Purification of the apolipoprotein B-67-containing low density lipoprotein particle and its affinity for the low density lipoprotein receptor

Francine K. Welty,[1] Leo Seman, and Frances T. Yen*

Lipid Metabolism Lab, Jean Mayer USDA Human Nutrition Center on Aging at Tufts University Boston, MA 02215, and INSERM U391,* Faculte des Sciences Pharmaceutiques et Biologiques, Universite de Rennes I, France

Abstract Naturally occurring mutant forms of apolipoprotein B (apoB)-100 may be able to provide valuable information on the structure-function relationships of apoB with the low density lipoprotein (LDL) receptor. ApoB-67, recently identified in a kindred displaying apoB levels 25% of normal (Welty et al. *J. Clin. Invest.* 1991. **87:** 1748–1754), is predicted to contain 3040 amino acids and therefore, contains part of the epitope for antibody 4G3, which blocks binding of LDL to the LDL-receptor. To determine whether the amino terminal 67% of apoB-100 is important for binding to the LDL receptor, the apoB-67-containing lipoprotein particle was purified from plasma by gradient ultracentrifugation. The fractions containing apoB-67 were in the density range 1.049–1.070 g/ml. These fractions were pooled and adsorbed onto an affinity chromatography column containing the monoclonal antibody, MB-47. The epitope for MB-47 is two nonlinear domains between amino acids 3429 to 3453 and 3507 to 3523; therefore, apoB-100 will bind to the MB-47 column but apoB-67 will not. The resulting apoB-67-containing particles were completely devoid of apoB-100. In competitive binding studies, the apoB-67 lipoprotein particle did not compete with ^{125}I-labeled apoB-100-containing LDL particles for binding, uptake, or degradation by normal human fibroblast monolayers. We conclude that the amino terminal 67% of apoB-100 in the naturally occurring lipoprotein particle does not appear to contain a functionally relevant epitope of the LDL-receptor binding domain.—**Welty, F. K., L. Seman, and F. T. Yen.** Purification of the apolipoprotein B-67-containing low density lipoprotein particle and its affinity for the low density lipoprotein receptor. *J. Lipid Res.* 1995. **36:** 2622-2629.

Supplementary key words apolipoprotein B • binding, competitive • hypolipoproteinemia • lipoprotein, LDL • receptor, LDL

Apolipoprotein (apo) B plays a central role in lipoprotein metabolism and exists in two isoforms in plasma, apoB-100 and apoB-48 (1), both of which are products of the same structural gene on chromosome 2 (2). ApoB-100 consists of a single 4536 amino acid polypeptide chain; apoB-48 consists of the amino terminal 48% of apoB-100 (3). ApoB-100 is synthesized by the liver and secreted in the form of very low density lipoprotein (VLDL) which is remodeled in plasma to form low density lipoprotein (LDL). ApoB-100 contains the LDL-receptor binding domain; therefore, VLDL remnants (intermediate density lipoprotein) and LDL are removed from the circulation by binding to specific hepatic LDL receptors (1). ApoB-48 is synthesized in the intestine and produced as a result of a premature stop codon at the apoB-100 codon 2153 by tissue-specific mRNA processing (4). ApoB-48 is secreted in the form of chylomicrons. As apoB-48 presumably lacks an LDL-receptor binding domain, chylomicron remnants are most likely taken up by the liver through receptors other than the LDL-receptor, e.g., the putative chylomicron remnant receptor that recognizes apoE (5, 6).

As a result of its lipophilic nature, large size (molecular weight of 550,000) and hydrophobicity, apoB-100 is very difficult to solubilize and, therefore, difficult to study. The knowledge of the primary structure of apoB-100 by sequencing has allowed the identification of structural and functional domains. Using a large panel of monoclonal antibodies, Pease et al. (7) concluded that the receptor binding region of apoB-100 lies between amino acids 2835 and 4081. Additional studies with monoclonal antibodies have identified two putative areas for the apoB receptor-binding domain at residues 3120–3156 and 3352–3371 (8–12). However, other areas of apoB may also be involved in binding to the LDL

Abbreviations: apo, apolipoprotein; DMEM, Dulbecco's modified Eagle's medium; VLDL, very low density lipoproteins; LDL, low density lipoprotein; HDL, high density lipoprotein; LpB-67, lipoprotein particles containing apoB-67; LDL-B-100, low density lipoprotein particles containing apoB-100; PAGE, polyacrylamide gel electrophoresis.

[1]To whom correspondence should be addressed.

Downloaded from www.jlr.org by guest, on June 18, 2012

ASBMB JOURNAL OF LIPID RESEARCH

receptor. Studies with monoclonal antibodies directed against epitopes between amino acids 1480–1693 and 2152–2377 of apoB-100 can block binding of apoB-100 to the LDL receptor, and antibodies with epitopes between amino acids 4082–4306 can increase binding of apoB-100 by threefold (13).

Mutant forms of apoB-100 can provide valuable information on the structure–function relationships of apoB, especially with the LDL receptor. Familial defective apoB-100 is due to an arginine to glutamine substitution at amino acid 3500 (14, 15). Recent ^{13}C NMR data suggest that this substitution induces a change in the local conformation that disrupts the receptor binding domain of apoB-100 and virtually abolishes the binding ability of LDL (16).

Truncated forms of apoB-100 may also be able to provide information on structure–function relationships of apoB-100. Some cases of familial hypobetalipoproteinemia, an autosomal codominant disorder characterized by low plasma concentrations of apoB and LDL-cholesterol, have been shown to be due to a truncated form of apoB-100 (17). Welty et al. (18) reported a kindred with hypobetalipoproteinemia due to deletion of an adenine at cDNA 9327; the mutation results in the production of a truncated form of apoB-100 containing the amino terminal 67% of apoB-100. In this study, we successfully purified apoB-67-containing lipoprotein (LpB-67) particles from plasma of several affected family members. The ability of unlabeled LpB-67 particles to compete with ^{125}I-labeled apoB-100-containing low density lipoprotein particles (^{125}I-LDL-B-100) for cellular binding, uptake, and degradation by cultured human fibroblasts was tested. No binding, uptake, or significant degradation of LpB-67 particles was observed. Thus, the amino terminal 67% of apoB-100 in the naturally occurring lipoprotein particle does not appear to contain a functionally relevant epitope of the LDL-receptor binding domain.

MATERIALS AND METHODS

Human subjects

The apoB-67 kindred was originally described by Welty et al. (18) who found that deletion of an adenine at CDNA 9327 converted a lysine to an arginine followed by a premature stop codon, thus producing the apoB-67 molecule. Affected subjects are heterozygous for the mutation. A family tree with each family member identified by a number and characterized according to apoB genotype has been published (18). [This study was approved by the Institutional Review Board at Tufts University, and informed consent was obtained.]

Plasma lipid and lipoprotein characterization

Sixty ml of fresh blood was obtained from four family members whose lipoproteins contained apoB-67: subjects II-2, II-5, II-10, and II-12 as previously identified (18). Blood was also obtained from normolipidemic subjects (unrelated to the apoB-67 subjects). Blood was collected into sterile tubes containing EDTA (0.1% final concentration). Plasma was immediately separated from red cells in a refrigerated centrifuge at 3,000 rpm for 30 min at 4°C. The following proteolytic inhibitors were then added to the plasma: probucol 0.1% (Sigma), sodium azide 0.1 mM, and aprotinin (Trasylol FBA) 2,000 kallikrein units per 10 ml of plasma. Plasma and lipoprotein fractions were assayed for total cholesterol and triglyceride with an Abbott Diagnostics ABA-200 bichromatic analyzer using enzymatic reagents (19, 20). Lipid assays were standardized through the Centers for Disease Control Lipid Standardization Program.

The apoB-100-containing low density lipoprotein (d 1.025–1.055 g/ml) fraction (LDL-B-100) was isolated from pooled fresh plasma from the normolipidemic subjects by sequential ultracentrifugation (21). This was the LDL-B-100 used in the competitive binding experiments described below. An LDL fraction (d 1.019–1.063 g/ml) was also isolated by sequential ultracentrifugation from plasma of one of the apoB-67 subjects. This was used in the SDS-(Gibco BRL, Gaithersburg, MD) polyacrylamide gel electrophoresis (PAGE) to illustrate the large excess of apoB-100 compared to apoB-67 in the LDL fraction of apoB-67 subjects.

Lipoprotein B-67 isolation and characterization

A gradient ultracentrifugation was performed on each of the plasma samples from the apoB-67 subjects. The plasma was adjusted to d 1.020 g/ml with potassium bromide (KBr) (Sigma, St. Louis, MO) and placed in a 37.5-ml quick-seal tube (Beckman, Palo Alto, CA). This was underlayered with 10 ml of d 1.21 g/ml KBr solution. The plasma was then overlayered with 9 ml of normal saline (d 1.006 g/ml). Centrifugation (Beckman rotor 50.3 Ti) was performed at 65,000 rpm × 18 h at 10°C. The bottom of the tube was punctured, and 5 ml was collected and discarded. One and one-half ml fractions were then collected. The density was determined by weighing 1 ml of each fraction. Apolipoproteins B-67 and B-100 were evaluated by SDS-PAGE using a Tris-glycine buffer system and stained with Coomassie Brilliant Blue as previously described (22, 23). Estimates of apoB-100 and apoB-67 were determined by gel scanning and peak integration using an LKB Ultrascan XL laser densitometer interfaced with an AT&T personal computer (LKB), a Canon J-1080A printer (LKB) and GSXL software (LKB-Paramus, NJ).

Downloaded from www.jlr.org by guest, on June 18, 2012

ASBMB JOURNAL OF LIPID RESEARCH JLR

Fractions containing apoB-67, in the density range 1.049–1.070 g/ml, were pooled and concentrated with an Amicon Centriprep-10 or 100 concentrator (Amicon Corp., Beverly, MA). The concentrator membranes were carefully washed to assure maximum recovery of apoB-67 particles. The fractions were dialyzed against two changes of 1 liter of equilibration buffer (50 mM Tris-HCl, pH 7.5, 0.9% NaCl, 0.01% EDTA) for the MB-47 column for 3 h each and then dialyzed against 1 liter overnight. The protein concentration of the lipoprotein fractions was determined by a modification of the Lowry technique using a bovine serum albumin (BSA) standard (24).

Production and use of the anti-MB-47 immunoaffinity column

The murine monoclonal antibody, MB-47, was a kind gift from Dr. Linda K. Curtiss, Department of Immunology, Research Institute of Scripps Clinic, La Jolla, CA 92037. The epitope for MB-47 is composed of two nonlinear domains between amino acids 3429 to 3453 and 3507 to 3523 (25–27); therefore, antibody MB-47 binds to apoB-100 but not to apoB-48 or apoB-67.

Thirty-five mg of MB-47 was covalently bound to 5 g (17.5 ml) of cyanogen bromide-activated Sepharose 4B (Pharmacia Biotechnology, Inc.) according to manufacturer's instructions. A 6-ml column of MB-47-Sepharose was prepared. The capacity of the column was 1.21 mg apoB/ml Sepharose. The column was washed with equilibration buffer (50 mM Tris-HCl, pH 7.5, containing 0.9% NaCl and 0.01% EDTA). To isolate LpB-67 particles, the pooled, concentrated lipoprotein fractions from the gradient ultracentrifugation from all four affected family members were adsorbed onto the MB-47 affinity column and incubated on the column for 60 min at 4°C. The LpB-67 was recovered free of LDL-B-100 particles (as determined by SDS-PAGE) in the wash step which consisted of 6 volumes of equilibration buffer in two sequential steps. Elution of bound LDL-B-100 was then attempted with 0.01 M Tris, pH 8.0, 0.5 M NaCl for a total of 6 column volumes. However, the affinity between apoB-100 and MB-47 was strong enough to resist column washing with 3 M NaSCN, as has been previously documented (12). The eluted apoB-67 lipoprotein particles were concentrated on an Amicon Centriprep-10 or 100 concentrator, transferred to a dialysis bag, and dialyzed against 2 changes of 0.15 M NaCl and 0.24 mm EDTA for 24 h.

Characterization of the apolipoprotein content of the LpB-67 preparation

The apolipoprotein content of LpB-67 was assessed by SDS-PAGE. ApoB was assayed in plasma and lipoprotein fractions with a noncompetitive, enzyme-linked immunosorbent assay using immunopurified polyclonal antibodies (28). The coefficient of variation for the apoB assay was less than 5% within runs and less than 10% between runs (28). The apoA-I content of lipoprotein fractions was measured by a polyclonal enzyme-linked immunosorbent assay, as previously described (29).

Studies with cultured fibroblasts

Low density lipoproteins (d 1.025–1.055 g/ml) were iodinated using the modified MacFarlane technique (30). Briefly, 700 µl 1 M glycine, pH 10.0, was added to 12 mg LDL (17 mg/ml) followed by addition of 3.5 mCi Na-^{125}I (Amersham, 100 mCi/ml). Fifty µl of ICl (33 mM in 1 N HCl) was injected into this mixture, which was then incubated at 4°C for 5 min. Free ^{125}I was removed by applying the radiolabeled LDL to two sequential PD-10 columns previously equilibrated with 0.15 M NaCl and 0.24 mM EDTA, pH 7.4. The ^{125}I-labeled LDL-B-100 was dialyzed against 500 ml 0.15 M NaCl and 0.24 mM EDTA for 3 h and against 4 liters of 0.15 M NaCl and 0.24 mM EDTA overnight. Ten µl of a 100-fold dilution was counted in the gamma counter and specific activity was calculated.

Binding, uptake, and degradation were performed as described by Goldstein, Basu, and Brown (31). Briefly, normal human foreskin fibroblasts (GM08333, NIGMS human genetic mutant cell repository, Camden, NJ) were plated at a density of 300,000 cells per plate in 36-mm dishes in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum for 3–4 days. On day 5, the cells were washed, and the medium was changed to DMEM containing 5% (v/v) lipoprotein-deficient serum, pH 7.4, to up-regulate the LDL-receptor. After 48 h in DMEM containing lipoprotein-deficient serum, when the cells had reached 85–90% confluency, the binding, uptake, and degradation experiments were performed. The cells were incubated with 2.5 µg of ^{125}I-labeled LDL-B-100 (1 ml/dish) in DMEM containing 10 mM HEPES, 0.2% BSA, pH 7.4, and increasing concentrations of unlabeled LpB-67 or unlabeled LDL-B-100 at 37°C for 5 h. The cells were then placed on ice and washed 3 times with 2 ml/dish ice-cold phosphate-buffered saline (PBS) containing 0.2% BSA, pH 7.4, with 10-min incubations between the second and third wash. This was followed by two rapid washes with ice-cold PBS, pH 7.4. The cell monolayers were then incubated at 4°C for 60 min with 10 mg/ml dextran sulfate in PBS, pH 7.4 (1 ml/dish). To determine the amount of binding, the medium was removed and counted for specifically bound ^{125}I-labeled LDL. The cell monolayers were recovered in 0.1 N NaOH (1 ml/dish) and counted. This represented internalized lipoproteins. Degradation products were measured in the media after trichloroacetic acid precipitation and chloroform

ASBMB JOURNAL OF LIPID RESEARCH

Downloaded from www.jlr.org by guest, on June 18, 2012

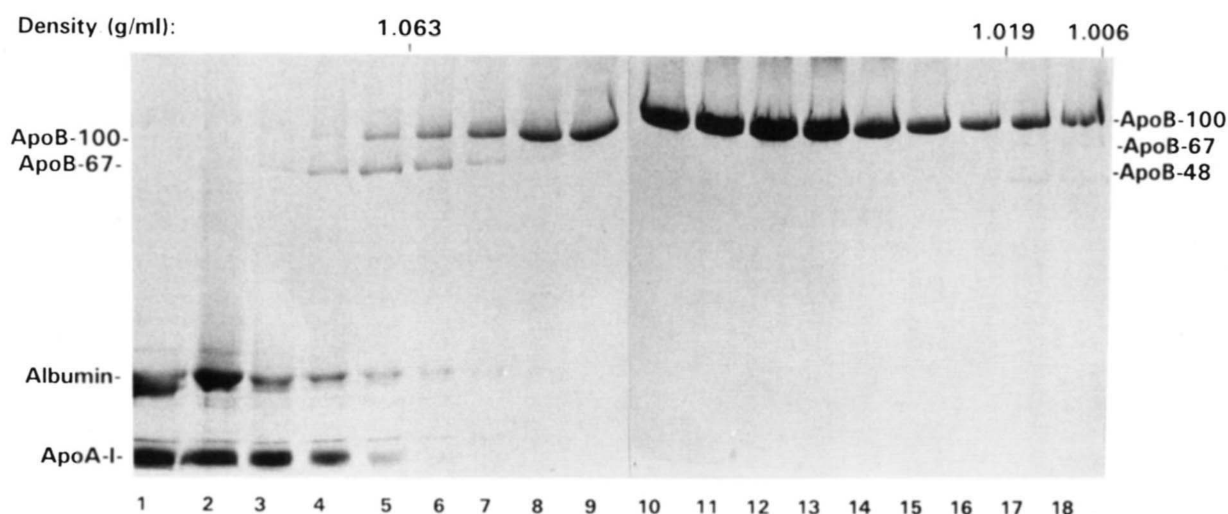


Fig. 1. SDS polyacrylamide gel electrophoresis of fractions from gradient ultracentifugation of plasma for subject II-5. Twenty ml of plasma was adjusted to d 1.020 g/ml with potassium bromide and underlayered with 10 ml of d 1.21 g/ml and overlayered with 9 ml normal saline (d 1.006 g/ml) in 37.5-ml quick-seal tubes. This was spun at 65,000 rpm × 18 h at 10°C. The bottom of the tube was punctured, the first 5 ml was discarded, and then 1.5-ml fractions were collected. Every other fraction was subjected to SDS PAGE as described under Materials and Methods.

extraction and counted for ^{125}I-labeled LDL (31). Dishes containing no cells were treated in exactly the same manner and used as controls for degradation. Data were calculated and expressed as amount of LDL protein bound, internalized, or degraded/mg of cell protein.

RESULTS

Gradient ultracentrifugation

Figure 1 shows the results of the gradient ultracentrifugation of plasma for subject II-5 and is representative of the results obtained for the other three subjects (II-2, II-10, and II-12). Scanning of the gels revealed approximately 4% of the LpB-67 in the VLDL density range (1.006 g/ml) and 96% of the LpB-67 in the LDL-HDL density range of 1.049–1.070 g/ml.

Isolation and characterization of LpB-67 by MB-47 immunoaffinity columns

To purify LpB-67 particles, an immunoaffinity column was prepared using antibodies directed against the MB-47 epitope. The pooled fractions in the density range of 1.049–1.070 g/ml from the gradient ultracentrifugation of all four subjects were circulated over an MB-47 affinity column. **Figure 2** shows the results of the SDS-PAGE of pooled fractions from the gradient ultracentrifugation (lane 2) and purified LpB-67 preparation (lanes 3 and 4). The particles not bound to the column contained only apoB-67 and no apoB-100 (Fig. 2, lanes 3 and 4), indicating that the column was effective in removing the LDL-B-100 particles from the LpB-67 particles in the pooled lipoprotein fractions. Lane 1 in Fig. 2 contains a representative LDL fraction from an apoB-67 subject and is shown to demonstrate the large excess of apoB-100 compared to apoB-67 in an LDL fraction.

The purified LpB-67 was collected in two aliquots: the first containing the first 3 column volumes (Fig. 2, lane

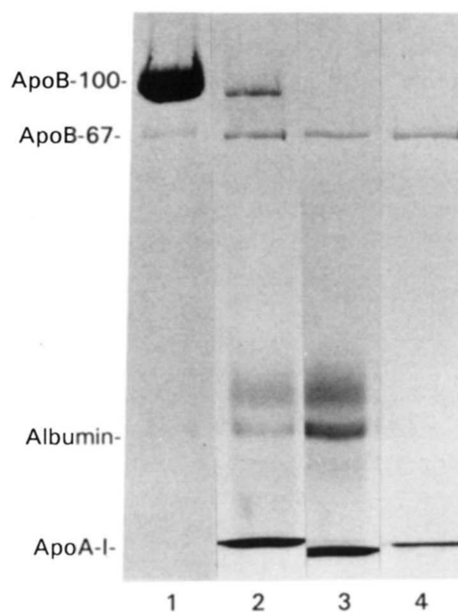
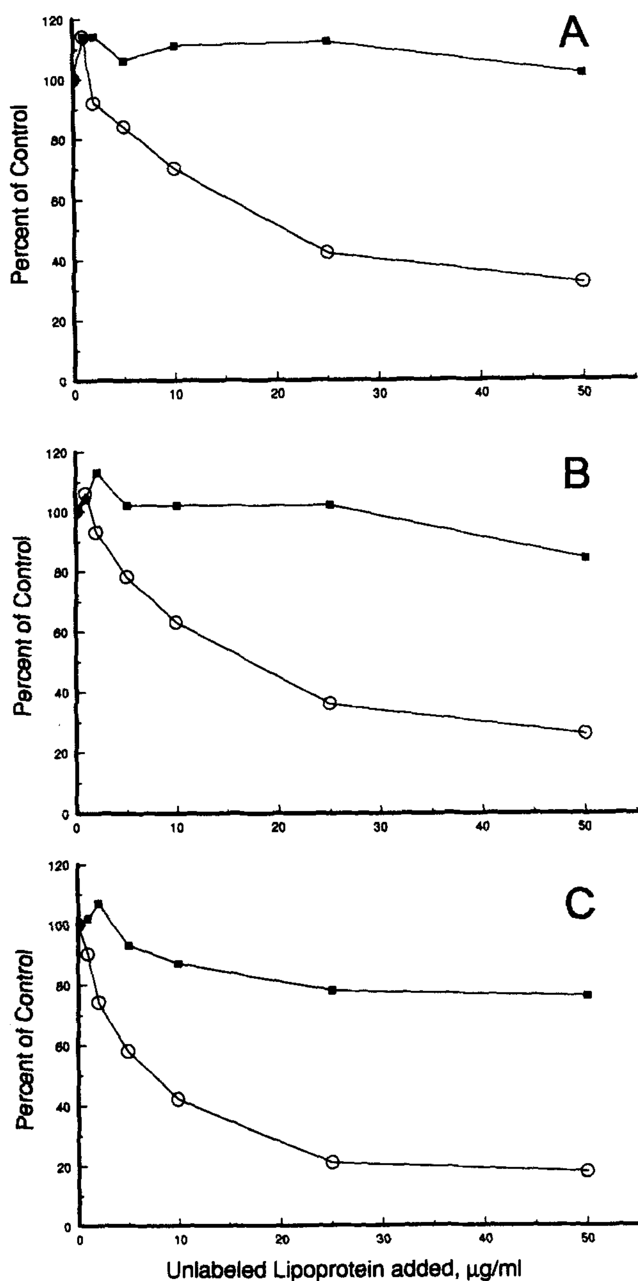


Fig. 2. SDS 4–12% polyacrylamide gel electrophoresis of fractions from the purification of apoB-67 lipoprotein particles. Lane 1 contains a representative LDL fraction from an apoB-67 subject and is shown to demonstrate the large excess of apoB-100 compared to apoB-67 in the LDL fraction. Lane 2 contains pooled fractions 4–7 from the gradient ultracentrifugation shown in Fig. 1. Lane 3 contains the first 3 column volumes from the MB47 immunoaffinity column. Lane 4 contains the last 3 column volumes from the MB47 immunoaffinity column.

Downloaded from www.jlr.org by guest, on June 18, 2012

ASBMB JOURNAL OF LIPID RESEARCH JLR

3) and the second containing the last 3 column volumes (Fig. 2, lane 4). The first 3 column volumes contained albumin whereas the last 3 column volumes had no albumin. Staining of the SDS polyacrylamide gel with Coomassie Brilliant Blue revealed an apoB-67 band and an apoA-I band. No apoB-100 or apoE was observed; this was confirmed by silver staining. The migration distances for apoB-67 and apoA-I were compared to prior studies using monoclonal antibodies to confirm the location of apoB-67 (18). The starting material (120 ml plasma) contained 1.2 mg LpB-67. Concentration of the LpB-67 particles with an Amicon filter and dialysis yielded 925 µg LpB-67. This represents a 77% recovery.



Ability of LpB-67 to compete with LpB-100 for binding, uptake, and degradation by the LDL receptor of cultured fibroblasts

The concentration of apoB-67 ranges from 0.5 to 1.0 mg/dl (1.7 to 3.4% of total apoB) in these human subjects. Even after pooling plasma from four subjects, the total yield was only 925 µg. This amount is too small to be radioactively labeled. Therefore, we chose to radioactively label the LDL from normal subjects (^{125}I-LDL-B-100) and compare purified, unlabeled LpB-67 preparations from the apoB-67 subjects and unlabeled LDL preparations (LDL-B-100) from normal subjects for their ability to compete with ^{125}I-labeled LDL-B-100 for uptake and degradation by cultured fibroblasts. The specific activity of ^{125}I-labeled LDL-B-100 was 226 cpm/ng.

Figure 3A demonstrates that LpB-67 did not compete with ^{125}I-labeled LDL-B-100 for binding to the LDL receptor even at very high protein concentrations of LpB-67 whereas excess unlabeled LDL-B-100 successfully competed with ^{125}I-labeled LDL-B-100. Uptake and degradation of ^{125}I-labeled LDL-B-100 by normal fibroblasts were also evaluated under identical conditions of competitive competition with unlabeled LpB-67 and LDL-B-100. Figure 3B demonstrates the inability of

Fig. 3. Ability of unlabeled lipoproteins B-67 (LpB-67) and unlabeled lipoprotein B-100 (LDL-B-100) to compete with ^{125}I-labeled LDL-B-100 for binding (A), uptake (B), and degradation (C) by the LDL receptor in cultured human fibroblasts. Normal human fibroblast monolayers were grown in 36-mm dishes in DMEM containing 10% fetal calf serum. Fibroblast apoB,E (LDL) receptor activity was up-regulated by a 48-h preincubation with DMEM containing 5% (v/v) lipoprotein-depleted serum. Normal human LDL was iodinated with the modified MacFarlane technique and then extensively dialyzed against 0.15 M NaCl containing 0.24 mM EDTA, pH 7.4. The specific activity of the ^{125}I-labeled LDL-B-100 was 226 cpm/ng of LDL protein. After 48 h in DMEM containing lipoprotein-depleted serum, when the cells had reached 85–90% confluency, the binding, uptake, and degradation experiments were performed. The cells were incubated with 2.5 µg of ^{125}I-labeled LDL-B-100 and increasing concentrations of unlabeled LpB-67 (squares) or unlabeled LDL-B-100 (circles) at 37°C for 5 h. The cells were washed as described in the Methods section. To determine the amount of ^{125}I-labeled LDL-B-100 bound, washed cell monolayers were incubated at 4°C for 60 min with 10 mg/ml dextran sulfate in PBS, pH 7.4 (1 ml/dish). The medium was then removed and counted for specifically bound ^{125}I-labeled LDL (panel A). The cell monolayers were recovered in 0.1 N NaOH (1 ml/dish) and counted. This represented internalized lipoproteins (panel B). Degradation products were measured as described in the Methods section (panel C). Dishes containing no cells were treated in exactly the same manner and used as controls for degradation. The data were expressed as amount of LDL protein degraded/mg of cell protein. The amounts of ^{125}I-labeled LDL-B-100 bound, internalized, and degraded in absence of competitor lipoproteins were (mean ± standard deviation, n = 4): 50.9 ± 9.4 ng/mg; 453.2 ± 41.4 ng/mg; and 2651.5 ± 245.7 ng/mg, respectively. Shown here is the percent of control binding (y axis) versus lipoprotein competitor (x axis) added to the medium.

Downloaded from www.jlr.org by guest, on June 18, 2012

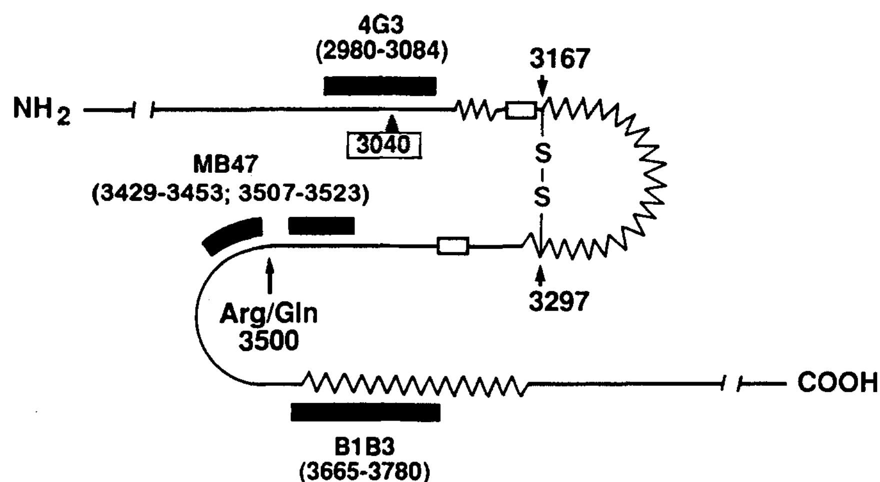


Fig. 4. The location of the carboxy-terminal amino acid of apoB-67 (amino acid 3040) in relation to the receptor-binding domain of apoB. The epitopes of monoclonal antibodies that totally or partially block binding of LDL to the LDL-receptor are indicated by solid and open boxes, respectively, above or below the polypeptide chain. ApoB-67 contains part of the epitiope for antibody 4G3. The two short stretches of positively charged amino acids are indicated by open boxes within the polypeptide chain; zigzag lines indicate regions of potential amphipathic B-sheet structure. The location of the Arg to Gln substitution associated with familial defective apoB-100 is indicated. (Modified with permission from Milne, R. et al., 1989. *J. Biol. Chem.* **264:** 19754–19760 [26]).

LpB-67 to compete with ^{125}I-labeled LDL-B-100 for uptake even at high protein concentrations of LpB-67 whereas unlabeled LDL-B-100 at 25 μg/ml reduced uptake of ^{125}I-labeled LDL-B-100 by 64%. Similarly, Figure 3C demonstrates a 79% reduction in degradation of ^{125}I-labeled LDL-B-100 in the presence of excess unlabeled LDL-B-100 whereas LpB-67 has no effect on degradation of ^{125}I-LDL-B-100 even at very high concentrations.

DISCUSSION

In this study, we were able to successfully purify apoB-67-containing lipoprotein particles completely from apoB-100- and apoE-containing lipoprotein particles. The purified apoB-67 lipoprotein particle was unable to compete with ^{125}I-labeled LDL-B-100 for cellular binding, uptake, or degradation by cultured human fibroblasts. It is important to note that the lipoproteins were added to the cell culture media on the basis of their protein concentration. At comparable protein concentrations, the molar concentration of LpB-67 particles was higher than the concentration of LDL-B-100-containing particles. In spite of the higher molar concentration of particles, there was still no binding, uptake, or degradation of LpB-67 compared to LDL-B-100.

These results add further information towards the definition of the LDL receptor binding domain of apoB-100. Based on DNA sequencing studies, apolipoprotein B-67 is predicted to contain 3,040 amino acids (18) and contains only part of the epitope for antibody 4G3 (25, 26), an antibody widely used over the past 10 years for blocking the binding of LDL to the LDL receptor (**Fig. 4**). Furthermore, apoB-67 terminates before epitopes for several other LDL receptor-blocking antibodies, 5E11, 3A10, MB47, and B1B3 (Fig. 4) and before the two short positively charged amino acid sequences that have been hypothesized to be involved in receptor binding (Fig. 4) (7–12, 26, 27). In addition, apoB-67 terminates before the missense mutation at residue 3500 that is associated with defective binding of LDL to the LDL receptor in familial defective apoB-100 (14, 15). Even though apoB-67 contains part of the epitope for antibody 4G3, the lack of these latter domains apparently accounts for the inability of the LpB-67 particle to compete with the apoB-100-containing LDL particle for binding to the LDL receptor. Whether this defective binding is due to a specific lack of receptor binding sequences or an incorrect folding remains to be established.

The present study provides a methodology that may be applied towards the purification of other truncated apoB proteins. The purification of naturally occurring truncated apoBs has been complicated by several factors. Truncated forms of apoB are usually present in very low concentration in plasma compared to apoB-100 (apoB-67 ranges from 1.7 to 3.4% of the total apoB); therefore, very large amounts of monoclonal antibodies are required to remove all the apoB-100. As we have previously shown that apoB-67-containing lipoprotein particles were smaller than apoB-100-containing lipo-

Downloaded from www.jlr.org by guest, on June 18, 2012

ASBMB JOURNAL OF LIPID RESEARCH

protein particles (18), we used a gradient ultracentrifugation as an initial step to separate LDL-B-100 and LpB-67 by density. In this manner, we were able to remove 92% of the LDL-B-100, therefore, decreasing the amount of apoB antibody required for the immunoaffinity column purification step.

The presence of multiple other apolipoproteins can also complicate the purification of truncated apoBs. A truncated apoB present in the VLDL fraction would be difficult to purify due to the presence of apoC, apoE, apoA-I, and apoA-IV, in addition to apoB-100 and apoB-48. Purification of a truncated apoB present in the HDL density range can be complicated by the presence of apoE. ApoA-I may also be present; however, this apolipoprotein should not interfere with binding to the LDL-receptor. In a previous study, it was shown that the truncated apoB form, apoB-75 (which terminates at amino acid 3387), in the density range 1.063–1.090 g/ml, was completely separated from apoB-100; however, apoE and apoA-I were present (32). The apoB-75-containing lipoprotein particles bound with greater affinity than apoB-100-containing lipoprotein particles to the LDL receptor on cultured human fibroblasts in vitro; however, the authors suggested that a possible role of apoE in mediating the enhanced binding of this particle to the LDL receptor could not be ruled out (32). The results of in vivo studies with stable isotopes, which demonstrated that apoB-75-containing lipoproteins were cleared from plasma more rapidly than apoB-100-containing lipoproteins, supports the results of the binding studies which suggested that apoB-75 bound with greater affinity than apoB-100 to the LDL receptor (32).

In summary, the results in the present study suggest that the proximal 3040 amino acids of apoB-100 are not sufficient to bind to the LDL-receptor. These results also provide confirmatory evidence for the monoclonal antibody studies which suggested that the proximal portion of the LDL-receptor recognition site is at residues 3120–3156 (8–12). The area between the termination of apoB-67 and apoB-75 (amino acids 3040–3387) contains both putative apoB receptor areas as defined by the monoclonal antibody studies (3120–3156 and 3352–3371). The lack of binding of the apoB-67 lipoprotein particle and the enhanced affinity of the apoB-75 lipoprotein particle for the LDL receptor compared to the apoB-100 lipoprotein particle suggest that this area is essential for binding of apoB-100 to the LDL receptor and may, in fact, enhance affinity for the LDL receptor. Whether this area confers appropriate conformation to the apoB particle or whether the actual sequences are necessary for binding to the LDL-receptor (or both) has not been determined. Future studies of mutant forms of apoB-100 should provide more information about the structure–function relationships of apoB-100, especially with the LDL-receptor.

We are very grateful to the apoB-67 kindred for their continued interest and participation in this research study. We are very grateful to Dr. Linda K. Curtiss, Department of Immunology, Research Institute of Scripps Clinic, La Jolla, California, for the kind gift of the murine monoclonal antibody, MB-47. This work was supported by grants from the National Heart, Lung, and Blood Institute: HL 02626 (F. K. Welty) and HL 25596 (Dr. P. Roheim, Department of Physiology at Louisiana State University Medical Center, New Orleans, Louisiana). [Dr. Welty was a Research and Teaching Scholar of the American College of Physicians.] We thank Dr. Stefania Lamon-Fava for reading the manuscript.

Manuscript received 20 June 1995 and in revised form 28 August 1995.

REFERENCES

1. Young, S. G. 1990. Recent progress in understanding apolipoprotein B. *Circulation.* **82:** 1574–1594.
2. Young, S. G., S. J. Bertics, T. M. Scott, B. W. Dubois, L. K. Curtiss, and J. L. Witztum. 1986. Parallel expression of the MB19 genetic polymorphism in apoprotein B-100 and apoprotein B-48: evidence that both apoproteins are products of the same gene. *J. Biol. Chem.* **261:** 2995–2998.
3. Powell, L. M., S. C. Wallis, R. J. Pease, Y. H. Edwards, T. J. Knott, and J. Scott. 1987. A novel form of tissue-specific RNA processing produces apolipoprotein B-48 in intestine. *Cell.* **50:** 831–840.
4. Chen, S. H., G. Habib, C. Y. Yang, Z. W. Gu, B. R. Lee, S. A. Weng, S. R. Silberman, S. J. Cai, J. P. Deslypere, M. Rosseneu, A. M. Gotto, Jr., W. H. Li, and L. Chan. 1987. Apolipoprotein B-48 is the product of a messenger RNA with an organ-specific in-frame stop codon. *Science.* **238:** 363–366.
5. Mahley, R. W. 1988. Apolipoprotein E: cholesterol transport protein with expanding role in cell biology. *Science.* **240:** 622–630.
6. Kowal, R. C., J. Herz, J. L. Goldstein, V. Esser, and M. S. Brown. 1989. Low density lipoprotein receptor-related protein mediates uptake of cholesteryl esters derived from apoprotein E-enriched lipoproteins. *Proc. Natl. Acad. Sci. USA.* **86:** 5810–5814.
7. Pease, R. J., R. W. Milne, W. K. Jessup, A. Law, P. Provost, J. C. Fruchart, R. T. Dean, Y. L. Marcel, and J. Scott. 1990. Use of bacterial expression cloning to localize the epitopes for a series of monoclonal antibodies against apolipoprotein B-100. *J. Biol. Chem.* **265:** 553–568.
8. Knott, T. J., R. J. Pease, L. M. Powell, S. C. Wallis, S. C. Rall, Jr., T. L. Innerarity, B. Blackhart, W. H. Taylor, Y. Marcel, R. Milne, D. Johnson, M. Fuller, A. J. Lusis, B. J. McCarthy, R. W. Mahley, B. Levy-Wilson, and J. Scott. 1986. Complete protein sequence and identification of structural domain of human apolipoprotein B. *Nature.* **323:** 734–738.
9. Yang, C. Y., S. H. Chen, S. H. Gianturco, W. A. Bradley, J. T. Sparrow, M. Tanimura, W. H. Li, D. A. Sparrow, H. De Loof, M. Rosseneu, F. S. Lee, Z. W. Gu, A. M. Gotto, Jr., and L. Chan. 1986. Sequence, structure, receptor-binding domains and internal repeats of human apolipoprotein B-100. *Nature.* **323:** 738–742.

ASBMB
JOURNAL OF LIPID RESEARCH
Downloaded from www.jlr.org by guest, on June 18, 2012

10. Marcel, Y. L., T. L. Innerarity, C. Spilman, R. W. Mahley, A. Protter, and R. W. Milne. 1987. Mapping of human apolipoprotein B antigenic determinants. *Arteriosclerosis.* **7:** 166–175.
11. Krul, E. S., S. Y. Kleinman, M. Kinoshita, B. Pfleger, K. Oida, A. Law, J. Scott, R. Pease, and G. Schonfeld. 1988. Regional specificities of monoclonal anti-human apolipoprotein B antibodies. *J. Lipid Res.* **29:** 937–947.
12. Young, S. G., J. L. Witztum, D. C. Casal, L. K. Curtiss, and S. Bernstein. 1986. Conservation of the low density lipoprotein receptor-binding domain of apoprotein B: demonstration by a new monoclonal antibody, MB47. *Arteriosclerosis.* **6:** 178–188.
13. Fantappie, S., A. Corsini, A. Sidoli, P. Uboldi, A. Granata, T. Zanelli, P. Rossi, S. Marcovina, R. Fumagalli, and A. L. Catapan. 1992. Monoclonal antibodies to human low density lipoprotein identify distinct areas on apolipoprotein B-100 relevant to the low density lipoprotein-receptor interaction. *J. Lipid Res.* **33:** 1111–1121.
14. Innerarity, T. L., K. H. Weisgraber, K. S. Arnold, R. W. Mahley, R. M. Krauss, G. L. Vega, and S. M. Grundy. 1987. Familial defective apolipoprotein B-100: low density lipoproteins with abnormal receptor binding. *Proc. Natl. Acad. Sci. USA.* **84:** 6919–6923.
15. Soria, L. F., E. H. Ludwig, H. R. G. Clarke, G. L. Vega, S. M. Grundy, and B. J. McCarthy. 1989. Association between a specific apolipoprotein B mutation and familial defective apolipoprotein B-100. *Proc. Natl. Acad. Sci. USA.* **86:** 587–591.
16. Lund-Katz, S., T. L. Innerarity, K. S. Arnold, L. K. Curtiss, and M. C. Phillips. 1991. ^{13}C NMR evidence that substitution of glutamine for arginine 3500 in familial defective apolipoprotein B-100 disrupts the conformation of the receptor-binding domain. *J. Biol. Chem.* **266:** 2701–2704.
17. Linton, M. F., R. V. Farese, Jr., and S. G. Young. 1993. Familial hypobetalipoproteinemia. *J. Lipid Res.* **34:** 521–541.
18. Welty, F. K., S. T. Hubl, V. R. Pierotti, and S. G. Young. 1991. A truncated species of apolipoprotein B (B67) in a kindred with familial hypobetalipoproteinemia. *J. Clin. Invest.* **87:** 1748–1754.
19. McNamara, J. R., and E. J. Schaefer. 1987. Automated enzymatic standardized lipid analysis for plasma and lipoprotein fractions. *Clin. Chim. Acta.* **166:** 1–8.
20. Cohn, J. S., J. R. McNamara, S. D. Krasinski, R. M. Russell, and E. J. Schaefer. 1989. Role of triglyceride-rich lipoproteins from the liver and intestine in the etiology of postprandial peaks in plasma triglyceride concentrations. *Metabolism.* **38:** 484–490.
21. Havel, R. J., H. A. Eder, and J. H. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **34:** 1345–1363.
22. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **227:** 680–685.
23. Cohn, J. S., J. R. McNamara, S. D. Cohn, J. M. Ordovas, and E. J. Schaefer. 1988. Plasma apolipoprotein changes in the triglyceride-rich lipoprotein fraction of human subjects fed a fat-rich meal. *J. Lipid Res.* **29:** 925–936.
24. Markwell, M. A., S. M. Haas, L. L. Bieber, and N. E. Tolbert. 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal. Biochem.* **87:** 206–210.
25. Milne, R. W., R. Theolis, Jr., R. B. Verdery, and Y. L. Marcel. 1983. Characterization of monoclonal antibodies against human low density lipoprotein. *Arteriosclerosis.* **3:** 23–30.
26. Milne, R. W., R. Theolis, Jr., R. Maurice, R. J. Pease, P. K. Weech, E. Rassart, J. C. Fruchart, J. Scott, and Y. L. Marcel. 1989. The use of monoclonal antibodies to localize the low density lipoprotein receptor-binding domain of apolipoprotein B. *J. Biol. Chem.* **264:** 19754–19760.
27. Young, S. G., R. K. Koduri, R. K. Austin, D. J. Bonnet, R. S. Smith, and L. K. Curtiss. 1994. Definition of a nonlinear conformational epitope for the apolipoprotein B-100-specific monoclonal antibody, MB47. *J. Lipid Res.* **35:** 399–407.
28. Ordovas, J. M., J. Peterson, P. Santaniello, J. S. Cohn, P. W. F. Wilson, and E. J. Schaefer. 1987. Enzyme-linked immunosorbent assay for human plasma apolipoprotein B. *J. Lipid Res.* **28:** 1216–1224.
29. Schaefer, E. J., and J. M. Ordovas. 1986. Metabolism of apolipoproteins A-I, A-II, and A-IV. *Methods Enzymol.* **129:** 420–443.
30. Bilheimer, D. W., S. Eisenberg, and R. I. Levy. 1972. The metabolism of very low density lipoprotein proteins. I. Preliminary in vitro and in vivo observations. *Biochim. Biophys. Acta.* **260:** 212–218.
31. Goldstein, J. L., S. K. Basu, and M. S. Brown. 1983. Receptor-mediated endocytosis of low-density lipoprotein in cultured cells. *Methods Enzymol.* **98:** 241–260.
32. Krul, E. S., K. G. Parhofer, P. H. R. Barrett, R. D. Wagner, and G. Schonfeld. 1992. ApoB-75, a truncation of apolipoprotein B associated with familial hypobetalipoproteinemia: genetic and kinetic studies. *J. Lipid Res.* **33:** 1037–1050.

Downloaded from www.jlr.org by guest, on June 18, 2012