Human very low density lipoprotein structure: interaction of the C apolipoproteins with apolipoprotein B-100

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Abstract Very low density lipoproteins (VLDL) are a heterogenous population of particles differing in size and composition. Heparin-Sepharose chromatography yields three VLDL subfractions. Two subfractions, $VLDL_{NR-1}$ and $VLDL_{NR-2}$, which are not retained by heparin, contain little or no detectable apolipoprotein (apo)E. According to negative stain electron microscopy, $VLDL_{NR-1}$ is slightly larger than $VLDL_{NR-2}$. The third fraction, $VLDL_R$, is composed of smaller particles that are retained by the heparin-Sepharose and contain apoE. The C apolipoproteins of the respective VLDL subfractions transfer to 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC) single bilayer vesicles giving three subfractions designated $VLDL_{NR-1}$-C, $VLDL_{NR-2}$-C, and $VLDL_R$-C. The protein, phospholipid, and cholesterol (free + esterified) contents decrease in the order $VLDL_R > VLDL_{NR-2} > VLDL_{NR-1}$. Triglyceride content decreases in the opposite order. POPC treatment of each VLDL subfraction increases the phospholipid and decreases the protein, triglyceride, and cholesteryl ester contents, while free cholesterol remains unchanged. According to immunological analysis of each subfraction with well-characterized monoclonal antibodies, the accessibility of some epitopes of apoB-100 on VLDL is changed by POPC treatment. Electron-microscopic analysis of POPC-treated VLDL subfraction reveals vacancies on the surfaces of each particle. $VLDL_{NR-1}$, $VLDL_{NR-2}$, and $VLDL_R$ are resistant to thrombin cleavage, whereas the lipoproteins lacking C apolipoproteins are not. Thrombin cleavage (8 h) of apoB-100 of $VLDL_{NR-2}$-C and $VLDL_R$-C gives two fragments, T_1 and T_2, that are converted to smaller fragments only after prolonged treatment. In contrast, apoB-100 of $VLDL_{NR-1}$-C is converted into small fragments after 8 h thrombin treatment. These results suggest that removal of apoCs affects the accessibility and conformation of apoB-100 in the individual VLDL subfractions in the region near residue 3249, which is the primary thrombin cleavage site and the epitope of monoclonal antibody 4C11.—**Yang, C-y., Z-W. Gu, N. Valentinova, H. J. Pownall, B. Lee, M. Yang, Y-h. Xie, J. R. Guyton, T. N. Vlasik, J-C. Fruchart, and A. M. Gotto, Jr.** Human very low density lipoprotein structure: interaction of the C apolipoproteins with apolipoprotein B-100. *J. Lipid Res.* 1993. **34:** 1311–1321.

Supplementary key words VLDL subfraction • thrombic cleavage • conformation of apoB-100 • 1-palmitoyl-2-oleoyl-phosphatidylcholine

The human plasma very low density lipoproteins (VLDL), which are secreted by the liver, differ in size and composition. VLDL are important because of their role in hypertriglyceridemia, triglyceride transport, and in the formation of low density lipoprotein (LDL). The structure and composition of VLDL subfractions are important determinants of its catabolism in vivo. The structural consequences of the interactions between the major VLDL apolipoproteins, apoB, apoE, and C apolipoproteins, may determine, in part, how the B-containing lipoproteins are recognized by lipolytic enzymes and cell surface receptors. When accessible and in the proper conformation, apoE and apoB-100 can target lipoproteins to cell surface receptors. However, normal VLDL, which contain both apoE and apoB-100, do not exhibit appreciable binding to the B, E-receptor. Thus, the receptor-binding activities of apoE and apoB-100 in VLDL are silent, presumably due to interactions of one or both of these proteins with the other components of VLDL. Intermediate density lipoproteins can be catabolized via the B, E-receptor although the relative importance of apoE and apoB-100 in this process is not clear (1). According to several reports, there is a subfraction of circulating VLDL with no detectable apoE (2–5). ApoE mediates the binding of VLDL particles to heparin and lipoprotein receptors on hepatocytes and other cells (6, 7). Ishikawa, Fielding, and Fielding (8) reported that the apoB conformation induced by the lipolysis of apoE-deficient VLDL was

Abbreviations: VLDL, very low density lipoproteins; LDL, low density lipoproteins; apo, apolipoprotein; POPC, 1-palmitoyl-2-oleoyl phosphatidylcholine; MAB, monoclonal antibody; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; EM, electron microscopy; ELISA, enzyme-linked immunosorbent assay.

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similar to that of native VLDL that contains apoE and that binds apoE and heparin.

The C apolipoproteins have a direct effect on the catabolism of VLDL. In vitro and in vivo, apoC-II activates lipoprotein lipase (9), whereas apoC-III is an in vitro inhibitor of lipoprotein lipase (10–12). Some studies have shown an increase in the apoC-III/apoC-II ratio in hypertriglyceridemic patients (13, 14). The apoC proteins might also influence the structure of apoB. Little is known about how VLDL subfractions might vary in apoC content, and the effects of apoC depletion on apoB structure have not been reported.

The present study extends the characterization of VLDL subfractions by using heparin-Sepharose chromatography to isolate three VLDL subfractions. One of these has a high affinity for heparin-Sepharose and contains a full complement of apoE. Two other fractions that do not bind to heparin-Sepharose are apoE-poor. Each of these fractions was modified by the removal of their respective complements of apoC proteins. These subfractions were characterized with respect to composition, affinities for monoclonal antibodies (MABs) of apoB, and accessibility thrombin digestion.

MATERIALS AND METHODS

Preparation of VLDL

Plasma from normal fasting subjects was obtained from The Methodist Hospital Blood Center. Aprotinin (0.55 unit/ml), sodium azide (5.00 μg/ml), and EDTA (5.00 μg/ml) were added to the plasma, and VLDL (*density* < 1.006 g/ml) was purified by ultracentrifugation (15) and concentrated within a dialysis membrane with Aquacide III (Calbiochem) to approximately 1 mg/ml protein.

Affinity chromatography of VLDL

Heparin-Sepharose was prepared with Sepharose CL 4B activated by cyanogenbromide coupling with heparin (Sigma, sodium salt, Grade I, porcine). Affinity chromatography of VLDL was conducted on a heparin-Sepharose column (1.6 × 40 cm) equilibrated in 0.15 M NaCl, 1 mM EDTA (pH 7.4), as previously described (4). The collection rate was 2 ml/fraction per 12 min. The portion of VLDL that was not retained emerged in two successive fractions, $VLDL_{NR-1}$ and $VLDL_{NR-2}$. The retained portion, $VLDL_R$, was displaced with 3 M NaCl. After spectrophotometric determinations at 220 nm and 280 nm (Beckman DU-6), the eluted fractions were pooled and concentrated.

Analysis of lipid and total protein content

Total protein content of the lipoproteins was determined by the method of Lowry et al. (16) with sodium dodecyl sulfate (SDS) at a final concentration of 0.1% (17). Triglyceride, cholesterol, and cholesteryl esters were determined enzymatically (Boehringer-Mannheim) (18). Phospholipid was determined enzymatically using a reagent kit supplied by Wako Company (Osaka, Japan).

Gel electrophoresis of apolipoproteins

Slab gel electrophoresis of apolipoproteins (10 μg) was performed at constant current in 3–10% or 5–25% gradient of polyacrylamide in Tris buffer (25 mM Tris-HCl, 0.2 M glycine, 0.1% SDS, pH 8.4) (19). The gel was fixed in methanol–ethanol solution for 30 min, stained for 1 h in Coomassie blue in methanol–acetic acid solution, and de-stained in 7% acetic acid solution. Bands were identified by comparison with pure apolipoprotein standards and standard thrombin cleavage products of LDL.

Preparation of VLDL without apoC proteins

ApoC proteins were transferred from VLDL to single bilayer vesicles of 1-palmitoyl-2-oleoyl phosphatidycholine (POPC) by a modification of the method of McKeone et al. (20). In brief, 100 mg of POPC (Sigma, St. Louis, MO) was sonicated with 5 ml of 10 mM Tris, 1 mM EDTA, 0.1 M NaCl, 1 mM NaN_3, pH 7.4, for 30 min, and centrifuged at 10,000 rpm for 30 min. The tenfold (by weight) excess of single-bilayer vesicle-phospholipid was mixed with VLDL at room temperature for 1 min and applied to a Sepharose CL4B column (1.6 × 40 cm) with Tris buffer. Two-milliliter fractions were collected and the protein and phospholipid, respectively, were monitored by measuring the absorbance at 280 nm and phosphorous analysis. Protein content was verified by SDS polyacrylamide gel electrophoresis (SDS-PAGE).

Electron microscopy

For electron microscopy (EM), Formvar- and carbon-coated copper grids were pretreated with 0.01% bovine serum albumin. Lipoproteins (unfixed) were then applied to the grids and negatively stained with 2.0% uranyl acetate. Grids were examined with a Jeol 200CX electron microscope. Magnifications were calibrated by using a Fullam diffraction grating replica. The reported particle radii are the means of 100 observations.

Antibodies

Monoclonal antibodies prepared against apoB were Mb43 (21), Mb47 (22), BL3, BL7, BIP45 (21, 23, 24), 4C11, 2G8 and peroxidase-conjugated 5F8* (25) (mouse antibodies) and B5 (26) (rat antibodies). The production and specificity of each have been previously described as indicated. Goat anti-apoB polyclonal antibodies were purchased from Alpha Biomedical Lab (Seattle, WA); goat anti-mouse and anti-rat peroxidase-conjugated IgG were from Jackson Immuno-Research Lab, Inc.

Analysis of apoB content in VLDL subfractions

For apoB quantification we used two different ELISA procedures, based on the application of both polyclonal and monoclonal antibodies prepared against apoB. OMEGA Lipid Fraction Control Serum (human; Technicon) with known apoB concentration (0.59 mg/ml) served as a standard. To dilute the antigen and the antibodies and to wash plates, the 0.15 M phosphate buffer saline, pH = 7.2, containing 0.5% BSA (Sigma), 0.1% Tween 20 (Sigma) (PBS-BSA-Tw) was used.

ApoB quantification with polyclonals. A 96-well microtiter plate (Corning) was coated with 100 μl per well of goat anti-apoB polyclonals (5 μg/ml in PBS) and incubated at 4°C overnight. After that the wells were washed twice with PBS+0.5% BSA and incubated with 275 μl of this solution for 1 h at room temperature to prevent VLDL unspecific binding. One hundred μl of serially diluted standard serum or VLDL sample to be tested (range 30–250 ng/ml of apoB) was added to each well and incubated for 2 h at room temperature. Unbound antigen was removed from plate (×5 washing) and the wells were filled with 100 μl of rabbit anti-apoB peroxidase-conjugated polyclonals at appropriate dilution (2 h at room temperature). The optimal dilution was chosen previously as a point of saturation for the dose-dependent binding of peroxidase-conjugated polyclonals with abundance of antigen on the plate.

After washing (×3 with PBS-BSA-Tw and ×2 with PBS only) wells were assayed for peroxidase activity. Substrate mixture (100 μl per well) contained 1 mg O-phenylenediamine (OPDA), 10 ml of 20 mM citrate buffer, pH 4.7, and 15 μl 30% H_2O_2; the reaction was stopped with 25 μl of 5 M H_2SO_4. ApoB concentration in VLDL samples was read from a calibration curve plotted for standard serum.

ApoB quantification with monoclonals. MAB 5F8* (peroxidase-conjugated) specific for T_4 fragment of thrombin-digested apoB was used for measurement of apoB content in VLDL subfractions by competitive ELISA.

Plates were coated with LDL (20 μg/ml in PBS, 100 μl per well) and incubated at 4°C overnight. After washing and incubation of plates with PBS-BSA (see above) 50 μl of VLDL sample or standard serum, serially diluted to apoB concentration 1.6–11.8 μg/ml, was mixed in the wells with 50 μl of 5F8* (0.02 μg/ml). Optimal concentration of 5F8* was determined previously as a middle point of S-curve for 5F8* dose-dependent binding with immobilized LDL. Plates were shaken thoroughly, stored for 2 h at room temperature, then washed and assayed for peroxidase activity (see above).

ApoB quantification with isopropanol precipitation method. To compare the values of apoB concentration, obtained by two different procedures, with the true apoB content in VLDL samples, we isolated apoB from the samples by isopropanol (27). Precipitates were delipidated with Folch reagent (chloroform–methanol 2:1), washed twice with 0.6 M GuHCl, 0.1% TFA, and redissolved in 10 mM Tris-HCl, pH = 8.5, containing 1 mM Na EDTA, 0.01% NaN_3, 1 mM PMSF, 100 mM SDS (28), with gentle stirring for 2 or 3 days. Volume of solution was adjusted to the volume of initial sample. Protein content was measured by the Lowry method (16). Resolubilized precipitates were applied to 5–25% SDS-PAGE gel electrophoresis to reveal any of apoE and apoC impurities.

Comparison of VLDL subfractions immunoreactivity

The competitive ELISA procedure used for comparison of VLDL subfraction reactivity with different MABs was similar to the procedure for 5F8* described above. Range of antigen dilution and optimal concentration of antibody were chosen for each MAB individually. After the incubation of samples with first antibody (anti-apoB, nonconjugated with peroxidase) the second antibody (peroxidase-conjugated goat anti-mouse or goat anti-rat IgG) at appropriate dilution was added (100 μl per well). Plates were incubated for 1 h at room temperature, washed five times, and assayed for peroxidase activity.

Thrombin digestion

To assess differential apoB-100 cleavage, limited thrombin (Boehringer) digestion of VLDL and its subfractions, of POPC-treated VLDL and its subfractions, and of LDL was performed at 37°C using 700 μg of lipoprotein in 1 ml of 10 mM Tris-HCl, 0.15 M NaCl, 0.01% NaN_3, pH 8.0, at an enzyme:apolipoprotein B-100 ratio of 1:100 (w/w) (29). After thrombin was added to lipoprotein, 0.1-ml aliquots were removed at various time intervals. Reactions were terminated by mixing the samples with 0.1 ml of buffer consisting of 10 mM Tris-HCl, 1.4% SDS, 1 mM EDTA, 6 M urea, 1.4% 2-mercaptoethanol, 20% sucrose, and 0.04% bromphenol blue. The samples were stored at −20°C until thawing and electrophoresis.

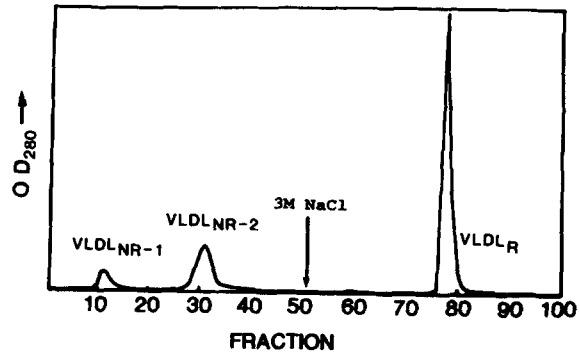

Fig. 1. Affinity chromatogram of VLDL subfractions using a heparin-Sepharose column (1.6 × 40 cm). $VLDL_{NR-1}$ and $VLDL_{NR-2}$ were eluted on 0.15 M NaCl, 1 mM EDTA, pH 7.4, and $VLDL_R$ was eluted with 3 M NaCl. Two-milliliter fractions were collected.

TABLE 1. Percent composition and size of VLDL subfractions before and after POPC treatment[a]

Composition and Size	$VLDL_{NR-1}$		$VLDL_{NR-2}$		$VLDL_R$	
	Original Subfraction	POPC Treated	Original Subfraction	POPC Treated	Original Subfraction	POPC Treated
Protein	7.2	6.5	9.5	7.3	11.3	10.6
Phospholipid	12.2	26.5	13.4	27.1	14.4	27.0
Triglyceride	65.7	55.9	61.32	55.0	58.7	51.8
Free cholesterol	3.7	3.9	3.8	3.1	3.5	3.1
Cholesteryl ester	11.2	7.3	12.0	7.5	12.2	7.6
Median particle diameter (nm)	46		44		39	

[a]Each value is the average from a duplicate experiment.

RESULTS

Isolation and chromatography of VLDL subfractions

VLDL isolated by Sepharose CL 4B column chromatography were applied to a heparin-Sepharose column. Three fractions were obtained. Similar results were obtained with VLDL isolated by ultracentrifugation. As this technique permits one to run many samples using higher VLDL concentrations, ultracentrifugation was used for VLDL isolation.

Separation of the VLDL subfractions by heparin-Sepharose chromatography is shown in **Fig. 1.** Subfractions $VLDL_{NR-1}$ and $VLDL_{NR-2}$ were separated on a low-salt (0.15 M NaCl) buffer system on the basis of affinity for heparin-Sepharose; $VLDL_R$ was displaced from the column by high-salt. The isolated peaks showed consistent chromatographic behavior on rechromatography under the same conditions. On average, across the eight subjects, VLDL were distributed among $VLDL_{NR-1}$ (11% range, 6–17%), $VLDL_{NR-2}$ (24% range, 14–34%), and $VLDL_R$ (65% range, 53–73%). Compositions of the three VLDL subfractions and POPC-treated subfractions are shown in **Table 1.** The protein, phospholipid, and cholesterol contents decreased in the order $VLDL_R > VLDL_{NR-2} > VLDL_{NR-1}$. Triglyceride content decreased in the opposite order. SDS-PAGE (gradient = 5–25%) showed that the $VLDL_{NR-1}$ and $VLDL_{NR-2}$ each contain apoB, C apolipoproteins, and a trace of apoE, and $VLDL_R$ contained B, C, and E apolipoproteins (**Fig. 2,** lanes 1–3).

Removal of C apolipoproteins from VLDL subfractions

VLDL subfractions without their C apolipoproteins, designated $VLDL_{NR-1}$-C, $VLDL_{NR-2}$-C, and $VLDL_R$-C, were obtained by separation on a Sepharose CL4B column after mixture of the subfraction with POPC vesicles. SDS-PAGE (Fig. 2, lanes 4–6) and compositional analysis (Table 1) indicated that this procedure exchanged over 95% of the C apolipoproteins of the VLDL for phospholipid in the POPC vesicles. At the same time there was a small decrease in the protein, triglyceride, and cholesteryl ester contents of the VLDL with the free cholesterol being little changed.

Electron microscopy

Negative-stain electron microscopy of the VLDL subfractions is shown in **Fig. 3.** Lipoprotein particle radii were calculated as the means of 100 observations. The mean particle diameters (nm) of $VLDL_{NR-1}$, $VLDL_{NR-2}$, and $VLDL_R$ were 46, 44, and 39 nm, respectively. Although there is an overlap in particle size among all subfractions, the $VLDL_{NR-1}$ and $VLDL_{NR-2}$ are composed of slightly larger particles. The overlap between $VLDL_{NR-1}$ and $VLDL_{NR-2}$ suggests that their separation may not have occurred simply because of particle size and that relative affinity for heparin-Sepharose may have played a role also. Aggregation was noted in all VLDL subfractions. In all cases, negative-stain electron microscopy of the VLDL subfraction after POPC treat-

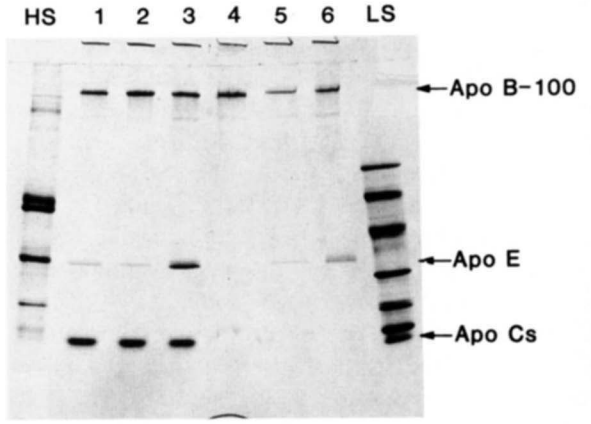

Fig. 2. The 5%–25% SDS-PAGE of VLDL and its subfractions. High and low molecular weight standards presented on left and right hand side, respectively. Lane 1: $VLDL_{NR-1}$; lane 2: $VLDL_{NR-2}$; lane 3: $VLDL_R$; lane 4: $VLDL_{NR-1}$-C; lane 5: $VLDL_{NR-2}$-C; and lane 6: $VLDL_R$-C.

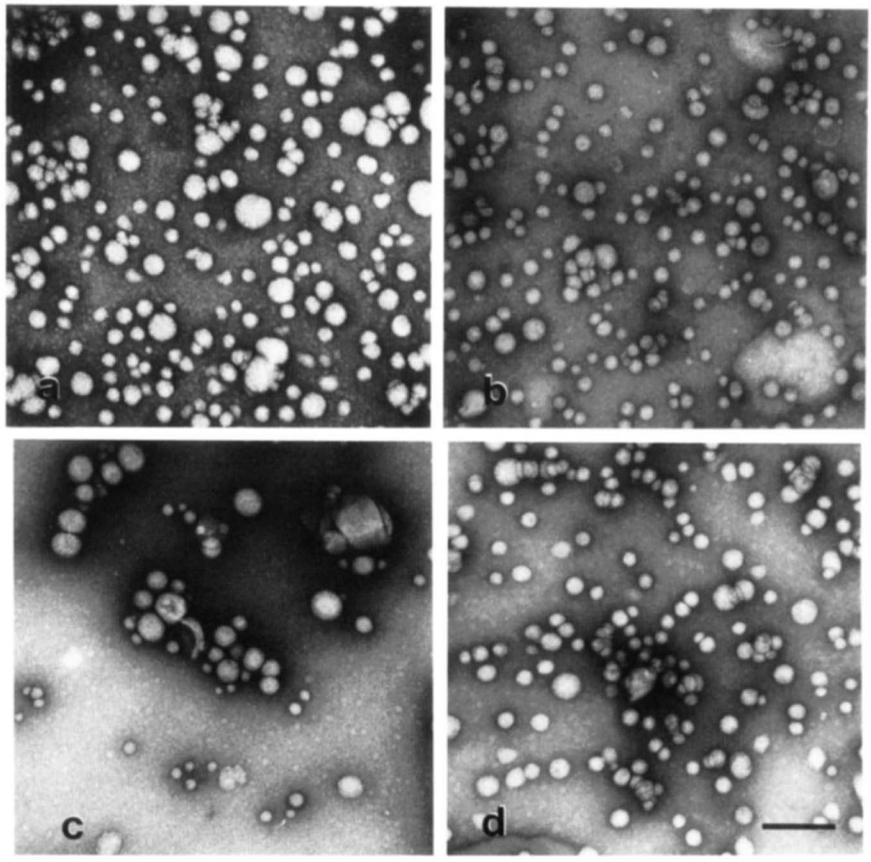

Fig. 3. Electron micrographs, visualized by negative stain microscopy using 2.0% uranyl acetate, of total VLDL (a) and VLDL subfractions $VLDL_R$ (b), $VLDL_{NR-1}$ (c), and $VLDL_{NR-2}$ (d). There was considerable overlap in particle size among all three subfractions. Areas of clumped lipoproteins were seen in all micrographs as an expected feature of negative staining. Bar = 200 nm.

ment revealed broken spherical lipoprotein particles. **Fig. 4** shows an example of $VLDL_{NR-2}$-C particles.

Quantitation of apoB in VLDL

To choose a procedure for measurement of apoB content in VLDL subfractions we compared the results obtained with three different methods of apoB quantification: *1)* sandwich-type ELISA with anti-apoB polyclonals; *2)* competitive ELISA with MAB 5F8* specific to T_4 fragment of thrombin-digested apoB; and *3)* apoB extraction by isopropanol followed by its delipidation, resolubilization, and measurement of protein concentration by the method of Lowry et al. (16). Complete apoB extraction was achieved; no apoB was found in supernatants by SDS-PAGE. Precipitates contained the apoB band with trace amounts of apoE in VLDL and $VLDL_R$ samples. The percentage of apoB-100 in VLDL subfractions detectable by polyclonal antibodies or MAB 5F8* was estimated as the ratio of apoB-100 concentrations measured immunologically to those measured by the Lowry method. **Table 2** shows that for each VLDL subfraction, different amounts of apoB-100 are recognized by polyclonal antibodies and a monoclonal antibody, 5F8. However, when the same antibody is used, the amount of immunologically detectable apoB-100 is the same in all VLDL subfractions.

Immunoreactivity of VLDL subfraction

Monoclonal antibodies prepared against various apoB epitopes were used to reveal structural differences between VLDL subfractions with and without apoC proteins. As shown by competitive ELISA in **Fig. 5,** VLDL subfractions and VLDL subfractions minus apoC have the same or very similar reactivities with MABs, BIP 45 (amino terminal, T_4), B5, BL7 (fragment T_3, residues 1854–1878 and 2239–2331, respectively), 2G8 (T_2), MB47 (receptor-binding domain, residues 3441–3569). A competitive ELISA with LDL showed that removal of the C apolipoproteins enhanced binding to MAB 4C11 (specific for fragment T_3) while diminishing that of Mb43 and BL3 (residues 4027–4081 and 4235–4355, respectively). For VLDL-C subfractions, the 50% displacement of

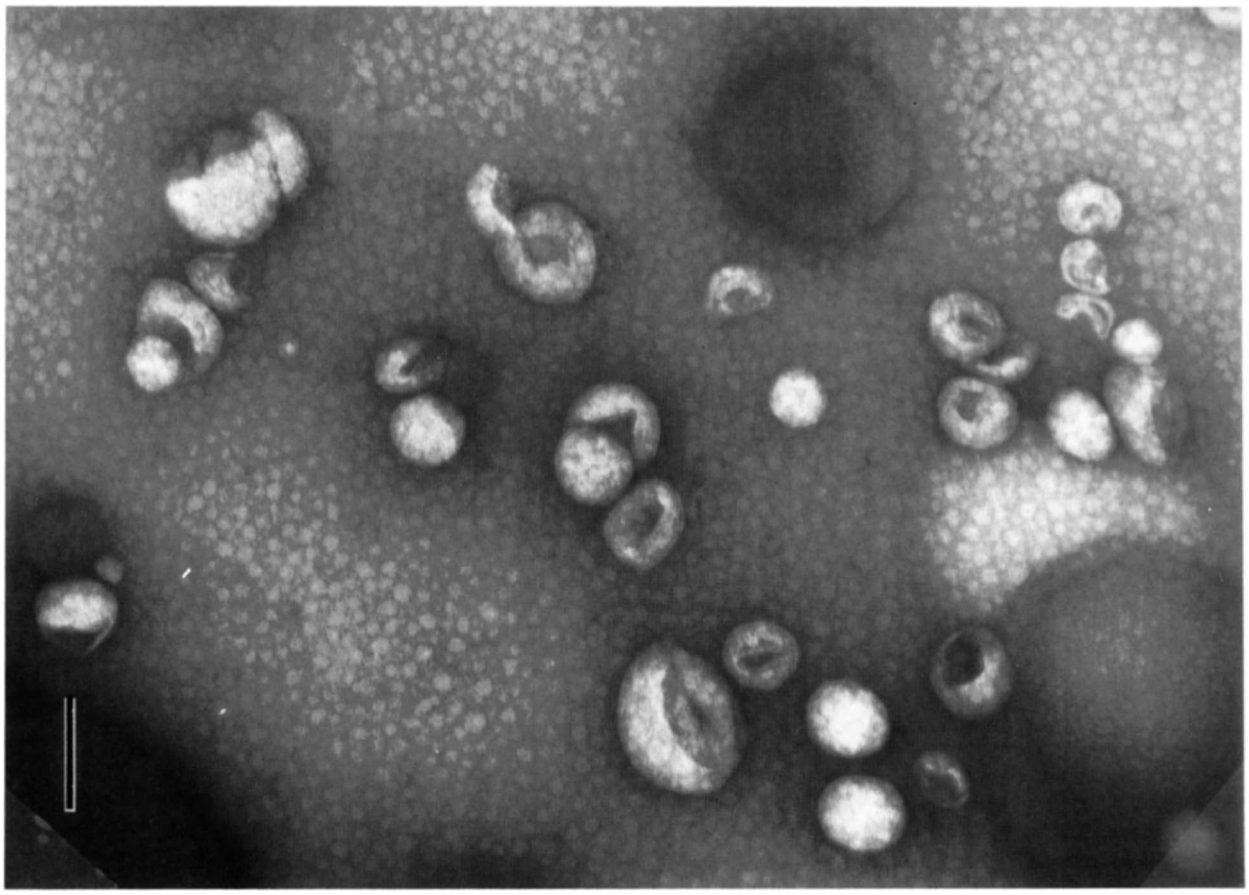

Fig. 4. Electron micrograph of $VLDL_{NR-2}$-C; see Fig. 3 legend for details. Bar = 65 nm.

maximal MAB binding with immobilized LDL was observed at 33 ± 6%, 204 ± 13%, and 173 ± 23%, respectively, with MAB 4C11, MB43, and BL3.

Thrombin digestion

The results, at different time points, of limited thrombin digestion of VLDL subfractions not treated with POPC and of apoC-depleted VLDL subfractions are shown in **Fig. 6** and **Fig. 7,** respectively. Thrombin cleavage of LDL (data not shown) was previously reported, i.e., cleavage at T_1, T_2, T_3, and T_4 (29).

Unfractionated VLDL was relatively resistant to thrombin cleavage. Exposure of unfractionated VLDL to thrombin yielded small amounts of T_1 and T_2 at long time intervals. The subfractions $VLDL_{NR-1}$, $VLDL_{NR-2}$, and $VLDL_R$ were similarly resistant, although $VLDL_R$ (Fig. 6a) and $VLDL_{NR-2}$ (Fig. 6b) yielded slightly more T_1 and T_2. $VLDL_{NR-1}$ was only slightly digested, and then only after an 84-h incubation, into a multi-fragment pattern (data not shown). Removal of the apoC protein had a dramatic effect on this pattern of reactivity. ApoB-100 of $VLDL_{NR-1}$-C (Fig. 7c) was cleaved completely during the first 8 h of thrombin treatment. ApoB-100 of $VLDL_{NR-2}$-C (Fig. 7b) and $VLDL_R$-C (Fig. 7a) were digested primarily into T_1 and T_2 after 8 h, and then into smaller fragments after 84 h and 72 h, respectively. These results suggest that removal of the C apolipoproteins alters the accessibility or structural conformation of apoB-100 in the various VLDL near the sites of thrombin cleavage.

TABLE 2. Percentage of apoB concentration detectable with 5F8* and polyclonals by ELISA tests[a]

Sample	n	5F8*	Polyclonals
VLDL	10	83 ± 8	152 ± 14
$VLDL_{NR-1}$	4	78 ± 12	141 ± 22
$VLDL_{NR-2}$	4	77 ± 9	139 ± 17
$VLDL_R$	6	81 ± 8	147 ± 15

[a]100%-protein measured by the method of Lowry et al. (16) in resolubilized precipitates.

DISCUSSION

Shelbourne and Quarfordt (2) first used heparin-Sepharose chromatography in the absence of divalent ions

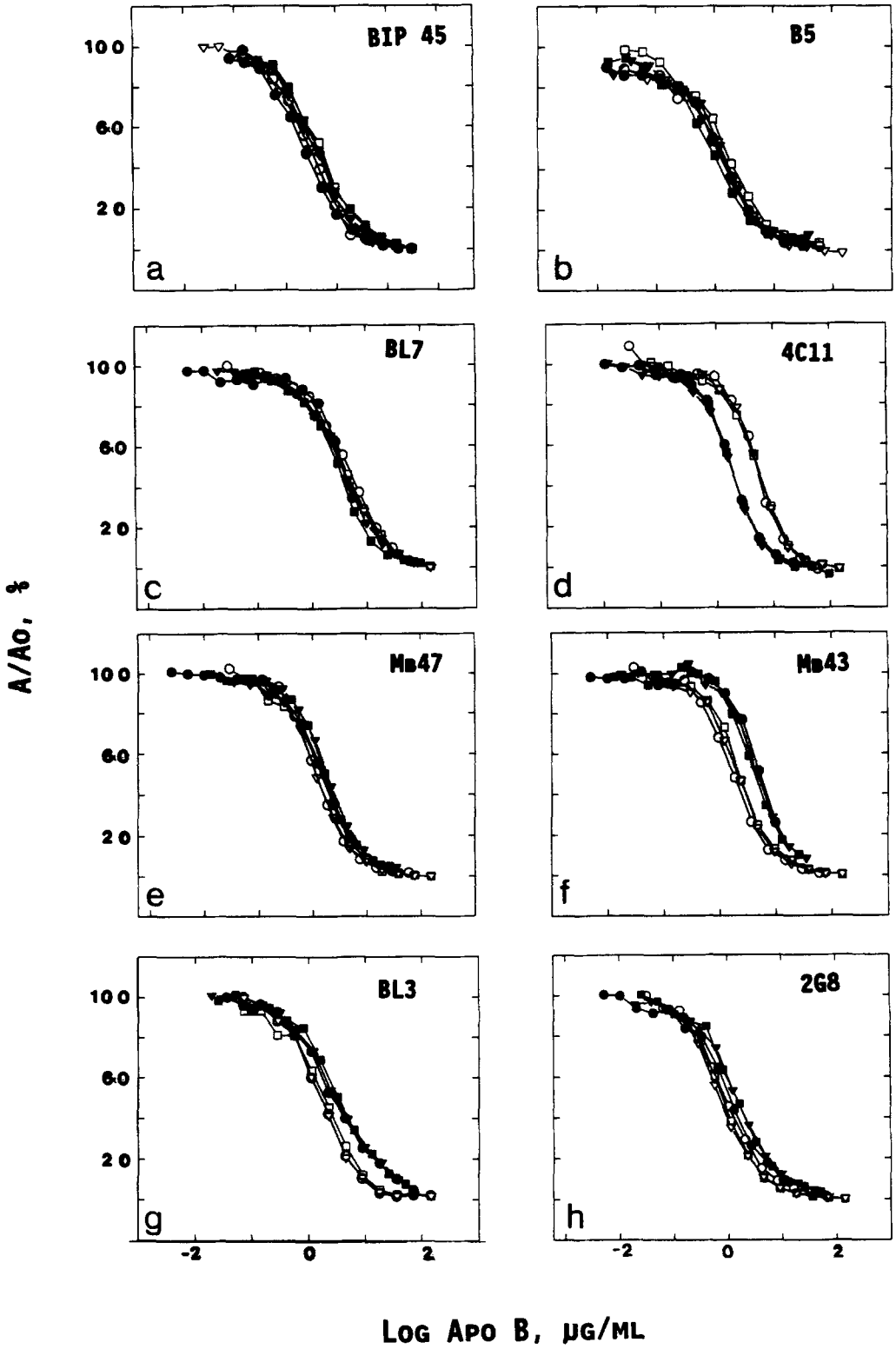

Fig. 5. Ability of $VLDL_{NR-1}$ (○), $VLDL_{NR-2}$ (▽), $VLDL_R$ (□), $VLDL_{NR-1}$-C (●), $VLDL_{NR-2}$-C (▼), and $VLDL_R$-C (■) to compete with immobilized LDL for MAB BIP45 (a), B5 (b), BL7 (c), 4C11 (d), Mb47 (e), Mb43 (f), BL3 (g), and 2G8 (h) binding. A and A_o represent the absorbance in the presence and in the absence of competitor, respectively.

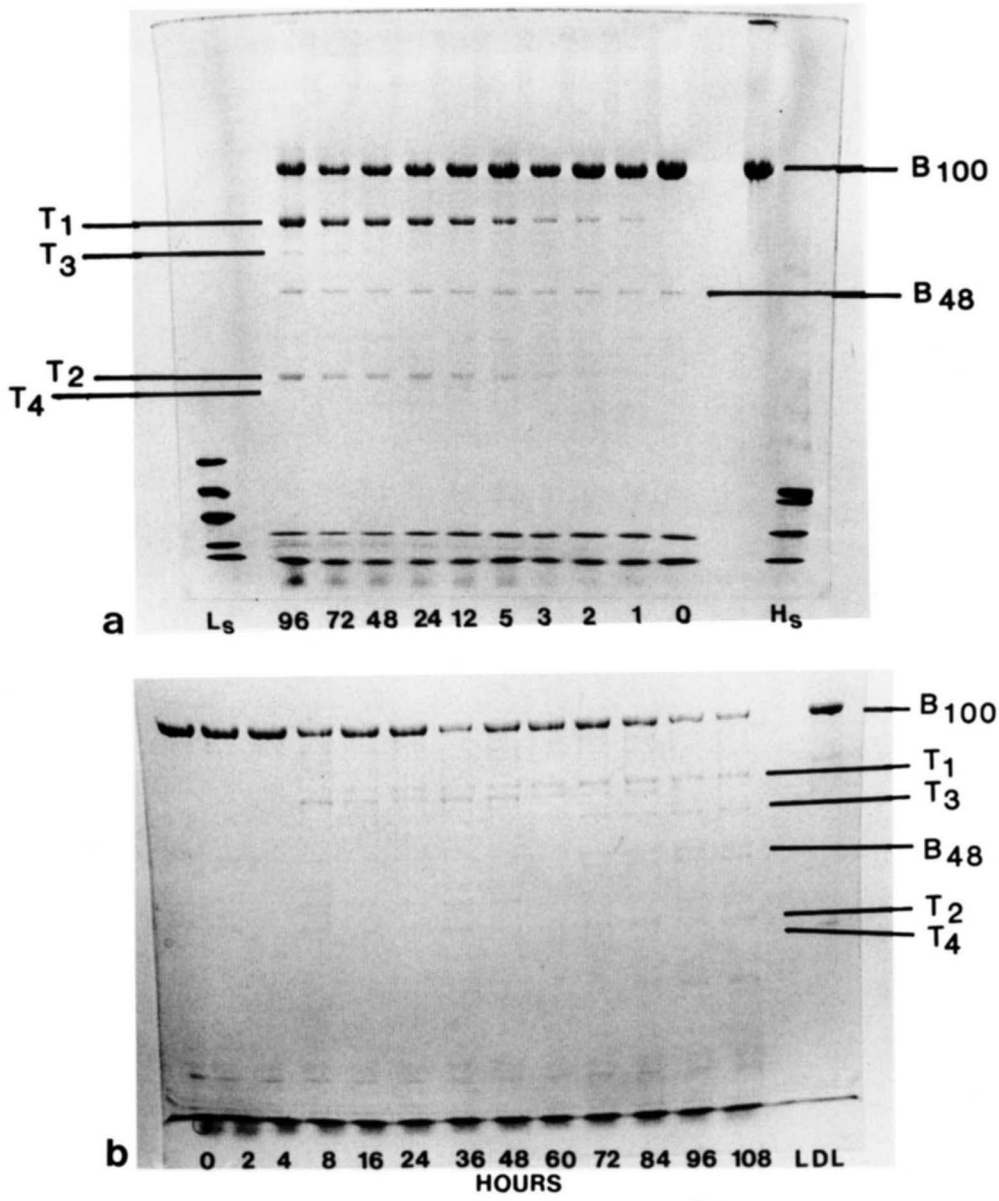

Fig. 6. Digestion at various time points of apoB-100 in VLDL subfractions $VLDL_R$ (a) and $VLDL_{NR-2}$ (b) by thrombin on 3–10% SDS-PAGE. On each of the x axes, zero is baseline (no enzyme addition) and the other numbers indicate incubation in hours. The known mobilities for apoB-100 and its fragments are indicated. Each well contains 35 μg protein for $VLDL_R$ and 20 μg protein for $VLDL_{NR-2}$, respectively.

to isolate two VLDL subfractions. In the presence of Mn^{2+}, heparin-Sepharose chromatography yielded four VLDL subfractions (3). VLDL subfractions containing apoE have also been separated from those without apoE on a heparin-agarose column (4). In the present study, a heparin-Sepharose affinity column permitted preparative separation of three VLDL subfractions. A retained fraction, $VLDL_R$, contained a full complement of B, C, and E apolipoproteins, and two nonretained fractions, $VLDL_{NR-1}$ and $VLDL_{NR-2}$, contained apoB and the C apolipoproteins, with only traces of apoE. The E-poor subfractions were distinguished by their differing particle sizes observed by electron microscopy and by their protein composition based on SDS-PAGE. Chemical composition among VLDL subfractions indicated that triglyceride, which composes most of the VLDL, is increased in the order of $VLDL_R$, $VLDL_{NR-2}$, and $VLDL_{NR-1}$, while the surface components, phospholipid, protein, and cholesterol, increase in the opposite order. These changes suggest that the size of VLDL increases in the same order. As expected, the nonabsorbed fractions are practically free of apoE. The small amounts of apoE seen on PAGE

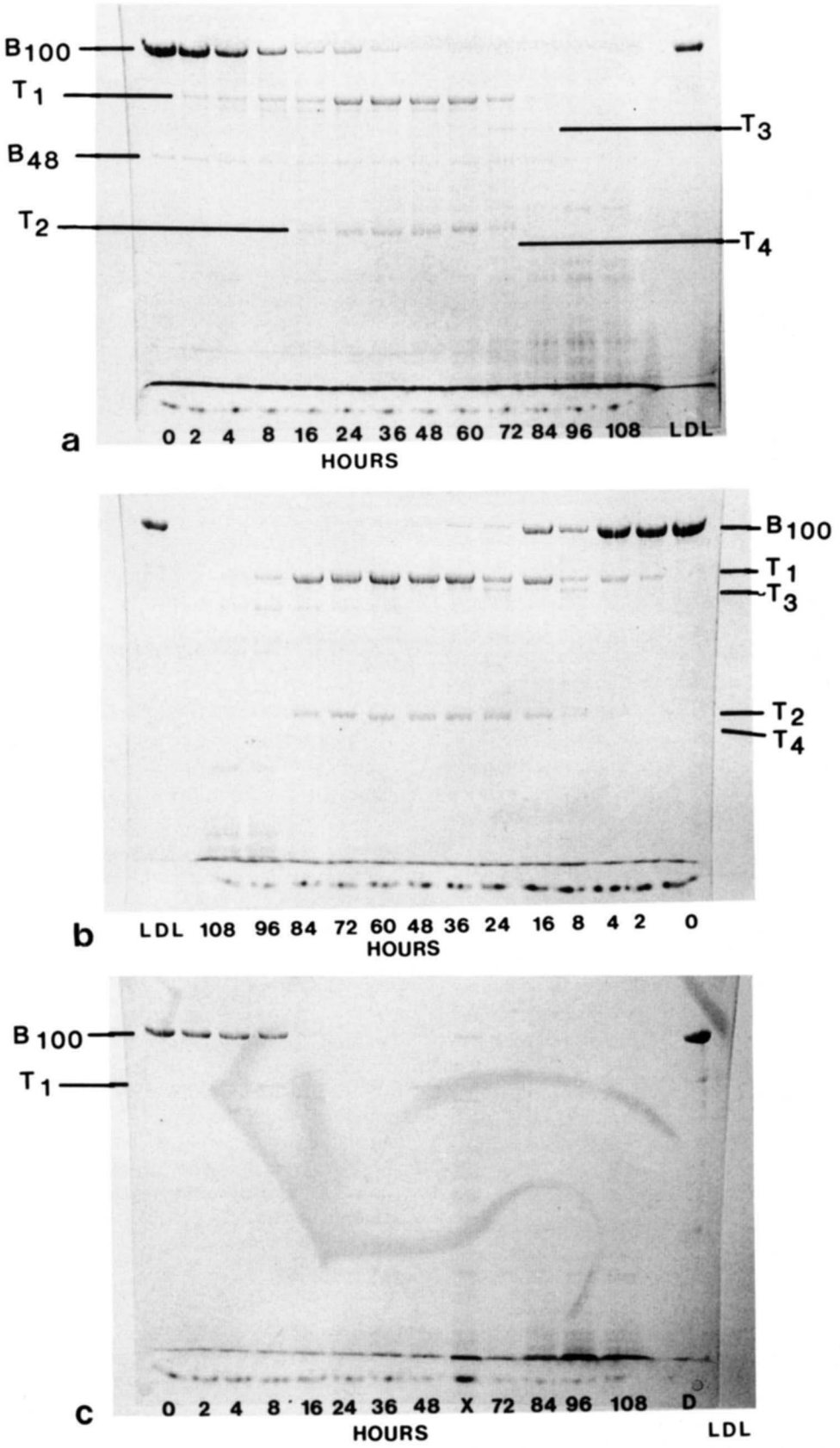

Fig. 7. Digestion at various time points of apoB-100 in POPC-treated (apoC-depleted) VLDL subfractions $VLDL_R$-C (a), $VLDL_{NR-2}$-C (b), and $VLDL_{NR-1}$-C (c) on 3–10% SDS-PAGE. On each of the x axes, zero is baseline (no enzyme addition) and the other numbers indicate incubation in hours. LDL is used as the standard for apoB-100. The known mobilities of apoB-100 and its fragments are indicated. Each well contains 20 μg protein.

may reflect particle aggregation, since aggregation could make the heparin-binding site of the lipoprotein inaccessible. Aggregation of particles was observed in all subfractions. However, aggregation of $VLDL_{NR-1}$ was slightly higher than that in $VLDL_{NR-2}$ or $VLDL_R$.

The POPC-treated VLDL had a higher concentration of phospholipid that is likely due to the transfer from the POPC vesicles. A portion of this likely replaced that occupied by the C apolipoproteins. However, a typical amino acid in an α-helix occupies about 15 $Å^2$ per amino acid residue whereas a phospholipid occupies about 65 $Å^2$ per molecule (30). Given mean molecular weights of 110 per residue and 750 per lipid, one can calculate that 50% more phospholipid by weight is required to occupy displaced protein. This is still much lower than the near doubling of the phospholipid content. We speculate that the excess phospholipid can insert into small spaces in the surface of VLDL that would be inaccessible to a whole protein.

According to an analysis of the electron micrographs, incubation of the VLDL with POPC vesicles did not have a statistically significant effect on particle size. However, the appearance of each particle was greatly altered. The distribution of stain on each sphere was uneven, suggesting that there were major differences in the surface composition. Given the increase in POPC content that occurs with loss of the C apolipoproteins, it is tempting to suggest that the surface contains distinct regions that are protein- or phospholipid-rich. Further studies will be needed to make a more definitive interpretation of these morphological changes.

The substitution of these POPC–apoB-100 interactions for those between the C apolipoproteins and apoB-100 has a dramatic effect on the physical properties of apoB-100, as assessed by both immunologic and enzymatic probes. According to a competitive ELISA, removal of the C apolipoproteins had no effect on MABs binding to some regions of apoB-100 whereas the reactivity of MABs to other regions was increased or decreased. For those that were unchanged, we conclude that the accessibility and conformation of apoB-100 is not affected by the presence of the C apolipoproteins. This included BIP45 (amino terminus), B5 (residues 1854–1878), BL7 (residues 2239–2331), and MB47, which binds at or near the receptor-binding domain. Conversely, with BL3 and Mb43, which recognize residues in the C terminus, VLDL were less reactive after the removal of the C proteins, suggesting that either the antigenic conformation of apoB-100 required C-proteins or that the POPC acquired during the C apolipoprotein transfer may partially mask some of these epitopes. In contrast, removal of the C proteins increased the binding of 4C11 to the VLDL indicating that the epitope for this antibody, which is located in T_3 (residues 1297–3249) is unmasked.

The kinetics of thrombin cleavage were also modified by removal of the C apolipoproteins. Thrombin cleavage of apoB-100 occurs at residues 1297 and 3249. A single cleavage at residue 3249 gives rise to amino and carboxyl fragments, T_1 and T_2, respectively. Cleavage of T_1 at residue 1297 forms additional amino and carboxyl fragments, T_3 and T_4, respectively (29). Whole VLDL and the VLDL subfractions, $VLDL_R$ $VLDL_{NR-1}$, and $VLDL_{NR-2}$, were relatively resistent to thrombin digestion. In contrast, after 8 h most of the apoB-100 of $VLDL_R$-C and $VLDL_{NR-2}$-C was cleaved into T_1 and T_2. The apoB-100 of $VLDL_{NR-1}$-C released only T_1 after 4 h and T_2 was barely detectable. These data suggest the following. The C apolipoproteins could inhibit thrombin digestion of apoB-100 in VLDL through alterations in the conformation of apoB-100 at or near residue 3249 via protein–protein interactions. Alternatively, the release of T_1 without T_2 from $VLDL_{NR-1}$-C but not from $VLDL_R$-C and $VLDL_{NR-2}$, suggests that there are differences in the affinities of VLDL subfractions for apoB-100. Each VLDL particle contains a mixture of proteins that may undergo physiologically important protein–protein interactions. In addition to the C apolipoproteins, each particle contains one copy of apoB-100 and multiple copies of apoE, which target the products of VLDL hydrolysis to cell surface B,E-receptors. Both receptor ligands are silent in VLDL but are activated in LDL and intermediate density lipoproteins, respectively. The mechanism by which the receptor ligands of apoB-100 and apoE in VLDL are silenced is important to our understanding of the turnover of apoB-100-containing lipoproteins. Collective consideration of our data supports the hypothesis that the C apolipoproteins modify the structure of apoB-100 in VLDL. Some of these structural changes may regulate the association of apoB-100-containing proteins with cell surface receptors. Studies testing this hypothesis are in progress.

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