

Intrahepatic assembly of very low density lipoproteins: immunologic characterization of apolipoprotein B in lipoproteins and hepatic membrane fractions and its intracellular distribution

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Abstract Monoclonal antibodies, prepared against rat apoB, were used to examine apoB structure in serum lipoproteins and characterize the forms and localization of apoB in liver membrane fractions and cultured hepatocytes. Of the several antibodies obtained, four, having separate epitopes, were characterized. Western blot analysis showed that three (DB11, F4, and LB14) antibodies recognized both apoB_L and apoB_S. One antibody (HB41) recognized only apoB_L. This antibody showed unusual properties. Competition ELISA assays showed that the epitope recognized by HB41 was more effectively expressed on low density lipoproteins (LDL) compared to very low density lipoproteins (VLDL). In addition, treatment of lipoproteins with detergents and sulfhydryl reducing agents also increased the expression of the HB41 epitope. Since HB41 has been found to inhibit LDL binding to hepatocyte receptors, these data indicate that the HB41 epitope is located on the carboxy-terminal side of the apoB_S junction (probably within the LDL receptor binding domain). Western blotting hepatic microsomal subfractions showed that in the rough and smooth microsomes, HB41 recognized only apoB_L, while in the Golgi it recognized both apoB_L and a protein having a molecular weight slightly smaller. In contrast, Western blotting with a polyclonal antibody known to recognize both apoB_L and apoB_S showed that, in rough and smooth microsomes, proteins in addition to apoB_L and apoB_S having molecular weights between 120,000 and 30,000 were recognized. These proteins, likely to be proteolytic fragments of apoB, were barely detectable in the Golgi. Additional biosynthetic studies show that the [³⁵S]methionine-labeled proteins smaller than apoB were immunoprecipitated from the rough microsome subfraction. Pulse-chase experiments show that these are produced with the same kinetics as full-size apoB_L and apoB_S, indicating that they are not incomplete nascent chains. Finally, immunofluorescence microscopy was used to determine the localization of monoclonal epitopes. ApoB monoclonal antibodies that recognized exclusively apoB_L (HB41) and apoB_L and apoB_S (DB11) produced an immunofluorescence pattern characteristic of the endoplasmic reticulum, but not the Golgi. **■** These data suggest that, in cultured rat hepatocytes, the majority of both molecular weight forms of apoB are localized in the endoplasmic reticulum, the initial site of VLDL

assembly. The additional finding that proteolytic fragments of apoB are enriched in the microsomal fraction suggests that if the proteolysis occurs during subcellular fractionation, immature apoB is susceptible to proteolysis. Moreover, if proteolysis occurred in vivo, the data suggest that degradation of apoB in the endoplasmic reticulum may, in part, determine the portion of apoB entering the VLDL assembly/secretion pathway. —Davis, R. A., A. B. Prewett, D. C. F. Chan, J. J. Thompson, R. A. Borchardt, and W. R. Gallaher. Intrahepatic assembly of very low density lipoproteins: immunologic characterization of apolipoprotein B in lipoproteins and hepatic membrane fractions and its intracellular distribution. *J. Lipid Res.* 1989. 30: 1185–1196.

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Apolipoprotein B (apoB) is an unusually large protein that is essential for the assembly and secretion of VLDL (1–4). In the rat there are two major molecular weight forms of apoB: one having a molecular weight of 550,000 (apoB_L), the other form (apoB_S) about 48% as large (1, 2). In both rats and humans, the large form is secreted by the liver, but not the intestine (1, 2). In humans, the small molecular weight form is secreted exclusively by the intestine, whereas in rats the small form is secreted by both the liver and intestine (1, 2). Recent evidence shows that

Abbreviations: VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; apoB, apolipoprotein B; PBS, phosphate-buffered saline; SDS, sodium dodecylsulfate; DTT, dithiothreitol; HAT, hypoxanthine-aminopterin-thymidine mixture; ELISA, enzyme-linked immunosorbent assay; PAGE, polyacrylamide gel electrophoresis.

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in humans the two different molecular weight forms of apoB are derived from a single gene via a unique RNA modification that results in the formation of a stop codon and therefore a truncated translation form (apoB-48) (5–7). As predicted by the mRNA, apoB-48 is comprised of the amino terminal 48 % of apoB-100 (6). Monoclonal antibody mapping studies show that essentially all antibodies that react with human apoB-48 also react with apoB-100, whereas some monoclonal antibodies recognize apoB-100, but not apoB-48 (8–11).

The tissue-specific expression of the two major molecular weight forms of apoB implies that functional differences may exist for the amino terminal and carboxyl terminal halves. Since apoB-48 appears to be the sole protein found in intestinal lipoproteins, the amino terminal 48 % of apoB-100 is probably sufficient for lipoprotein assembly. The carboxyl terminal half of apoB-100 contains what is thought to be the unique domain responsible for recognition by the LDL receptor (3, 4). Monoclonal antibodies specific for apoB-100 block the binding of LDL to the LDL receptor on human fibroblasts (12). The epitopes recognized by these antibodies have been mapped to the LDL receptor binding domain which is located near the midpoint of the carboxyl terminal half of apoB-100 (12, 13). The additional finding that this epitope is expressed on LDL but not on VLDL has led to the suggestion that loss of lipid via lipolysis exposes the cryptic antibody epitope (and presumably the LDL receptor binding domain) (13). Variable expression of the LDL receptor binding domain on apoB-100 may play an important physiologic role via directing plasma clearance of lipolyzed VLDL particles.

To gain an understanding of rat apoB structure, we embarked on the goal of isolating monoclonal antibodies against rat apoB. These antibodies have proved useful in delineating some of the rather unique molecular features exhibited by rat apoB in serum lipoproteins, hepatic microsomal membrane fractions, and cultured hepatocytes.

MATERIALS AND METHODS

Chemicals, culture supplies, and reagents were obtained from suppliers as described (14). Male Sprague-Dawley rats were obtained from Charles River Breeding Laboratories (Wilmington, MA) and were fed chow and water ad libitum. After the rats were anesthetized with ether, the livers were excised and homogenized. Proteolytic inhibitors were added and the liver homogenate was separated into rough, smooth, and Golgi microsomal membrane fractions as described in detail (14). Individual fractions were characterized using marker enzymes as previously described (14).

BALB/c female mice were obtained from Southern Animal Farms (Prattville, AL) and fed chow and water ad libitum. They were injected intraperitoneally with 0.5

ml of Freund's complete adjuvant containing 500 μ g of apoB_L which was purified by chromatography on Sepharose CL 6B following its solubilization delipidation as described (14). After 2 weeks, the mice were boosted with 500 μ g of apoB_L in incomplete Freund's followed by a final injection of 500 μ g of apoB in 0.9 % saline. Three days later, the mice were killed by cervical dislocation and their spleens were removed, dissected, and minced using a garlic press.

Preparation of hybridomas

The BALB/c mouse myeloma line, P3x63 E.g.8.653, a nonproducing, nonsecreting recipient plasmacytoma, was obtained from J. Kearney of the University of Alabama, Birmingham. Suspension cultures were grown in 75-cm² plastic flasks containing Dulbecco's modified minimum essential medium (DMEM) and 10 % (v/v) fetal calf serum (GIBCO). Cells were seeded at 1×10^5 /ml and passed when at $2\text{--}3 \times 10^6$ /ml. Before each fusion, the cells were checked for continued sensitivity to hypoxanthine-aminopterin-thymidine (HAT-modified DMEM) and found to be stably sensitive.

Normal peritoneal macrophages were used as feeder layer cultures after initial fusion and during subcloning of hybridomas. These were obtained by killing a BALB/c mouse, injecting 5 ml of PBS intraperitoneally with a 21-gauge needle, massaging the distended abdomen for 2 min, and then withdrawing the saline. The cells were then centrifuged at low speed, washed DMEM containing 10 % fetal calf serum, and plated at a density of 10^3 cells per well in 96-well culture dishes.

Production of monoclonal antibodies

Erythrocyte-free splenocytes ($10^8\text{--}10^9$) obtained from the inoculated mice described above were mixed with 0.1 times the number of myeloma cells by gentle suspension of a mixed cell pellet for 5 min in 50 % polyethylene glycol 4000 (E. Merck, Darmstadt, Germany). The cells were gently resuspended in DMEM without serum, and then washed further by resuspension in DMEM containing 10 % fetal calf serum. The cells were plated in 96-well tissue culture plates over a lawn of 10^4 peritoneal macrophages/well, which had been plated 24 h previously. At 24 h after fusion, HAT medium was added (0.1 mM thymidine/0.1 mM hypoxanthine/0.8 μ M aminopterin) and the plates were incubated undisturbed for 2 weeks except for addition of two additional drops of HAT medium at 1 week.

Stocks of monoclonal antibody were readily obtained by harvesting the supernatant from hybridoma cultures after a minimum of 4 days in culture. In some cases, selected cultures in which antibody production appeared to be best late in the growth cycle were allowed to overgrow to up to 10^7 cells/ml and then the cells were discarded after harvesting the medium. In other instances,

particularly for immunoprecipitation from hepatocyte cell culture medium, ascites fluid was used as a source of antibody. BALB/c mice were primed with Pristane (Aldrich) 1 week prior to intraperitoneal injection of 10^7 freshly harvested hybridoma cells. When the mice became abdominally distended they were monitored daily, and the ascites fluid was collected by drainage of the peritoneal cavity with an 18-gauge needle.

Lipoprotein preparation

Lipoproteins were prepared by ultracentrifugation of serum containing 0.02% sodium azide, 0.02% EDTA, and 0.01% BHT, according to a modification of the method of Havel, Eder, and Bragdon (15), using a Beckman L5-50 centrifuge and SW40 rotor. The plasma or lipoproteins were adjusted to the appropriate density by addition of KBr. Fractions collected included $d < 1.006$ g/ml (VLDL), $1.006 < d < 1.063$ g/ml (LDL), and $1.063 < d < 1.21$ g/ml (HDL), as well as a total lipoprotein fraction, $d < 1.21$ g/ml. The lipoprotein preparations were dialyzed against 10 mM Dulbecco's phosphate-buffered saline, pH 7.4, containing 0.02% EDTA and 0.02% sodium azide. Protein determinations were made by the methods of Lowry et al. (16) and Bradford (17), using bovine serum albumin as standard.

ELISA and competitive ELISA

Lipoproteins (0.05 ml of a $d < 1.21$ g/ml fraction obtained from rat serum) containing 0.025 mg of total protein were added to each well of a 96-well polystyrene microtiter plate (Immunlon 2, Dynatech, Alexandria, VA) in 0.2 M carbonate buffer, pH 9.5, and the plate was incubated overnight at 4°C. After washing 3 times in PBS containing 0.05% Tween 20 (PBS-T), the plates could be stored wet at 4°C for weeks before use without any change in immunoreactivity (data not shown). In a given assay, a dilution of antibody was added at 37°C for 45 min, followed by washing and addition of rabbit anti-mouse IgG antibody for 45 min, washing again, and adding substrate. The enzyme substrate was 100 μ l of 2, 2'-azine-di(3-ethyl-benzthiazole sulfate) (ABTS) at 1 mg/ml in 0.015% hydrogen peroxide-0.2 M PBS. Absorbance of the reaction was read using a Bio-Rad ELISA reader. Details of each ELISA assay are described in figure and table legends.

Electrophoresis and immunoblotting

Polyacrylamide gel electrophoresis (PAGE) in linear 3.5–10% gradients employed the discontinuous buffer system of Laemmli (18). Electrophoresis and immunoblotting were performed using the techniques of Towbin et al. (19). Molecular weights were estimated by the relative migration of standards (high molecular weight obtained from Sigma Chemical Co., St. Louis, MO) using 2–20% linear gradients of polyacrylamide.

Pulse-chase studies using cultured rat hepatocytes

Hepatocytes were subjected to pulse-chase studies using [35 S]methionine, as described in detail (14). Briefly, following a 10-min pulse with translation grade [35 S]methionine, cells were chased with unlabeled methionine, harvested at the indicated times, and fractionated into rough, smooth, and Golgi microsomal membrane fractions. The individual fractions were subjected to immunoprecipitation and, following separation on SDS/PAGE, the gels were autoradiographed.

Indirect immunofluorescent microscopy

To ensure specificity of immunologic reactivity, monoclonal antibody IgGs were used (DB11 and HB41). Mouse IgGs were purified from hybridoma culture medium using a Bio-Rad MAPS IgG purification HPLC column. Rat hepatocytes, prepared as described in detail (14), were plated on glass cover-slips coated with poly-L-lysine. Cells were washed 3 times with PBS and then fixed in methanol (-20°C) for 5 min, followed by a wash in acetone (-20°C). The slides were washed again with PBS and then incubated with the indicated IgG for 1 h at 37°C in an incubator. The slides were washed again with PBS, and then incubated with a fluorescein-conjugated goat anti-mouse IgG for 1 h at 37°C. After washing with PBS, the slides were treated with anti-fade (*p*-diaminobenzene) and examined using a microscope equipped with a fluorescent light source and a camera as described (20).

RESULTS

Monoclonal antibodies to four epitopes expressed by apoB

A series of 21 hybridomas was obtained from a series of five fusions of P3x63 A8.653 cells with splenocytes derived from mice immunized with delipidated rat apoB. Of these, 11 were confirmed as apoB-specific by immunoblotting. From the entire series of 21, 7 were consistently high producers of monoclonal antibody with comparable ELISA titers, and were subjected to ELISA co-titrations to determine the number of epitopes of apoB represented in the series. Evidence for five distinct epitopes was obtained, but only some were verifiable by immunoblotting as specific for apoB. These latter are shown in Table 1. Of the four monoclonal antibodies in this co-titration experiment, 5E12 and LB14 do not result in additive binding, and thus appear to represent closely apposed or identical antigenic determinants. Antibodies DB11 and F4 result in additive binding in combination with any antibody other than themselves. This series of four monoclonals therefore recognizes three separate epitopes on apoB.

An additional monoclonal antibody, HB41, behaved quite differently in ELISA such that it could not be co-

TABLE 1. Co-titration of anti-rat apoB monoclonal antibodies

First Antibody	Second Antibody (Absorbance at 414 nm)			
	5E12	LB14	DB11	F4
5E12	(0.722)	0.792	1.226	1.139
LB14	0.838	(0.771)	1.171	1.085
DB11	1.205	1.195	(0.577)	0.935
F4	1.169	1.555	1.002	(0.475)

Increasing amounts of the indicated monoclonal antibodies were added to 96-well culture dishes which were pre-coated with $d < 1.21$ g/ml total lipoprotein fraction obtained from rat serum. From this experiment, the amount of antibody needed to give maximum reaction by ELISA was determined. This amount was used to examine whether the reaction of one monoclonal antibody interfered with the reaction of another to the $d < 1.21$ g/ml lipoprotein coat on the plate. This was determined by first adding one antibody to the pre-coated 96-well dish, incubating for 1 h at 37°C, and then washing. The second antibody was then added and incubated with the $d < 1.21$ g/ml lipoprotein-coated plate, previously incubated with the first antibody. A goat anti-mouse IgG-horseradish peroxidase-conjugated antibody was then added and the absorbance was determined as described. Each value represents the mean of three individual determinations.

titrated with the above series. However, its unique immunoblotting (Fig. 1) supports the assignment of unique epitope, for a total of four distinct epitopes among the series of antibodies confirmable as specific for rat apoB.

The immunoglobulin isotypes of antibodies representative of these four epitopes were determined by double diffusion Ouchterlony analysis, using standard anti-mouse immunoglobulin reagents. HB41 and F4 were found to be IgG1, kappa; while DB11 and LB14 were found to be IgG2a, kappa.

Specificity of monoclonal antibodies for the individual molecular weight forms of apoB

To examine expression of the monoclonal antibody epitopes by the different molecular weight forms of apoB, a $d < 1.21$ g/ml fraction of rat serum was electrophoresed and subjected to immunoblotting (Fig. 1). The results show that three of the epitopes were found on both molecular weight forms. The fourth, represented by monoclonal HB41, reacted exclusively with the large molecular form. (A 100-fold increase in the concentration of HB41 used in Fig. 1 failed to show any reactivity with the small molecular weight form; data not shown.)

Reactivity of monoclonal antibodies with lipoproteins

The recognition of VLDL and LDL particles by the four monoclonal antibodies was examined by ELISA. Since VLDL and LDL coat the plastic plates with markedly different efficiency (i.e., VLDL showed a reduced ability to adhere to the plastic dish), a competitive ELISA using a constant coat of rat $d < 1.21$ g/ml serum fraction, and a constant amount of monoclonal antibody

was developed. After reaction of the antibody with a dilution series of VLDL or LDL in suspension, the mixture was allowed to react with the lipoproteins ($d < 1.21$ g/ml) coated on the plastic 96-well dish. The antibody that was free to bind to the coated dish was then quantitated by ELISA. It should be pointed out that for a specific antibody, the same solution of antibody, lipoprotein competitor, and the same $d < 1.21$ g/ml coated 96-well plate were used. Thus, a direct comparison can be made in regard to the relative abilities of VLDL versus LDL to bind to the particular antibody. The results of the competitive ELISA are plotted as a fraction of control (absence of competing lipoprotein) versus apoB concentration of the competing lipoproteins (either VLDL or LDL) (Fig. 2). VLDL and LDL showed similar ability to compete with LB14, suggesting that the epitope recognized by LB14 is equally expressed by both VLDL and LDL. DB11 is less sensitive to competition, but is competed by both VLDL and LDL. F4 is competed with a much more shallow slope, suggesting that its epitope is not as efficiently expressed by both VLDL and LDL. In contrast, HB41 is

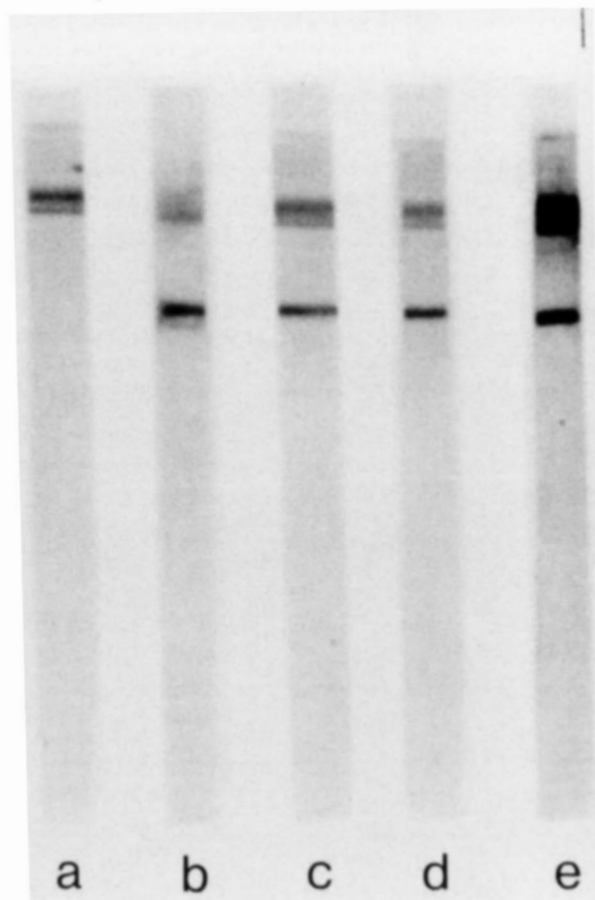


Fig. 1. Western blot analysis of rat serum lipoproteins by monoclonal and polyclonal antibodies. A total lipoprotein fraction ($d < 1.21$ g/ml) was electrophoresed using a 2–20% linear gradient of polyacrylamide. The gels were electroblotted onto nitrocellulose paper and reacted with the indicated antibody, followed by the peroxidase-conjugated IgG. Lane a, HB41; b, LB14; c, F4; d, DB11; e, polyclonal rabbit antiserum.

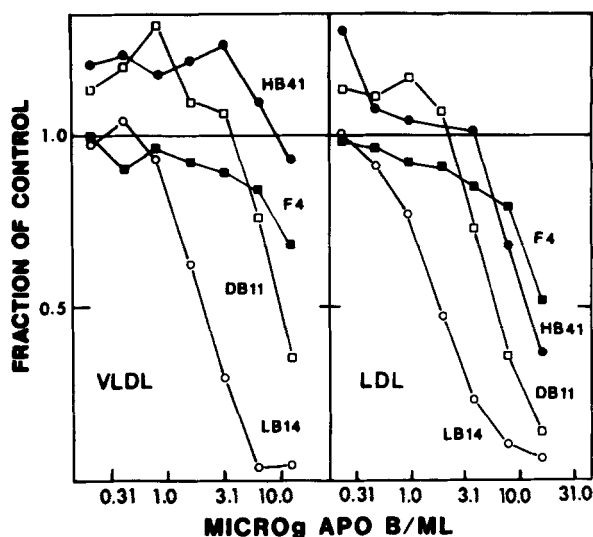


Fig. 2. Ability of VLDL and LDL to compete in ELISA assay. Microtiter 96-well plastic dishes were coated with the same $d < 1.21$ g/ml lipoprotein fraction obtained from rat serum. Aliquots obtained from the same pool of serum VLDL and LDL containing the indicated concentration of apoB were added to the incubation medium containing the indicated monoclonal antibody, which was obtained as an aliquot from the same pool of IgG. The amount of apoB (both apoB_s and apoB_L) was quantitated by determining the mass of total protein in each fraction, determining the relative portion of each apoprotein (by densitometry of Coomassie blue staining of SDS-PAGE) and calculating the mass (assuming equal staining with the dye). Following 2 h incubation at 37°C, the antibody/lipoprotein solution was added to the 96-well plastic plate precoated with $d < 1.21$ g/ml lipoproteins. The amount of antibody retained by the plastic dishes was then determined using a horseradish peroxidase-conjugated goat anti-mouse IgG. Values are expressed as a fraction of the control (no competitive lipoprotein added). Since the same solutions of antibodies, lipoprotein competitors, and $d < 1.21$ g/ml lipoprotein coat were used for each reading, for a given antibody, the results obtained with VLDL are directly comparable to the results obtained with LDL. Furthermore, it should be noted that the ratio of apoB_L/apoB_s was 1:1 for VLDL and 3:1 for LDL. Each value gives the mean of three individual determinations. Variation of each determination was less than 5%.

displaced with markedly different efficiency by VLDL and LDL: only very poorly by VLDL (6% inhibition of binding) versus competition by LDL comparable with other monoclonal antibodies (65% inhibition of binding). This experiment has been repeated three times (using different lipoprotein preparations) and the same results were obtained. Thus, there was about a tenfold difference between the ability of VLDL compared to LDL to be recognized by HB41. In some cases, addition of competing lipoprotein produced an enhancement of binding (i.e., > 1.0) due to the precipitation of antigen-antibody complexes and their sticking to the plastic dish. Densitometric quantitation of the relative amount of apoB_L and apoB_s showed that, compared to VLDL, LDL contained threefold enrichment of apoB_L. Thus, while some of the preferential reactivity of HB41 with LDL (compared to VLDL) is caused by an enrichment of apoB_L in LDL, this threefold difference cannot account for the ten-

fold difference in binding. The marked difference in the ability of VLDL and LDL to displace binding of HB41 is consistent with its epitope being expressed on LDL, while being obscured on VLDL.

Sensitivity of the HB41 epitope to SDS and DTT

Expression of the HB41 epitope was sensitive to both SDS and DTT. Addition of SDS (up to a final concentration of 0.005%) increased the expression of the HB41 epitope by 80% (Fig. 3). In marked contrast, adding the same concentration of SDS had essentially no effect on the expression of the epitopes recognized by all the other monoclonal antibodies. Concentrations of SDS of 0.01% or higher decreased the ELISA reading for all the antibodies tested. These data show that at least some HB41 epitopes are cryptic and can be exposed by treatment with SDS. Additional data show that the nonionic detergent Triton X-100 (0.1%) also increased the reactivity of VLDL toward HB41 to the same extent as that observed with SDS (data not shown).

Similar to its sensitivity toward detergents, the HB41 epitope was also uniquely sensitive to DTT (Fig. 4). Addition of DTT increased the reactivity of lipoproteins toward HB41, whereas no effect was seen on the reactivity toward the other monoclonals (data for LB14 reactivity are shown in Fig. 4). These data suggest that the epitope recognized by HB41 requires disulfide bond reduction for expression.

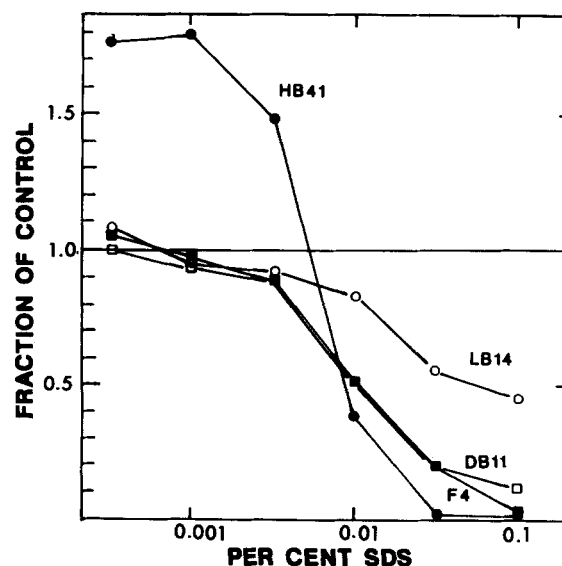


Fig. 3. Effect of SDS on reactivity of monoclonal antibodies. ELISA assays were performed as described in Fig. 2, except no competitive lipoproteins were added. SDS was added at the indicated concentration prior to adding the indicated antibody. Results are presented as the fraction of the control value (antibody addition without SDS).

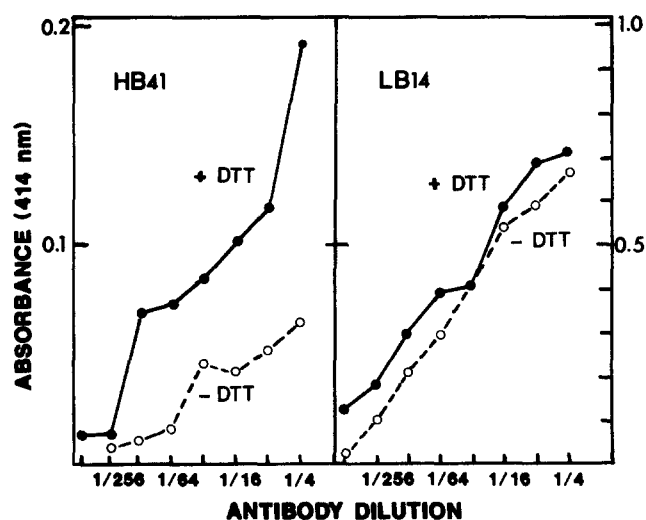


Fig. 4. Effect of DTT on reactivity of monoclonal antibodies. ELISA assays were performed as described in Fig. 3, except 5 mM DTT was added instead of SDS. Results are expressed as a fraction of control (no DTT added).

Antibody recognition of intrahepatic forms of apoB

To examine the intracellular forms of apoB recognized by the antibodies, rat liver was fractionated into subcellular microsomal membrane fractions as described (14). The enrichment of specific marker enzymes is shown in Table 2. The marker enzyme data are consistent with the designation of each fraction. Two antibodies were used for these studies: a rabbit polyclonal antiserum that recognized both molecular weight forms of apoB having both native and SDS-denatured conformations, and the apoB_L-specific monoclonal: HB41. In the rough and smooth microsomes the polyclonal antiserum recognized both apoB_L and apoB_S and several minor proteins having molecular weights between apoB_L and apoB_S, as well as several additional proteins having molecular weights between 120,000 and 30,000 (Fig. 5). One particular 60,000 dalton protein was barely detectable in the rough microsomes, greatly enriched in the smooth microsomes, and

essentially absent from the Golgi. In the Golgi fraction, the polyclonal antiserum recognized mainly apoB_L and apoB_S and a protein slightly smaller than apoB_L. In experiments directed toward examining the topography of apoB in the endoplasmic reticulum, we performed Western blot analyses of 60 different preparations of liver subcellular fractions. While the relative intensities of the bands were quite variable, in essentially all experiments bands corresponding in molecular weights of 40,000 to 60,000 were clearly present. Thus, while the relative abundance and molecular weights of the small peptides were somewhat variable from preparation to preparation, they were always found.

In the rough and smooth membrane fractions, the HB41 monoclonal antibody reacted essentially only with apoB_L (Fig. 5). In the Golgi fraction, HB41 also reacted with a protein slightly smaller than apoB_L (which is thought to be PII) (21). Control experiments using uninjected rabbit serum, monoclonal antibody (against chicken ovalbumin) that did not react with rat liver proteins, and the second antibodies only (horseradish peroxidase-linked goat anti-rabbit and mouse IgG) showed no detectable reactivity to apoB_L, apoB_S, or the 60,000 dalton protein (present mainly in the smooth microsomal fraction; data not shown).

The protein slightly smaller than apoB_L, which was recognized by both the polyclonal antiserum and HB41 (Fig. 5), is likely to be PII, described by Reuben et al. (21) as a proteolytic cleavage product of apoB_L produced distal to the Golgi in the secretion pathway. This protein (PII) contains the HB41 epitope, is a major form found in the $d < 1.21$ g/ml total lipoprotein fraction obtained from rat serum, but is not found in rough or smooth microsomal fractions (Fig. 5). The presence of PII in the Golgi fraction, but not the rough or smooth microsomes, suggests that the Golgi fraction is contaminated with endosomes while the rough and smooth microsomes are not. The existence of forms of apoB having molecular weights smaller than mature secreted apoB and sharing antigenic epitopes recognized by the polyclonal antibody is pro-

TABLE 2. Characterization of microsomal membrane fractions

Fraction	% Distribution of Protein	% Distribution of RNA	Fold-Enrichment ^a			
			UDP-Galactosyl Transferase	Glucose-6-Phosphatase	Acid Phosphatase	5'-Nucleotidase
Rough	54	77	1.5	4.1	2.3	1.7
Smooth	40	22	2.5	3.5	1.2	6.7
Golgi	6	1	56.3	1.9	3.1	11.1

Livers were homogenized and separated into membrane fractions by density gradient ultracentrifugation. Each fraction was subjected to protein, RNA, and enzyme activity analysis as described (14). Values represent mean of three individual determinations.

^aFold-enrichment was calculated as the specific activity of the designated fraction \times the specific activity of the total homogenate.

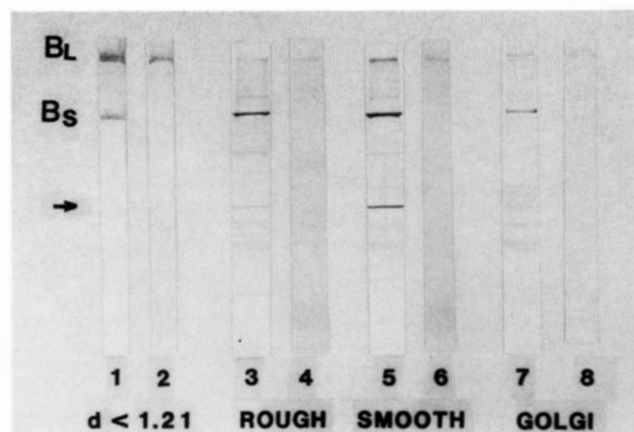


Fig. 5. Western blot analysis of hepatic microsomal fractions with apoB-specific antibodies. Livers from rats fed *ad libitum*, taken between 9 and 11 AM, were homogenized in the presence of proteolytic inhibitors, and microsomal membrane fractions (rough, smooth, and Golgi) were obtained by sucrose density ultracentrifugation. An aliquot of each fraction (rough and smooth, 0.4 mg; Golgi, 0.1 mg) which represented 3, 3.6, and 20% of the total fraction, respectively) was applied. Antibodies used were rabbit polyclonal antiserum (odd-numbered lanes) and apoB_L-specific HB41 (even-numbered lanes). Detection was obtained as described in Fig. 1. A *d* < 1.21 g/ml total lipoprotein fraction obtained from rat serum was used as a control (lanes 1 and 2). The migration of apoB_L and apoB_S is given. The arrow indicates the migration of a 67,000 dalton protein standard.

bably the result of proteolysis. The presence of these peptides in the rough and smooth microsomes suggests that they might be formed from apoB in the secretory pathway. We, therefore, examined whether these peptides would be biosynthetically labeled with [³⁵S]methionine.

Immunoprecipitation of [³⁵S]methionine-labeled apoB

Hepatocytes were pulsed for 10 min with [³⁵S]methionine and then chased with unlabeled methionine. Cells were harvested at 0, 10, 30, 45, 90, and 180 min, separated into membrane fractions, and immunoprecipitated with polyclonal antiserum. The rate constants and quantitation of the relative incorporation of [³⁵S]methionine into apoB and albumin have been reported (14). The autoradiographs of the immunoprecipitates showed that, in addition to apoB_L and apoB_S, two distinct proteins were labeled: one having an apparent molecular weight of 120,000 and another having an apparent molecular weight of 60,000 (**Fig. 6**). There were other proteins labeled to a lesser degree. Moreover, during the chase period, the 120,000 dalton band disappeared at a rate comparable to that displayed by both molecular weight forms of apoB (**Fig. 6**). In contrast, the band having a molecular weight of approximately 60,000 showed little, if any, reduction in intensity during the chase period (**Fig. 6**). These data show that neither the 120,000 dalton or 60,000 dalton proteins are incomplete nascent chains of either apoB_L or apoB_S.

Co-localization of apoB polyclonal and monoclonal epitopes in the endoplasmic reticulum of cultured rat hepatocytes

Previous studies using subcellular fractionation and immunoprecipitation show that in cultured rat hepatocytes most of the newly synthesized apoB_L and apoB_S is located in the rough and smooth microsomal fractions (14). We examined the localization of apoB_L and apoB_S in cultured rat hepatocytes using immunofluorescence. Two monoclonals were used: one that recognized both apoB_L and apoB_S (DB11) and one that only recognized apoB_L (HB41).

Cultured rat hepatocytes were fixed with organic solvents (to remove neutral lipids) and then reacted with a purified IgGs obtained from the culture medium of the designated hybridoma cell. Preliminary studies (ELISA

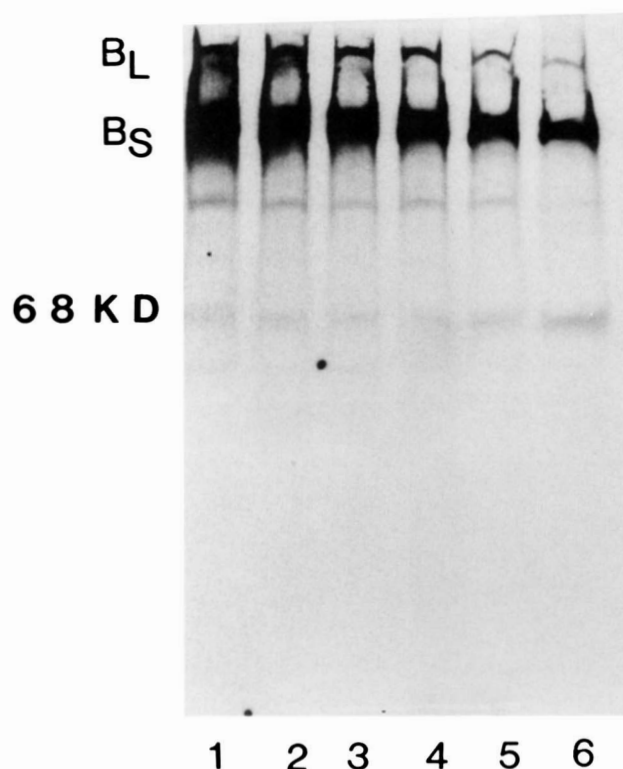


Fig. 6. Immunoprecipitation of pulse/chase [³⁵S]methionine-labeled rough microsomes. Cultured rat hepatocytes were pulsed for 10 min with 100 μ Ci/ml [³⁵S]methionine. The cells were washed and then chased with unlabeled culture medium containing a 1000-fold excess of unlabeled methionine. Cells were harvested and then fractionated into rough, smooth, and Golgi subfractions. The individual fractions were added to buffers containing 0.1% SDS, boiled, cooled to room temperature, and immunoprecipitated with rabbit polyclonal antiserum specific for apoB (see Western blot in Fig. 5). Following separation on a 2–20% linear gradient of polyacrylamide, the gels were dried and subjected to autoradiography for 2 weeks. The designation of standards of apoB_L and apoB_S are shown. An estimation of 68,000 daltons (determined from the migration of protein standards) is also shown. The following samples are shown: lane 1, 0-min time point; lane 2, 10-min chase; lane 3, 20-min chase; lane 4, 45-min chase; lane 5, 90-min chase; lane 6, 180-min chase.

assay) show that this treatment increases the expression of the monoclonal antibody epitopes. The cells were then treated with fluorescein-conjugated second antibody. Fluorescence microscopy afforded a distribution of apoB epitopes in cultured rat hepatocytes. Most of the immunofluorescence pattern produced with DB11, which recognizes both apoB_L and apoB_S, was localized throughout the cytoplasm (**Fig. 7A**), characteristic of the fluorescence produced by antibodies to the endoplasmic reticulum

(22). In addition, the immunofluorescence pattern produced by apoB_L-specific monoclonal IgG HB41 (**Fig. 7B**) was the same as that produced by DB11. To show that our immunofluorescence microscopy technique and reagents could discriminate Golgi, hepatocytes from the same preparation were reacted with a monoclonal antibody found to be specific for Golgi (characterized by D. C. F. Chan). The immunofluorescence pattern of Golgi-specific antibody was clearly different from that produced with

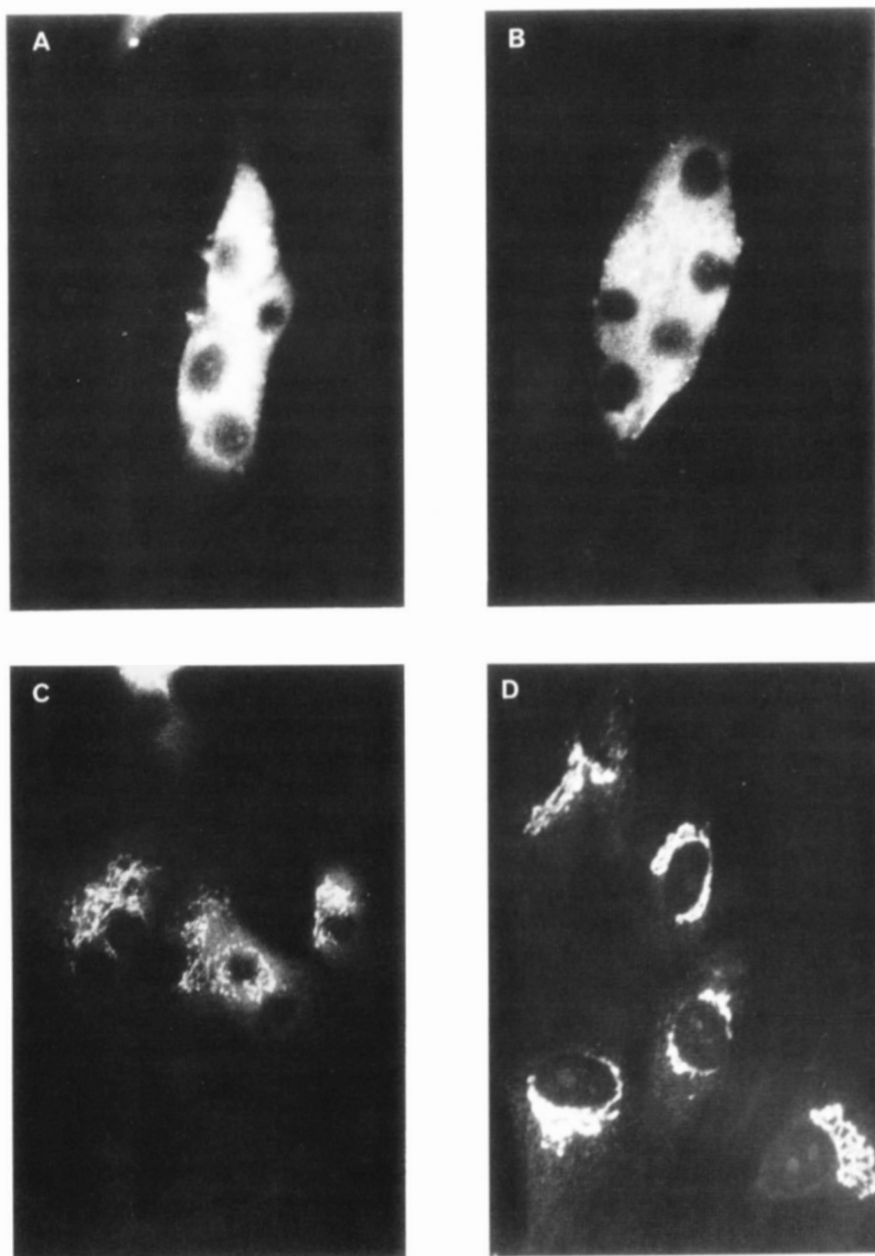


Fig. 7. Immunofluorescence microscopy of using monoclonal antibodies. Immunofluorescence studies were carried out as described in Methods. Cultured rat hepatocytes were reacted with the following monoclonal IgGs: DB11 (frame A), HB41 (frame B), and Golgi specific monoclonal 6A3 (frame C). For further comparison, the Golgi-specific monoclonal antibody 6A3 was also reacted with human skin fibroblasts (frame D).

either apoB antibody (Fig. 7C). Finally, the Golgi-specific antibody was reacted with human fibroblasts (Fig. 7D), again producing a pattern characteristic of Golgi (22). Thus, the Golgi-specific antibody recognizes a protein that is expressed by both fibroblasts and liver. Moreover, the data show that in cultured rat hepatocytes, epitopes expressed by both apoB_L and apoB_S are localized to the endoplasmic reticulum.

DISCUSSION

The major goal of this research was to produce monoclonal antibody reagents with which to probe the structure and localization of apoB in lipoproteins, hepatic membranes, and in liver cells. Consistent with previous studies using monoclonal antibodies directed against human apoB (8–11), all antibodies that recognize small molecular weight apoB also recognize large molecular weight apoB (i.e., LB14, F4, and DB11). In contrast, a monoclonal antibody (HB41) was obtained that recognized large molecular weight apoB, but did not react with small molecular weight apoB. These data are consistent with sequence analysis which shows that the small molecular weight form of apoB encompasses the amino-terminal domain which extends approximately to the midpoint of apoB_L (6). Additional data show that the apoB_L-specific antibody (HB41) recognizes a cryptic domain in VLDL, which upon treatment with detergents (SDS and Triton X-100) becomes accessible. Furthermore, the HB41 epitope show the unusual characteristic of being sensitive to DTT: treatment of lipoproteins causes a substantial increase in the expression of the HB41 epitope. Studies of the LDL receptor binding domain of apoB using monoclonal antibodies that recognize epitopes in this portion of apoB-100 show that some of the epitopes are poorly expressed on VLDL and are expressed more efficiently on LDL (13). Detailed analysis of the amino acid sequence of human apoB-100 suggests that the LDL receptor binding domain is likely to lie on the carboxyl terminal third of the molecule encompassing a thrombin cleavage site (the T2/T3 junction) (3, 4, 12, 13). Monoclonal antibody epitopes lying within this area of human apoB-100 show a dependency on lipid for expression (12) and are thought to be encompassed by an intramolecular disulfide bond (4). However, studies on pig apoB indicate that the putative disulfide bridge is not required for recognition by the LDL receptor (23). Studies characterizing the lipoprotein receptors on cultured rat hepatocytes show that HB41 blocks the specific binding of ¹²⁵I-labeled LDL (L. Junker and R. Davis, unpublished data). While we have not yet determined the amino acid sequence of the HB41 epitope, the combined data suggest that it lies proximal to or within the LDL receptor binding domain

on apoB_L. Furthermore, in regard to apoB structure/function relationships, the differential expression of the HB41 epitope on VLDL and LDL, probably in response to lipid (Figs. 2 and 3) mimics the expression of the LDL receptor binding domain epitopes reported for human apoB-100 (13).

The specificity of the monoclonal antibody (HB41) allowed us to examine the expression by intracellular membranes of the apoB_L epitope. Consistent with previous results obtained using cultured rat hepatocytes, apoB was found in the rough, smooth, and Golgi microsomal fractions (Fig. 5). However, there were consistent differences in the molecular weight of the peptides that reacted with different antibodies. In the rough and smooth microsomal fractions there were two major proteins that reacted with the polyclonal antibody: apoB_L and apoB_S. Moreover, the smooth microsomes were found to be enriched (relative to the Golgi fraction) with several peptides smaller than apoB which react with the apoB-specific antiserum. Since no PII, a major plasma form produced from apoB_L prior to secretion in the Golgi (21), was detected in either the rough or smooth microsomal fractions, it is unlikely that the rough and smooth microsomal fractions are contaminated with endosomes. In marked contrast, the Golgi fractions contained a different spectrum of immunoreactive proteins: proteins the size of apoB_L, PII, and apoB_S were detected by the polyclonal antibody, whereas the 60,000 dalton protein was not (Fig. 5). Reuben et al. (21) failed to detect PII in the Golgi fraction, and concluded that the proteolytic cleavage producing PII may have occurred beyond the Golgi but prior to secretion. Our finding of an immunoreactive protein similar in size to PII in the Golgi membrane fraction might be due to our different method of isolation. Also, it is possible that the different Golgi membrane fractions may have contained different amounts of multivesicular bodies (24), and that some of the PII might represent the endocytotic pathway rather than the secretion pathway.

To determine the origin of the truncated forms of apoB, fluorograms of apoB immunoprecipitated from pulse-chase experiments (Fig. 6 of ref. 14) were analyzed. In addition to apoB_L and apoB_S, two major and several minor labeled proteins were present. One having an apparent molecular weight of 120,000 disappeared with kinetics similar to that for apoB. Another having a molecular weight close to 60,000, displayed a much slower rate of decrease during the chase period. These data indicate that neither of the small peptides immunoprecipitated by the polyclonal antiserum are likely to be precursors to mature apoB. It is more likely that the small peptides are either small translation products of the apoB mRNA, produced by modification of the initial transcription product, or that they are proteolytic products. [³⁵S]Methionine-labeled peptides smaller than apoB that are specifically

immunoprecipitated by apoB-specific antibodies appear in the autoradiograms reported by Reuben et al. (Fig. 5 of ref. 21), suggesting that these peptides are not unique to our experimental systems.

Since the 60,000 dalton protein found in the rough and smooth microsomal fraction reacted only with the polyclonal antiserum (not the apoB_L-specific HB41 monoclonal antibody), this peptide is a truncated form of apoB which does not contain the HB41 epitope. Since it is not possible to completely rule out proteolysis (even when several proteolytic inhibitors have been added to the buffers), we cannot determine whether the truncated forms of apoB are produced *in vivo* or during the membrane isolation step. However, the presence in the rough and smooth, but not the Golgi, microsomal fractions of the major truncated form of apoB (having a molecular weight of 60,000) suggests that apoB in the early portion of the secretory pathway (i.e., rough and smooth endoplasmic reticulum) is susceptible to proteolysis, whereas in the distal portion (i.e., Golgi) it is not. Since in the same membrane fractions there are no detectable immunoreactive forms of albumin, apoE, or apoA-IV, which are smaller than their mature secretory forms (data not shown), the proteolysis of apoB appears selective.

In previous studies, we have found that only a portion of *de novo* synthesized apoB is secreted; the remainder appears to be degraded intracellularly (14). We have proposed that intracellular degradation is one mechanism responsible for determining apoB secretion rates (14). While the finding of a selective presence of proteolytic fragments of apoB in the rough and smooth, but not the Golgi, microsomes is consistent with our hypothesis that the site of apoB degradation is in the endoplasmic reticulum, more data are needed to prove this point.

Previous pulse-chase studies showed that, in cultured rat hepatocytes, the rate of movement out of the endoplasmic reticulum is rate-limiting for secretion (14). Furthermore, quantitation of [³⁵S]methionine steady-state labeled apoB in subcellular fractions obtained by density gradient ultracentrifugation showed that most of the label was in the rough and smooth microsomal fraction (14). In contrast, other hepatocyte culture models (i.e., HepG2 (25) and estrogen-stimulated chicken cells (26)) display kinetics consistent with the proposal that movement out of the Golgi is rate-limiting for secretion. The controversy regarding the rate-limiting step for secretion includes both apoB and albumin. The kinetics displayed by cultured rat hepatocytes showing that movement out of the endoplasmic reticulum is the slowest step is consistent with the kinetics displayed by most secretory proteins (27) and several other culture models including rat hepatoma cells (28) and human HepG2 cells (29). Since two different results have been obtained using the same line of hepatoma cells (i.e., HepG2 (25, 29)), the

differences cannot be ascribed to different culture models.

A major goal of this study was use of the specific nature of monoclonal antibodies to probe the intrahepatic localization of apoB. To rule out the possibility that membrane fraction cross-contamination (an inherent short-coming of cell fractionation techniques) may have been responsible for the intrahepatic distribution of *de novo* synthesized apoB showing that the majority was in the endoplasmic reticulum of cultured rat hepatocytes, we used immunofluorescence microscopy. The immunofluorescence data obtained using monoclonal antibodies that recognize both apoB_L and apoB_S (DB11) and the apoB_L-specific monoclonal HB41 showed that the majority of apoB epitopes produce a fluorescence distribution consistent with the endoplasmic reticulum but not the Golgi (Fig. 7A, B). With the proviso that apoB epitopes are uniformly accessible to the antibody reagents, our findings are consistent with the proposal that most of the apoB in cultured rat hepatocytes resides in the endoplasmic reticulum. Furthermore, using the same methods but a Golgi-specific monoclonal antibody, we were able to obtain a fluorescence pattern characteristic of Golgi (Fig. 7C). Thus, our methods can discriminate the immunofluorescence pattern of a Golgi-specific epitope.

Earlier studies by Alexander, Hamilton, and Havel (30) show that immunoreactive apoB was detected in the rough endoplasmic reticulum unassociated with VLDL particles. Moreover, apoB was found associated with VLDL in junctional complex between the rough and smooth endoplasmic reticulum (30). Their data showed, for the first time, that VLDL is assembled with the endoplasmic reticulum. The molecular processes through which apoB orchestrates the assembly of a VLDL in the endoplasmic reticulum remain poorly defined. Our findings that in cultured rat hepatocytes movement out of the endoplasmic reticulum is the slowest step in VLDL secretion (14) and that most of the apoB is located in the endoplasmic reticulum are consistent with hypothesis that assembly of the VLDL particle (in the endoplasmic reticulum) is rate-limiting for secretion. The additional finding of this study showing the selective presence of proteolytically cleaved apoB fragments in the rough and smooth, but not the Golgi, microsomal fractions suggests that a form of apoB residing in the endoplasmic reticulum is accessible to proteolysis. Attempts to inhibit this proteolysis with proteolytic inhibitors have been unsuccessful (data not shown). If the proteolysis takes place during the membrane isolation, the susceptibility of this form of apoB to proteolysis implies a unique molecular feature of the immature form of apoB present in the endoplasmic reticulum. If, on the other hand, the proteolysis takes place *in vivo*, degradation of this form of apoB may play a fundamental role in regulating VLDL assembly/secretion. ■

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