15% SDS-polyacrylamide slab gels. 50 μ g of protein was loaded onto each lane. (Lane 1) VLDL isolated from a normal individual; (lane 2) VLDL from subject 2 (see following article [18]); (lane 3) VLDL of subject 18.

We considered the possibility that apo B-37 was the result of an unusual protease(s) present in H.J.B. plasma. To test this possibility, 125 I-LDL isolated from a normal individual was mixed with fresh H.J.B. plasma to which no inhibitors had been added. The lipoproteins were then ultracentrifugally isolated from the plasma and analyzed by SDS-PAGE. Apo B-37 was present on the stained gel. However, an autoradiogram demonstrated that no apo B-37 was generated from the 125 I-LDL during the incubation with H.J.B. plasma (data not shown).

Because multiple proteolytic inhibitors were immediately added to the plasma, and because the lipoproteins were isolated in a sterile manner, proteolytic breakdown products such as apo B-74 and B-26 were very rarely observed on stained gels or autoradiograms. A faint apo B band ("apo B-90") was observed immediately below the B-100 band in several preparations of H.J.B. VLDL (see Fig. 1 A, lane 2), but its amount appeared to be variable in the different preparations of H.J.B. lipoproteins. This band, which we presumed to be a minor degradation product, was not observed in other family members with apo B-37.

Characterization of the apo B-37 protein. To document that the band identified as apo B-37 was indeed an apo B species and to map it in relationship to apo B-100, apo B-48, and the proteolytic fragments of apo B-100, we utilized a panel of 18 apo B-specific monoclonal antibodies. These antibodies were characterized for their ability to bind to (a) apo B-100 and apo B-48 of chylomicrons isolated from a normal subject, (b) apo B-37 contained in H.J.B. lipoproteins, and (c) the thrombin fragments of apo B-100 (Table I). Knott et al. (31) have established that thrombin fragment T4 is the amino-terminal fragment of apo B-100, and that T2 is the carboxyl-terminal fragment, while T3 is in the middle of the molecule. Thrombin fragment T1 contains both T4 and T3.

All of the monoclonals used had been selected because of their ability to bind to apo B-100 (22, 24, 25). 10 antibodies (class A—see Table 1) bound to thrombin fragment T4. Each of these antibodies also bound to apo B-37, as well as to apo B-48. For example, Fig. 3, A and B demonstrates the binding of two T4-specific antibodies, MB3 and MB19, to apo B-48 and

Table I. Characterization of Apo B-specific Monoclonal Antibodies

Monoclonal antibody	B-100 thrombin fragments				Apo B-48	Apo B-37
	T1	T2	T3	T4		
Class A						
MB1	×			×	×	×
MB2	×			×	×	×
MB3	×			×	×	×
MB11	×			×	×	×
MB18	×			×	×	×
MB19	×			×	×	×
MB20	×			×	×	×
MB24	×			×	×	×
MB16	×			×	×	×
OXB1	×			×	×	×
Class B						
2D8	×		×		×	×
Class C						
MB44	×		×			
MB6	×		×			
MB8	×		×			
MB7	×		×			
MB10	×		×			
Class D						
MB47		×				
MB43		×				

Binding of apo B-100-specific monoclonal antibodies to apo B-48 of normal chylomicrons and to apo B-37 in H.J.B. lipoproteins was determined by Western blot analysis. Binding of monoclonal antibodies to the thrombin fragments was determined by solid-phase RIA and was confirmed in certain cases by Western blot analysis. In this table, × designates binding of the monoclonal antibody to the thrombin fragments, apo B-48, or apo B-37. Class A antibodies bind to thrombin fragment T4. (Thrombin fragment T1 includes T3 and T4.) Class B antibodies bind to thrombin fragment T3 and to apo B-48. Class C antibodies bind to thrombin fragment T3, but do not bind to apo B-48. Class D antibodies bind to thrombin fragment T2. Antibodies MB1, MB2, and MB19 detect a common form of genetic polymorphism in apo B-100 and apo B-48 (9). Antibodies MB1, MB2, MB3, MB11, MB18, MB19, MB20, and MB24 do not block binding of LDL to the cellular LDL receptor. When added in excess, antibodies MB43 and MB44 block ~ 50% of LDL uptake; MB47 blocks 100% of LDL uptake (25).

apo B-37. These data suggest that apo B-37 contains amino-terminal domains of apo B-100.

Monoclonal antibodies MB47 and MB43 (class D) bound to thrombin fragment T2, suggesting that their epitopes were in the carboxyl-terminal region of apo B. They did not bind to apo B-48, nor did they bind to apo B-37 (Fig. 3 C).

To further map apo B-37 in relation to apo B-100 and B-48, we utilized six monoclonal antibodies that bound to thrombin fragment T3, which is in the middle of the apo B-100 molecule. Only one of the six T3-specific antibodies, 2D8 (class B), bound to apo B-37 or apo B-48 (Fig. 4 A). This antibody, supplied to us by Drs. Milne and Marcel, is known to bind to T3 relatively near the T4-T3 junction (personal communication from Dr. Ross Milne). The other five T3-specific monoclonal antibodies (class C) did not bind to apo B-37 or apo B-48. Fig. 4 B dem-

Figure 3. Western blots demonstrating binding of three apo B-specific monoclonal antibodies to apo B-100, apo B-48, and apo B-37. Apo-proteins of delipidated lipoprotein preparations were separated on 3–15% SDS-polyacrylamide slab gels; Western blots were performed as previously described (10, 22). (A, B, and C) Western blots using antibodies MB3, MB19, and MB43, respectively. In each panel, lane 1 is normal chylomicrons; lane 2, H.J.B. VLDL; lane 3, normal HDL; lane 4, H.J.B. HDL. Antibodies MB3 and MB19 bind to the amino-terminal region of apo B-100 (thrombin fragment T4); antibody MB43 binds to the carboxyl-terminal region of apo B-100 (thrombin fragment T2). The apparent apo B-90 band observed in lane 2 of A and B is presumed to be a degradation product of apo B-100 (see Results and legend of Fig. 1).

onstrates the binding of one such antibody, MB6. Thus, a portion of apo B-37 must extend into the T3 region of apo B-100.

Apo B-37 in the lipoproteins isolated from subjects 2 and 18 (see following article) was tested in a similar fashion and was

Figure 4. Western blots demonstrating binding of two apo B-specific monoclonal antibodies to apo B-100, apo B-48, and apo B-37. Apo-proteins of delipidated lipoprotein preparations were separated on 3–15% SDS-polyacrylamide slab gels, and Western blots were performed as previously described (10, 22). (A) Western blot using antibody 2D8, an antibody that binds to thrombin fragment T3. This antibody was generously provided by Drs. Milne and Marcel. (Lane 1) Normal chylomicrons; (lane 2) H.J.B. HDL isolated in the absence of proteolytic inhibitors. (See Results and Fig. 5 for discussion of “apo B-11”). (B) Western blot using antibody MB6, which also binds to thrombin fragment T3. (Lane 1) Normal chylomicrons; (lane 2) H.J.B. VLDL; (lane 3) normal HDL; (lane 4) H.J.B. HDL. The apo B-90 band observed in lane 2 is presumed to be a degradation product of apo B-100 (see Results and legend to Fig. 1).

found to react with the panel of monoclonal antibodies exactly as did apo B-37 in H.J.B. lipoproteins. Thus, the monoclonal antibody-binding data suggested that apo B-37 was most likely an amino-terminal fragment of apo B-100. Amino-terminal amino acid sequence analysis of purified apo B-37 purified from H.J.B. HDL by preparative gels supported this idea. We analyzed the first 13 amino acid residues of apo B-37; they were: Glu-Glu-Glu-Met-Leu-Glu-Asn-Val-Ser-Leu-Val-Cys-Pro. This sequence of apo B-37 is identical to that reported by Protter et al. (32) for the amino-terminal sequence of apo B-100.

Though the monoclonal antibody and amino acid sequence data suggested that apo B-37 was simply an amino-terminal fragment of apo B-100, we considered the possibility that apo B-37 might nevertheless contain some domains from the carboxyl-terminal region of apo B-100. Such domains might conceivably be included in apo B-37 if apo B-37 was the product of a messenger RNA that had undergone alternative splicing. Small carboxyl-terminal domains might be overlooked by our panel of monoclonal antibodies. We considered it less likely that such a domain would be missed by a polyclonal antiserum. Therefore, using purified apo B-37 as the immunogen, we generated a rabbit antiserum to apo B-37. On Western blots, this antiserum bound to apo B-100, apo B-48, and apo B-37. It bound strongly to thrombin fragment T4, weakly to fragment T3, but it did not bind thrombin fragment T2 at all. Similarly, an antiserum against T4 bound apo B-37 strongly, an antiserum against T3 bound apo B-37 weakly, and an antiserum against T2 did not bind apo B-37 at all (data not shown). The antisera evidence therefore strongly supports the idea that apo B-37 is simply an amino terminal fragment of apo B-100.

Cardin et al. (19) have provided evidence that apo B-74 and apo B-26 of LDL are kallikrein-mediated proteolytic cleavage products of apo B-100. Apo B-26 contains the amino-terminal domain of apo B-100 whereas apo B-74 contains the carboxyl-terminal domain (32). When HDL was isolated from plasma in the presence of proteolytic inhibitors, only apo B-37 was apparent in HDL by analytical SDS-PAGE. When HDL was isolated from plasma in the absence of proteolytic inhibitors, apo B-26 and a lower molecular weight species, “apo B-11” (58,000 mol wt), were observed, in addition to the apo B-37. The apo B-26 band was evident even though no apo B-74 or apo B-100 were de-

etectable on the stained gels. We hypothesized that apo B-37 could be cleaved into apo B-26 and apo B-11 fragments in exactly the same way that apo B-100 is cleaved into apo B-26 and apo B-74 fragments. Western blot studies provided strong support for this hypothesis. H.J.B. HDL isolated in the absence of proteolytic inhibitors was run on SDS-PAGE, then transferred to nitrocellulose membranes. Monoclonal antibody MB19, which binds to thrombin cleavage product T4 and apo B-26 of LDL, bound both apo B-37 and apo B-26 of H.J.B. HDL (Fig. 5 A). Similarly, the polyclonal antiserum against T4 bound to apo B-37 and apo B-26 (Fig. 5 B). Antibody 2D8, which binds apo B-74 and T3, bound to apo B-37 and the smaller molecular weight fragment, apo B-11 (Fig. 5 C). The antiserum against T3 also bound to apo B-37 and apo B-11 (Fig. 5 D). Neither antibody 2D8 nor the T3-specific antiserum bound to apo B-26. Thus we conclude that apo B-26 and apo B-11 are complementary fragments of apo B-37, just as apo B-26 and apo B-74 are complementary fragments of apo B-100.

All of our data support the idea that apo B-37 in H.J.B. lipoproteins contains only the amino-terminal domains of apo B-100. This includes thrombin fragment T4, and a 58,000-mol wt portion of T3. The proposed relationship of apo B-37 to apo B-100, to apo B-48, to the kallikrein cleavage fragments apo B-26 and apo B-74, and to the thrombin cleavage products of B-100 is shown in Fig. 6.

Distribution of apo B in H.J.B. plasma. As noted in Table I, antibody MB47, which binds to thrombin fragment T2, does

Figure 5. Western blots demonstrating binding of apo B-specific antibodies to H.J.B. HDL. H.J.B. HDL was isolated from plasma in the absence of proteolytic inhibitors; the apoproteins were separated by electrophoresis on 3–15% polyacrylamide slab gels. Western blots were then performed, as previously described (10, 22). (A) Binding of monoclonal antibody MB19; (B) a rabbit antiserum specific for thrombin cleavage product T4 (anti-T4); (C) monoclonal antibody 2D8; (D) a rabbit antiserum specific for thrombin cleavage product T3 (anti-T3). The T4- and T3-specific antisera were provided by Dr. T. Innerarity; monoclonal antibody 2D8 was provided by Dr. Milne and Dr. Marcel.

Figure 6. Binding of monoclonal antibodies to different species of apoprotein B. We tested a panel of 18 apo B-specific monoclonal antibodies for binding to (a) apo B-26 and apo B-74 (kallikrein-mediated breakdown products of apo B-100), (b) the thrombin cleavage products of apo B-100 (T₄, T₃, T₂), (c) apo B-48 of normal chylomicrons, and (d) apo B-37 of H.J.B. lipoproteins. Binding of individual monoclonal antibodies and classification of the individual antibodies into groups A, B, C, and D is found in Table I. 10 of the 18 monoclonal antibodies (group A) bound to apo B-26, T₄, apo B-48, and apo B-37. Monoclonal antibody 2D8 (group B) bound to apo B-74, T₃, apo B-48, and apo B-37. (As illustrated in Fig. 5, this antibody bound to the apo B-11 fragment of apo B-37.) Five monoclonal antibodies (group C) bound to apo B-74 and T₃, but did not bind to apo B-48 or apo B-37. Two monoclonal antibodies (group D) bound to apo B-74 and T₂ but not to apo B-48 or apo B-37.

not bind to apo B-48 or apo B-37. We used this antibody in a solid-phase RIA to measure the concentration of apo B-100 in H.J.B. plasma, and to measure the distribution of apo B-100 in the different density fractions of his plasma. In H.J.B. whole plasma, the concentration of apo B-100 was only 0.375 mg/dl. This concentration is < 1% of that found in normal plasma and is consistent with the data presented in the following article (18) that H.J.B. is actually a compound heterozygote for familial hypobetalipoproteinemia, i.e., he has one allele leading to apo B-37, and another allele leading to greatly reduced plasma concentrations of the normal apo B species, apo B-100. 50% of the total apo B-100 in H.J.B. plasma was in the 1.006 g/ml top fraction, 75% was in the 1.025 g/ml top fraction, and > 95% was in the 1.063 g/ml top fraction.

An RIA using antibody MB3 (which binds apo B-100, apo B-48, and apo B-37) was used to measure the distribution of total apo B immunoreactivity in H.J.B. plasma. The total apo B concentration in plasma was 3 mg/dl. In contrast to the results of the RIA using antibody MB47, only 15% of the total MB3 immunoreactivity was in the $d = 1.006$ g/ml top fraction. > 75% of the total MB3 immunoreactivity was in the $d = 1.063$ g/ml bottom fraction. Because SDS-PAGE did not detect apo B-100 or apo B-48 in the $d = 1.063$ g/ml bottom fraction, all of this immunoreactivity was due to apo B-37. Thus, < 15% of the total apo B in H.J.B. plasma was apo B-100. The remainder was apo B-48 and apo B-37. In contrast to the findings with H.J.B. plasma, only 5% of the total apo B in control plasma was in the $d = 1.006$ g/ml top fraction, yet > 95% was in the $d = 1.063$ g/ml top fraction. These results for normal plasma are consistent with previous studies (26, 33).

Characterization of apo B-37 in the HDL density fraction. The SDS-polyacrylamide gels and Western blots indicated that apo B-37 was virtually the only apo B species present in the HDL density range (Fig. 1, A and B). The apo B-37 in H.J.B. HDL was easily detected in solid-phase RIAs using monoclonal antibodies capable of binding apo B-37. Fig. 7 demonstrates the ability of H.J.B. HDL, but not normal HDL, to compete with

Figure 7. Ability of H.J.B. HDL and two control HDL preparations to compete with LDL for binding to three different apo B-specific monoclonal antibodies in a solid-phase RIA. The RIAs were performed exactly as previously described (9, 25). 96-well microtiter plates were coated with LDL-apo B isolated from a normal control subject. Remaining binding sites were blocked with BSA. Increasing concentrations of competitor and a fixed concentration of monoclonal antibody were then added to the wells. After an overnight incubation, the plates were washed, and binding of the monoclonal antibody to the immobilized LDL-apo B was assessed with ^{125}I -goat anti-mouse Ig. In these

studies, we tested the ability of four lipoprotein preparations to compete with the immobilized LDL-apo B for binding to the monoclonal antibodies: normal LDL isolated from a control subject (*open squares*); H.J.B. HDL (*solid circles*); HDL isolated from a control subject (*solid triangles*); HDL isolated from a second control subject (*solid squares*). Each competitor was added to the assay on the basis of its protein content, which was determined chemically (21). (A) Ability of these competitors to bind antibody MB19; (B) antibody MB3; (C) antibody MB47.

normal LDL for binding to monoclonal antibodies MB19 and MB3. Because antibody MB47 does not recognize apo B-37 (Table I), it was not surprising that H.J.B. HDL did not compete with LDL for binding to antibody MB47.

Several studies were performed to characterize the apo B-37 lipoprotein particle in the HDL density range. First, the electrophoretic mobility of H.J.B. HDL was analyzed in 1% agarose gels. After lipid staining, normal HDL appeared as one broad band, whereas H.J.B. HDL had two. Like normal HDL, the predominant band in H.J.B.-HDL had alpha mobility (Fig. 8 A). However, unlike normal HDL, there was a second broad band in the beta and pre-beta region. To test this hypothesis that this band was due to an apo B-37-containing lipoprotein, we performed a Western blot of the gel, using antibody MB19, which binds to apo B-37. Normal HDL contained only a trace of apo B-immunoreactive material in the beta and pre-beta region, whereas H.J.B. HDL had a significant amount (Fig. 8 B).

The position of this apo B band on the autoradiogram corresponded to the abnormal band observed with the lipid stain. Antibody MB47 (which does not bind to apo B-37) did not detect this band (data not shown).

We also analyzed the size distribution of H.J.B. HDL and control HDL preparations on nondenaturing 4–30% gradient polyacrylamide gels (Fig. 9). Like normal HDL, H.J.B. HDL contained a broad band of smaller particles that migrated far into the gel. However, in addition, the H.J.B.-HDL contained a second broad band of larger particles. This second broad band was never observed in normal subjects. Although the particles in this second band were larger than normal HDL particles, they were still significantly smaller than the particles found in normal LDL. Western blots were used to analyze the apoprotein content of the different sized particles in normal and H.J.B. HDL. As expected, only the smaller particles in both H.J.B. and normal HDL bound to a monoclonal antibody specific for apo A-I (Fig.

A. Lipid Stain

B. Western Blot-MB19

Figure 8. Analysis of H.J.B. HDL and control HDL by electrophoresis on 1% agarose gels. In this experiment, we used 1% agarose gels and the Corning electrophoresis system, as described in Methods. Electrophoresis of 0.001-ml samples was performed exactly according to manufacturer's instructions. (A) Gel stained for lipid with fat red 7B. (Lane 1) H.J.B. plasma. As explained in the following article (18), the concentration of LDL in H.J.B. plasma is markedly reduced and the concentration of HDL in plasma is moderately reduced. (Lane 2) Plasma from a normolipidemic control subject; (lane 3) H.J.B. HDL, 15 mg/ml; (lane 4) control HDL, 15 mg/ml; (lane 5) normal LDL, 3 mg/ml. (B) Western blot of a 1% agarose gel, using antibody MB19. Transfer of the lipopro-

teins from the gel to a sheet of nitrocellulose membrane was accomplished by laying a moist sheet of nitrocellulose membrane on the gel for 18 h at 4°C. The Western blot procedure was then performed exactly as previously described (10, 22). (Lane 1) Plasma from a normolipidemic control subject; (lane 2) VLDL from a control subject, 1 mg/ml; (lane 3) LDL from a control subject, 3 mg/ml; (lane 4) HDL from a control subject, 15 mg/ml; (lane 5) H.J.B. HDL, 15 mg/ml.

Figure 9. Analysis of H.J.B. HDL and control HDL on 4–30% gradient polyacrylamide gels. Pharmacia Fine Chemicals (PAA 4/30) gels were used, and the gels were run under nondenaturing conditions (i.e., absence of SDS), exactly as previously described (27, 28). The gel shown was stained with silver, as described in Methods. 10 μ g of each sample was loaded on the gel. (Lane 1) Normal LDL isolated from a control subject; (lane 2) H.J.B. HDL; (lane 3) HDL isolated from a control subject. The position of Pharmacia Fine Chemicals high molecular weight markers is indicated by arrows. K, thousands.

10 C). The larger particles in H.J.B. HDL bound to monoclonal antibody MB19 (Fig. 10 B), but not antibody MB47 (Fig. 10 A). Identical data were obtained with the HDL fractions isolated from two other family members having apo B-37, subjects 2 and 18 (data not shown). Thus, the apo B-37-containing particles in HDL, designated “Lp-B37,” are much larger than apo A-I-containing HDL particles.

To determine the density spectrum of the apo A-I-containing particles and Lp-B37 particles in HDL, we used discontinuous

Figure 10. Western blots analysis of H.J.B. HDL and control HDL on 4–30% gradient polyacrylamide gels. Pharmacia Fine Chemicals (PAA 4/30) gels were used, and the gels were run under nondenaturing conditions (i.e., absence of SDS), exactly as previously described (27, 28). 10 μ g of each sample was loaded onto the gels. The size-separated lipoprotein particles were electrophoretically transferred to sheets of nitrocellulose membrane, and Western blots were performed exactly as previously described (10, 22). For each panel, lane 1 is LDL isolated from a normal control subject; lane 2, H.J.B.-HDL; lane 3, HDL isolated from a normal control subject. (A) Binding of monoclonal antibody MB47. The faint binding observed in lane 2 was observed only after a prolonged exposure of the autoradiogram, and we presume it was nonspecific binding, in that MB47 did not bind to apo B-37 (Table I). MB47 binding to H.J.B. HDL was not observed in subsequent experiments. (B) Binding of antibody MB19; (C) binding of monoclonal antibody A-I-10, an apo A-I-specific antibody.

Figure 11. Density spectrum of apo A-I and apo B immunoreactivity in H.J.B. HDL. 5 mg of H.J.B. HDL was subjected to discontinuous salt gradient ultracentrifugation, as described in Methods. The salt gradients were then unloaded into 13 fractions. The density of each fraction was determined using a densitometer. The fractions were then dialyzed against PBS. The apo B content of each fraction was determined by RIA (10, 25). In the experiment shown here, the apo B content was determined in an assay using MB3 (solid triangles) and an assay using MB19 (solid circles). The apo A-I content (empty circles) was determined in a monoclonal antibody-based immunoassay. Exactly the same results were obtained in two separate experiments. HDL samples obtained from two other family members with apo B-37, subjects 2 and 18 (see following article), yielded identical results. Control HDL preparations yielded an apo A-I peak; no apo B was detectable in any of the 13 fractions.

salt gradient ultracentrifugation. These studies indicated that the apo A-I particles and Lp-B37 particles in H.J.B. HDL had essentially the same peak hydrated density, ranging from 1.10 to 1.15 g/ml (Fig. 11). HDL preparations from two other subjects with apo B-37 (subjects 2 and 18) yielded identical results. Apo B was not detectable in the density gradient fractions from normal HDL. Thus, both the apo A-I particles and the apo B-37 particles in HDL were quite dense. Consistent with this observation, HDL₃ (the most dense HDL subfraction), but no HDL₂ (a more buoyant subfraction), was found on analytical ultracentrifugation of H.J.B. plasma (data not shown).

Discussion

In 1979, Steinberg and colleagues (17) reported a unique kindred with hypobetalipoproteinemia. Affected subjects from this kindred were characterized by severe hypobetalipoproteinemia and normotriglyceridemia. Interestingly, H.J.B. and two of his siblings with markedly reduced LDL cholesterol levels were free of any adverse clinical consequences of low LDL levels. In the original investigations, the etiology of the low apo B levels was not elucidated.

In a recent reexamination of this kindred, we discovered the presence of an abnormal apo B species present in the plasma of H.J.B. and other members of this kindred. This report represents the first description of the existence of an abnormal apo B species in humans. In this article, we characterize the properties of the

abnormal apo B species, apo B-37. In the following article (18), we demonstrate that apo B-37 is the product of one of two apo B alleles, and we show that the presence of this abnormal apo B-37 allele is invariably associated with hypobetalipoproteinemia. We also document the existence of a second abnormal apo B allele, which is associated with markedly reduced plasma concentrations of apo B-100. We trace the inheritance of both abnormal apo B alleles over three generations in the H.J.B. kindred.

Apo B-100 is particularly subject to proteolytic degradation, both by specific enzymes (19) and as the result of oxidative processes (34, 35). We initially considered the possibility that apo B-37 was merely an artifact produced during the isolation of the lipoproteins. However, several lines of evidence indicate that apo B-37 is not an artifact. First, proteolytic inhibitors were immediately added to all plasma samples, and the lipoproteins were prepared in a strictly sterile manner. In our experience, these precautions greatly reduce proteolytic breakdown products of apo B. In spite of these precautions, apo B-37 consistently appeared as a major apo B band in lipoproteins of H.J.B., the proband, as well as in lipoproteins of other family members with hypobetalipoproteinemia. Apo B-37 was never found in the lipoprotein fractions of control subjects. We briefly considered the possibility that an enzyme in H.J.B. plasma might cleave normal apo B-100, producing apo B-37. However, when we incubated normal ^{125}I -labeled-LDL with fresh H.J.B. plasma, no apo B-37 was produced. A third line of evidence indicating that apo B-37 is not an artifact produced during lipoprotein isolation is that this apolipoprotein was found on lipoprotein particles that have no other apo B fragments and have properties distinct from those of particles containing apo B-100 or apo B-48. Apo B-37 was the sole apo B species on a lipoprotein particle found in the HDL density range. Perhaps the strongest argument against the notion that apo B-37 is an artifact produced during lipoprotein isolation is our genetic studies on the H.J.B. kindred (18), which demonstrate that apo B-37 is the sole apo B product of one of the two apo B alleles and that the abnormal allele is transmitted vertically over three generations.

Here, we show that the abnormal apo B species, apo B-37, is an amino-terminal fragment of apo B-100. The first 13 residues of the amino-terminal amino acid sequence were the same as that reported for apo B-100 (32), and the data derived from the panel of 18 monoclonal antibodies suggested that apo B-37 does not contain domains from the carboxyl-terminal region of apo B-100. All monoclonal antibodies that bound to T4, an amino-terminal fragment of apo B-100, bound to apo B-37. Antibody 2D8, which binds T3 near the T4-T3 junction, bound to apo B-37, but none of the other T3-specific or T2-specific monoclonal antibodies bound to apo B-37. These data, together with the other immunochemical studies with the polyclonal antisera to apo B-37 and to the individual thrombin fragments, strongly support the idea that apo B-37 contains only amino-terminal domains of B-100.

Because apo B-37 contains only amino-terminal domains of apo B-100, it is tempting to speculate that apo B-37 is the result of a point mutation or an insertion or deletion leading to a termination signal on one of the apo B alleles. Alternatively, it is possible that a point mutation might uncover a site for proteolytic cleavage of apo B-100. If this were the case, one might expect to find evidence for the complementary carboxyl-terminal apo B fragment, an "apo B-63 fragment." We found no evidence for such a fragment. However, our failure to find such a com-

plementary carboxyl-terminal fragment cannot exclude this possibility, in as much as the proteolytic cleavage might occur intracellularly, and/or the carboxyl-terminal fragment might be rapidly degraded (either intra- or extracellularly).

We have demonstrated that the normal apo B species, apo B-100 and apo B-48, are the products of a single apo B gene (10). To date, the mechanism of the formation of the lower molecular weight species, apo B-48, is not completely understood. However, it is conceivable that a defect in the "apo B-37 allele" could interfere with this mechanism, leading to the formation of apo B-37, rather than apo B-48. If this were the case, one would have to postulate that such a defect in the apo B-37 allele also blocked the formation of apo B-100, in that studies presented in the following article (18) indicate that apo B-37 is the sole product of one of the apo B alleles. Knowledge of the complementary DNA (cDNA) sequence and the deduced amino acid sequence of normal apo B-100 is now emerging from several laboratories (31, 32, 36–38). Studies to determine the molecular basis of the abnormality in the allele encoding for apo B-37 are currently underway.

Although apo B-37 shares some properties of apo B-48 and apo B-100, it clearly has its own distinct properties. Like apo B-100 and apo B-48, apo B-37 was found in the VLDL density range, suggesting that it retains at least some capacity to have a structural role in a buoyant, triglyceride-rich lipoprotein particle. Normally, apo B-48 is found only in chylomicrons, with a trace amount extending into the VLDL and IDL density classes. In normal subjects, ~ 5% of apo B-100 is found in VLDL, with nearly all of the remainder in the IDL and LDL density range (26, 33). A small amount of apo B-100 can also be found in Lp(a) particles in the 1.070–1.090 g/ml density range (39, 40). Unlike the normal distribution of apo B, > 75% of the apo B in H.J.B. plasma was in the HDL density fraction. All of the apo B in H.J.B. HDL was apo B-37. In HDL, apo B-37 was found on a distinct lipoprotein particle, Lp-B37, that apparently contains little, if any, apo A-I, and had a peak density of 1.12–1.15 g/ml (i.e., the HDL₃ density range). Thus, Lp-B37 particles have distinct properties different from those of particles containing either apo B-100 or apo B-48.

Recently, we have been able to purify Lp-B37 particles from H.J.B. HDL using immunoaffinity columns (Young, S. G., F. Peralta, and J. L. Witztum, unpublished observations). Studies to determine the physical and chemical characteristics of these particles are now underway. In particular, isolation of these naturally occurring particles should enable us to determine whether apo B-37 retains the capacity to bind to the LDL-receptor and/or the ability to be cleared by the chylomicron remnant pathway.

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Note added in proof. Recently, we tested an apo B-specific monoclonal antibody, D7.2, developed by and generously provided to us by Dr. Y. Kleinman and Dr. G. Schonfeld of Washington University School of Medicine in St. Louis. This unique antibody binds to thrombin fragments T1 and T3, and to apo B-48. In this regard, it is similar to antibody 2D8 (Table I and Fig. 6). However, unlike antibody 2D8, antibody D7.2 does not bind to apo B-37. It therefore seems likely that antibody D7.2 binds to an epitope located between the carboxyl terminus of apo B-37 and the carboxyl terminus of apo B-48 (see Fig. 6).

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