Topography of apolipoprotein B in subcellular fractions of rabbit liver probed with a panel of monoclonal antibodies

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Abstract We have used a panel of anti-rabbit apolipoprotein B monoclonal antibodies in a competitive ELISA to probe the availability of apoB in rough endoplasmic reticulum, smooth endoplasmic reticulum, *cis*-enriched Golgi, and *trans*-enriched Golgi fractions from rabbit liver. The ability of each subcellular fraction to inhibit binding of monoclonal antibody to immobilized low density lipoprotein (LDL)-apoB was determined and compared with the expected inhibition based on the apoB content of the fraction. The vesicles remained closed during ELISA, demonstrated by monitoring loss of radiolabeled secretory proteins from the lumen and by measuring leakage of albumin from the vesicles. In control experiments, vesicles were permeabilized using 0.4% taurocholate. All epitopes of apoB were fully expressed in closed *trans*-Golgi vesicles, indicating that the membrane-bound apoB is at the cytosolic side of this fraction. In the smooth endoplasmic reticulum the epitopes were expressed between 55 and 70%, suggesting that the two pools of apoB may exist in these membranes. These results suggest that newly synthesized apoB has two possible fates. It may be incorporated into the cytosolic side of the endoplasmic reticulum from where it moves to the cytosolic side of the Golgi membrane, or newly synthesized apoB may be translocated to the lumenal surface of the endoplasmic reticulum membrane followed by assembly with lipids for secretion.—**Wilkinson, J., J. A. Higgins, P. Groot, E. Gherardi, and D. Bowyer.** Topography of apolipoprotein B in subcellular fractions of rabbit liver probed with a panel of monoclonal antibodies. *J. Lipid Res.* 1993. **34:** 815–825.

Supplementary key words smooth endoplasmic reticulum • rough endoplasmic reticulum • Golgi fractions • membrane • lumen • ELISA

Apolipoprotein B (apoB) is the major nonexchangable protein of very low density lipoproteins (VLDL) and is essential for their assembly and secretion by liver. ApoB is the ligand for the LDL receptor and therefore plays a crucial role in cholesterol homeostasis. Although apoB has a signal sequence and is synthesized by bound ribosomes, it does not behave as a typical secreted protein. In rat liver, apoB is present in a membrane-associated pool and a lumenal pool in the endoplasmic reticulum (1–7) and in the Golgi compartment (7). Recently we have demonstrated that apoB is also present in membrane-bound and lumenal pools in endoplasmic reticulum and Golgi fractions of rabbit liver (8). ApoB appears to be produced in excess and the protein not packaged with VLDL for secretion is degraded intracellularly (1, 6, 9–12).

ApoB is a large and hydrophobic protein and its insertion into and translocation across the endoplasmic reticulum membrane, its assembly with lipids to form VLDL, and its transit through the secretory compartment pose special problems. To examine the mechanisms involved in apoB translocation we have now investigated the topography of apoB in isolated endoplasmic reticulum and Golgi fractions. For these studies we have used a panel of monoclonal antibodies to probe the availability of apoB in intact subcellular fractions and in subcellular fractions permeabilized with taurocholate. The monoclonal antibodies used recognize epitopes throughout the length of the apoB molecule. That for MAC 31 is in the N-terminal 48%, that for MAC 22 in the N-terminal 48–74%, and those for MAC 27, 28, and 29 are in the C-terminal 25% (13).

Abbreviations: VLDL, very low density lipoproteins; LDL, low density lipoproteins; SER, smooth endoplasmic reticulum; RER, rough endoplasmic reticulum; TER, total microsomes; *cis*-Golgi, *cis*-enriched Golgi; *trans*-Golgi, *trans*-enriched Golgi.

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MATERIALS AND METHODS

Fractions

Subcellular fractions, rough endoplasmic reticulum (RER), smooth endoplasmic reticulum (SER), *cis*-enriched Golgi (*cis*-Golgi) and *trans*-enriched Golgi (*trans*-Golgi), and total microsomes (TER), were prepared from livers of rabbits (dwarf lop-ear or small New Zealand white, approximately 2 kg body weight) as described previously (8) using methods exactly similar to those developed for rat liver (14–18) except that a cocktail of protease inhibitors was added to the homogenate and the subcellular fractions during preparation to minimize proteolysis (7). Electron microscopy, cytochemistry, immunochemistry, and functional studies indicate that these fractions consist of membrane vesicles or intact organelles with the same orientation as in situ (that is, the outside of the vesicle corresponds to the cytosolic side of the organelle) (14, 16, 17, 19–25). Each vesicular fraction was opened and separated into the membrane component (membrane fraction) and the lumenal contents (content fraction) which are derived from the cisternal contents of the organelle in situ (7, 15–18).

Antibodies

Primary antibodies were sheep anti-rabbit apoB (13), sheep anti-human apoB (Boerhinger), goat anti-human albumin (Sigma), and a panel of monoclonal antibodies (MAC 22, MAC 27, MAC 28, MAC 29, and MAC 31) raised against rabbit apoB (13). Nonimmune sheep serum (Scottish Antibodies Ltd.) was used in control experiments in place of sheep anti-apoB.

Secondary antibodies were donkey anti-sheep IgG (Scottish Antibodies Ltd.) for immunoprecipitation, donkey anti-sheep IgG coupled to alkaline phosphatase (Sigma) for studies with polyclonal antibodies, and goat anti-rat IgG coupled to alkaline phosphatase (Sigma) for studies with monoclonal antibodies.

Standards

LDL standards (d 1.019–1.063 g/ml) were prepared from rabbit blood as described previously (8). These were shown by SDS-PAGE to contain only one major band corresponding to apoB and a very faint band corresponding to apoE.

Enzyme-linked immunosorbent assay (ELISA) of apoB

LDL (0.53 μg apoB per well) in 0.1 M bicarbonate buffer, pH 9.6, were placed in the wells of ELISA plates (Nunc). The plates were sealed in plastic bags and left at room temperature overnight. The wells were aspirated and washed four times with wash buffer [Tris-buffered saline, pH 7.4, containing 0.05% (v/v) Tween 20 and 0.5% (w/v) RIA grade bovine serum albumin (BSA)] followed by blocking with Tris-buffered saline containing 3% RIA grade BSA for 2 h. The wells were aspirated and 100 μl of primary antibody (dilutions up to 1:200000) in Tris-buffered saline containing 1% RIA grade BSA (incubation buffer) was added together with 50 μl buffer, 50 μl LDL (0.1–150 μg/ml) or 50 μl unknown fraction (plasma or subcellular fraction) and incubated overnight with occasional shaking. The wells were aspirated and washed four times with wash buffer and 150 μl of secondary antibody diluted 1 to 1000 in incubation buffer was added. The plates were incubated at room temperature with shaking for 4 h; the wells were aspirated and washed four times in wash buffer followed by a wash in 0.1 M glycine buffer, pH 10.4, containing 1 mM $MgCl_2$ and 1 mM $ZnCl_2$. Bound alkaline phosphatase was measured by addition of *p*-nitro-phenylphosphate (Sigma). In initial experiments the linearity of the reaction was determined and in subsequent experiments the reaction was terminated after 30 min by addition of 50 μl of 0.1 M EDTA; the color was read immediately at 405 nm on a Denley plate reader.

Antibody dilution curves were prepared using a range of primary antibody concentrations. Antibody dilutions that gave 75–50% of the maximum binding to immobilized LDL were selected for subsequent assays.

Competition curves were prepared using a range of LDL-apoB concentrations. In some experiments 0.4% taurocholate was added to the incubation buffer.

Determination of apoB in unknown samples was performed as above. A competition curve was always performed simultaneously. In some experiments 0.4% taurocholate was added to the incubation buffer to permeabilize the subcellular fractions.

Determination of the integrity of subcellular fractions during ELISA

Leakage of lumenal radiolabelled proteins. The lumenal proteins were preferentially labeled by intraportal injection of [^{3}H]leucine (50 μC/kg) 30 min before removal of the liver and preparation of subcellular fractions (15, 16, 26). After ELISA the subcellular fractions from triplicate wells were removed, pooled, diluted to 10 ml with 0.25 M sucrose, and centrifuged at 100000 *g* for 45 min. Aliquots of the supernatants and the resuspended pellets were precipitated with an equal volume of 15% trichloroacetic acid. The precipitates were isolated by centrifugation, dissolved in tissue solubilizer (Beckman), and counted. In control experiments 0.4% taurocholate was added to the incubation buffer for ELISA, or subcellular fractions were suspended at the same concentration in 0.25 M sucrose and treated in the same way omitting the ELISA incubation step.

Leakage of lumenal albumin. The amount of albumin that had leaked from subcellular fractions during incubation in the absence (nonpermeabilized vesicles) and presence

(permeabilized vesicles) of 0.4% taurocholate was determined by ELISA. The method used was as described above for ELISA of apoB except that crystalline human albumin (15 ng/well) replaced immobilized LDL, gelatin replaced BSA in all buffers, and the primary antibody was goat anti-human albumin. In preliminary experiments it was demonstrated by immunoblotting that the anti-human antibody cross-reacts with rabbit serum albumin.

Immunoprecipitation of apoB from lumenal content fractions

The association of radiolabeled lipids with apoB in the content fractions from subcellular fractions was determined by immunoprecipitation as described previously (18). Briefly, lumenal lipids were prelabeled with [^{3}H]palmitic acid by intraportal injection 30 min prior to removal of the liver and preparation of subcellular fractions (15, 16, 18). Content fractions were prepared (8), dialyzed, concentrated by ultrafiltration, and the apoB was immunoprecipitated. The lipids were extracted and radioactivity was determined by scintillation counting.

RESULTS

Preparation of subcellular fractions

The preparation and characterization of subcellular fractions from rabbit liver have been described in detail elsewhere (8). The methods used were based on those originally developed for rat liver and yielded essentially similar fractions (14, 15–18). The purity of fractions was determined by assay of galactosyltransferase as a marker for Golgi membranes, and NADPH-cytochrome c reductase as a marker for the endoplasmic reticulum. Although marker enzymes have a primary location in one cell component, they may also be detected in lower amounts in other membranes. For example, Howell, Ito, and Palade (19) have demonstrated that the specific activity of supposed endoplasmic reticulum marker enzymes in the Golgi preparations can be as high as 30% of the activity in endoplasmic reticulum preparations. Immunocytochemical studies have also shown that morphologically recognizable Golgi preparations contain NADPH cytochrome c reductase and 5' nucleotidase (20). However, assuming that NADPH-cytochrome c is exclusively in the endoplasmic reticulum and galactosyltransferase is exclusively in the Golgi elements, it is possible to calculate the maximum cross-contamination between fractions (**Table 1**). Thus, the maximum contribution of protein from the endoplasmic reticulum to the *trans*-Golgi was 7.0% and to the *cis*-Golgi, 30.4%; the maximum amount of Golgi-derived protein in the RER was 4.4%, the TER, 3.9%, and the SER, 14.5%. The significance of this in relation to the experimental results is discussed below.

TABLE 1. Cross-contamination among subcellular fractions

Subcellular Fraction	Contaminating Fraction: Endoplasmic Reticulum	Contaminating Fraction: Golgi
	%	
Total microsomes		3.9
Smooth endoplasmic reticulum		14.5
Rough endoplasmic reticulum		4.4
trans-Golgi	7.0	
cis-Golgi	30.4	

Subcellular fractions were prepared (8) and the maximum cross-contamination among fractions was determined using NADPH-cytochrome c reductase as a marker for endoplasmic reticulum and galactosyltransferase as a marker for Golgi. The maximum percent of each fraction protein contributed by the contaminating organelle was calculated from data in ref. 8.

Determination of apoB by ELISA

Similar competition curves were obtained for LDL apoB assayed in the absence and the presence of taurocholate (**Fig. 1**). The amount of unbound LDL-apoB that competed 50% with a fixed amount of immobilized apoB (0.53 μg/well) was 0.035 μg LDL apoB/well with MAC 28, 0.3 μg LDL apoB/well with MAC 31, 0.6 μg LDL apoB/well with MAC 27, 1.0 μg LDL apoB/well with MAC 29, and 1.58 μg LDL apoB/well with MAC 22. Fifty percent inhibition occurred at 0.6 μg LDL apoB/well with the polyclonal antibody. The expression of epitopes, therefore, differs between bound and unbound LDL apoB. Those for MAC 28 and MAC 31 are shielded in the immobilized LDL, while those for MAC 29 and MAC 22 are more available in immobilized LDL.

In initial experiments the ability of subcellular fractions to compete with immobilized LDL-apoB was determined. Competition curves for all subcellular fractions in the presence and absence of taurocholate were parallel with the LDL competition curves performed at the same time. Data for MAC 28 are shown in **Fig. 2.** Similar results were obtained for all antibodies.

The apoB content of subcellular fractions measured in the presence of taurocholate using the polyclonal antiserum was comparable with the values obtained using a radioimmunoassay in the presence of 0.5% Triton and 0.5% deoxycholate (**Table 2**) as described previously. The proportion of apoB in the membrane and content fractions was determined by RIA as described previously (8) and is shown in Table 2.

Expression of epitopes of apoB at the cytosolic surface of subcellular fractions

The ability of apoB in subcellular fractions to compete with immobilized LDL was determined by ELISA

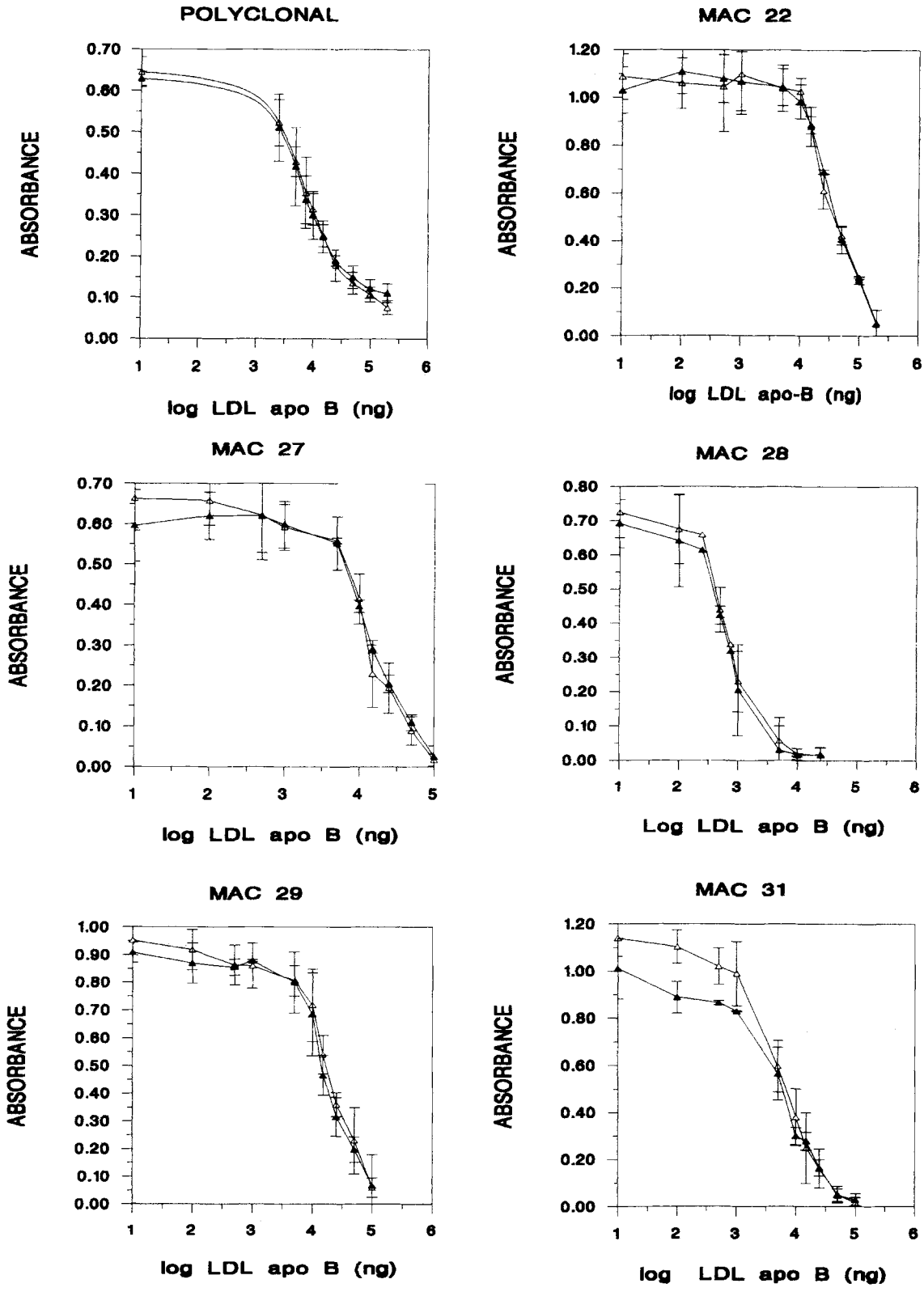

Fig. 1. Competition curves for assay of LDL apoB by monoclonal and polyclonal anti-rabbit apoB. ELISA assays were performed and competition curves were generated as described in Methods; (△), presence of taurocholate; (▲) absence of taurocholate. The absorbance at 405 nm is plotted against the log of the concentration of the stock LDL apoB (ng/ml). Because 50 μl of LDL suspension was used in each assay, the amount of apoB in each well is one-twentieth of the concentration used.

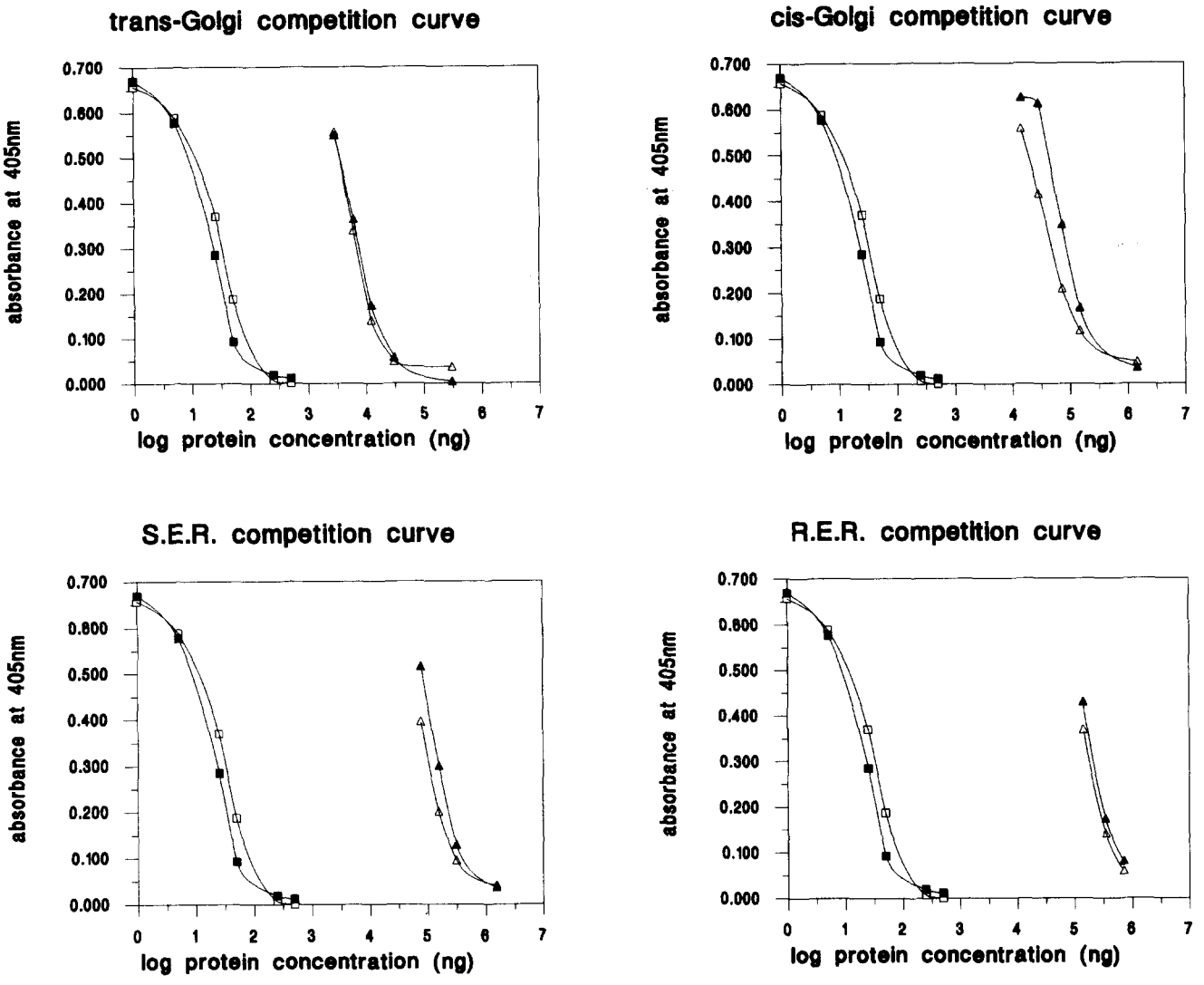

Fig. 2. Ability of subcellular fractions to compete with immobilized LDL for MAC 28. ELISA assays were performed and competition curves were generated for LDL, RER, SER, *trans*-Golgi, and *cis*-Golgi as described in Methods. All curves were generated at the same time to allow comparison. The absorbance at 405 nm is plotted against the log of the concentration of the protein (LDL apoB or subcellular fraction) (ng/ml). Open symbols denote the presence of taurocholate (0.4%); solid symbols denote the absence of taurocholate; LDL-apoB (□, ■); subcellular fraction (△, ▲).

TABLE 2. Determination of apolipoprotein B in subcellular fractions by radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA)

Assay	Rough Endoplasmic Reticulum	Smooth Endoplasmic Reticulum	*trans*-Golgi	*cis*-Golgi
		μg apoB/mg of fraction protein		
ELISA	1.23 ± 0.85	2.24 ± 0.69	11.85 ± 2.10	2.77 ± 0.78
RIA				
Total subcellular fraction	1.65 ± 0.32	2.43 ± 0.37	12.93 ± 0.46	3.00 ± 0.80
Membrane fractions	1.03 ± 0.14	1.01 ± 0.28	2.94 ± 0.24	1.48 ± 0.36
Content fractions	0.71 ± 0.23	1.01 ± 0.34	6.51 ± 1.33	1.00 ± 0.58

The apoB content of subcellular fractions was determined by RIA as described previously (8) using a polyclonal sheep anti-rabbit apoB and by ELISA as described in Methods using the same antibody. The values are the average of three separate experiments with two to five values determined in each experiment and averaged. Results are μg apoB per mg of fraction protein ± standard deviation. The apoB in the total homogenate determined by RIA was 0.66 ± 0.061 and by ELISA was 0.57 ± 0.06 μg/mg protein.

(**Table 3**). The amount of apoB in each fraction and the proportion of this in the membrane component were calculated from the data in Table 2. The % inhibition that would be expected if all of the membrane bound pool of apoB is fully exposed at the surface of the vesicles was calculated from the competition curves for LDL shown in Fig. 1. This was compared with the actual competition observed (Table 3, **Fig. 3**). The ratio of actual inhibition to theoretical inhibition is an indicator of the expression of an epitope at the cytosolic surface of the subcellular fraction. A ratio of one or more indicates that the epitope is fully exposed and a ratio of less than one indicates that the epitope is partly shielded or in more than one pool. A ratio of zero (or no inhibition) indicates that the epitope is not exposed at the cytosolic surface of the vesicle.

All five epitopes of apoB were expressed in the *trans*-Golgi fraction. In each case there was no significant difference between the inhibition observed and that expected. In contrast, all epitopes in the SER were partly shielded showing ratios of observed inhibition/theoretical inhibition of between 55 and 70%. In the RER, all epitopes except that for MAC 22 were partly shielded, whilst in the *cis*-Golgi all except that for MAC 31 were similarly partly obscured. Considered simply, these observations suggest that in the RER and SER and *cis*-Golgi membranes apoB is either in two pools, one at the cytosolic surface of the membrane and one at the cisternal surface, or that the topography of the protein differs among the components of the secretory pathway.

In taurocholate-permeabilized vesicles, both surfaces of the membrane are exposed in addition to apoB in the lumen of the vesicles (Table 3 and Fig. 3). In the presence of taurocholate there was generally an increase in the expression of each epitope in each fraction. In the case of MAC 31 and MAC 22, the epitopes were fully expressed in permeabilized vesicles. However, in the case of MAC 29, MAC 27, and MAC 28, epitope expression varied among different fractions, suggesting that the epitopes are differently expressed in the membrane and content fractions and are not fully unmasked by detergent treatment.

TABLE 3. Availability of epitopes of apolipoprotein B to a panel of anti-apoB monoclonal antibodies

	Closed Vesicles (minus detergent)			Opened Vesicles (plus detergent)		
	% Inhibition	% Expected Inhibition	Ratio	% Inhibition	% Expected Inhibition	Ratio
RER						
MAC 31	19.14 ± 3.05	58.42*	0.33 ± 0.05	72.70 ± 4.82	72.80	0.99 ± 0.07
MAC 28	48.68 ± 4.57	97.11*	0.50 ± 0.05	64.82 ± 7.38	97.71**	0.66 ± 0.07
MAC 29	15.08 ± 3.00	22.09***	0.68 ± 0.14	37.18 ± 4.92	32.43	1.14 ± 0.15
MAC 27	19.65 ± 3.31	33.60**	0.58 ± 0.10	24.12 ± 2.29	52.11	0.41 ± 0.04
MAC 22	15.74 ± 5.84	12.61	1.25 ± 0.46	16.38 ± 5.13	23.15	0.70 ± 0.11
SER						
MAC 31	50.93 ± 3.73	90.59*	0.56 ± 0.04	93.98 ± 6.96	91.88	1.02 ± 0.08
MAC 28	63.61 ± 4.56	96.63*	0.66 ± 0.05	76.94 ± 4.61	98.62**	0.78 ± 0.05
MAC 29	37.65 ± 2.54	66.29*	0.57 ± 0.04	58.40 ± 2.15	81.62*	0.72 ± 0.02
MAC 27	48.85 ± 8.44	72.40***	0.67 ± 0.12	74.37 ± 5.71	89.82***	0.83 ± 0.05
MAC 22	26.68 ± 3.67	37.96**	0.70 ± 0.09	57.05 ± 6.54	66.66	0.86 ± 0.12
cis-Golgi						
MAC 31	98.40 ± 2.26	93.31	1.05 ± 0.02	97.98 ± 3.30	97.36	1.00 ± 0.04
MAC 28	26.40 ± 8.13	87.94*	0.30 ± 0.09	69.37 ± 9.04	89.43***	0.78 ± 0.10
MAC 29	44.43 ± 5.55	77.34*	0.57 ± 0.07	59.25 ± 4.25	85.94*	0.69 ± 0.04
MAC 27	52.80 ± 2.23	81.20*	0.66 ± 0.03	74.04 ± 7.79	92.46	0.80 ± 0.08
MAC 22	36.94 ± 4.75	48.63***	0.76 ± 0.10	69.05 ± 2.85	71.75	0.96 ± 0.04
trans-Golgi						
MAC 31	63.78 ± 6.17	58.41	1.09 ± 0.11	89.07 ± 5.46	86.84	1.03 ± 0.06
MAC 28	44.64 ± 2.99	42.12	1.06 ± 0.07	51.89 ± 3.02	76.09*	0.69 ± 0.04
MAC 29	16.99 ± 3.69	21.54	0.79 ± 0.17	47.78 ± 6.43	51.35	0.93 ± 0.12
MAC 27	41.50 ± 3.87	33.20	1.25 ± 0.12	73.38 ± 5.69	72.85	1.00 ± 0.08
MAC 22	16.14 ± 5.35	12.34	1.31 ± 0.43	48.07 ± 5.12	42.12	1.14 ± 0.12

The ability of subcellular fractions to compete with immobilized LDL-apoB for a limited amount of monoclonal antibody was determined as described in Methods. In some experiments, no detergent was added (Closed Vesicles) and in others 0.4% taurocholate was added to the incubation buffer (Opened Vesicles). The % inhibition of binding of antibody to immobilized LDL was determined. In each assay a competition curve was prepared using LDL. In preliminary experiments a range of dilutions of subcellular fraction was used to determine the concentration required to record inhibition on the linear part of the competition curve (see Fig. 2). Subsequent assays were performed with a fixed amount of subcellular fraction. In taurocholate-permeabilized vesicles, both surfaces of the membrane are exposed in addition to apoB in the lumen. Thus, the expected inhibition for open vesicles is that which would occur if the total apoB determined as in Table 2 competes on an equivalent basis to LDL-apoB. The expected inhibition for open vesicles was calculated in the same way but assuming that only the fraction of apoB in the membrane determined as in Table 1 is available to compete. The ratio is the observed % inhibition/calculated % inhibition. Results are means of four to eight separate assays ± standard deviation.
*, $P < 0.2\%$; **, $P < 1.0\%$; ***, $P < 5.0\%$; all other values, $P < 5.0\%$.

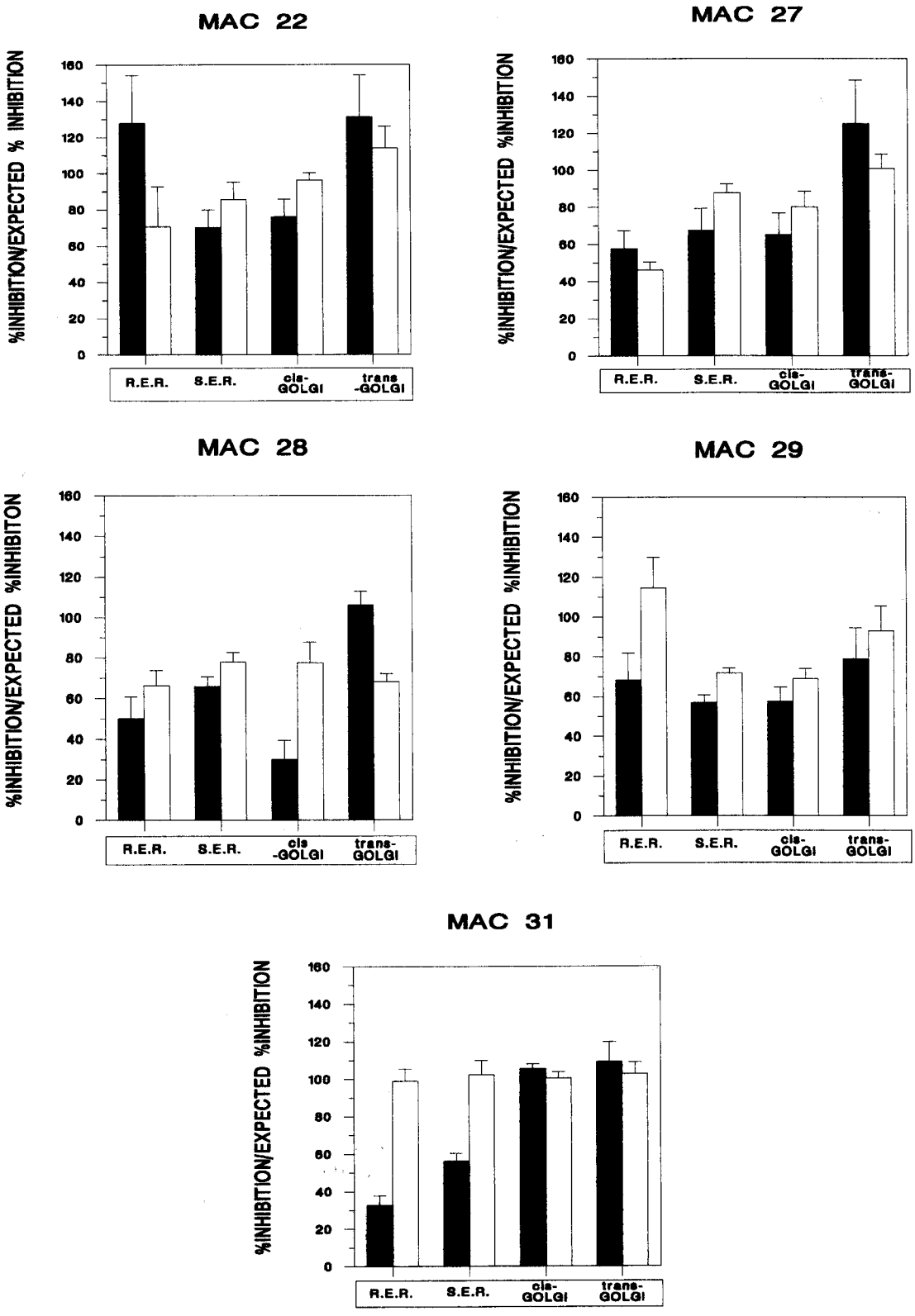

Fig. 3. Determination of the expression of apoB in subcellular fractions. The ability of subcellular fractions (RER, SER, *cis*-Golgi, and *trans*-Golgi) to inhibit binding of each monoclonal antibody to immobilized LDL was determined as described in Methods. The 'expected inhibition' was calculated from the amount of protein used in each assay and the apoB content of the fractions as described in the legend to Table 2 and the text. The percent of the observed inhibition/expected inhibition is plotted for closed vesicles (■) and vesicles permeabilized with 0.4% taurocholate (□). Values plotted are means of four to eight determinations ± standard deviation.

TABLE 4. Leakage of [^{3}H]leucine-labeled secretory proteins during ELISA

Fraction	Control	ELISA plus Taurocholate	ELISA minus Taurocholate
		% dpm released	
Rough microsomes	13.37	76.8	16.37
Smooth microsomes	11.13	74.11	14.68
trans-Golgi	14.70	74.88	16.55
cis-Golgi	10.39	73.39	12.45

Subcellular fractions were prepared from rabbit liver after injection of [^{3}H]leucine into the portal vein as described in Methods. The fractions were incubated on multiwell plates as for ELISA assay with and without taurocholate using MAC 22 as primary antibody. The fractions were removed from the wells after incubation and the loss of ^{3}H-labeled proteins from the vesicles was determined as described in Methods. In the control an equivalent amount of subcellular fraction was resuspended in 0.25 M sucrose and centrifuged, and the loss of ^{3}H-labeled protein was determined in the same way. Similar results were obtained using the MACs 27, 28, 29, and 31, and the polyclonal antibody.

Demonstration that subcellular fractions remained closed and impermeable during ELISA

Loss of radiolabeled protein contents. During ELISA in the presence of taurocholate, leakage of [^{3}H]leucine-labeled secretory protein contents from subcellular fractions was approximately 75%. The 25% remaining bound may be integral or adsorbed membrane protein (26). When intact microsomes were washed with 0.25 M sucrose, between 10 and 15% of the labeled protein was lost. This was probably newly synthesized cytosolic protein adsorbed to the outside of the vesicles. During ELISA there was no significant increase in the loss of the labeled protein, indicating that the vesicles remained closed (**Table 4**).

Loss of albumin from the vesicle contents. In the presence of taurocholate, 15 and 19 ng of albumin per mg of fraction protein were detected in microsomes and Golgi, respectively. This is the amount of albumin released from the vesicles or detectable in open vesicles. In the absence of taurocholate, albumin was not detectable indicating that the vesicles remain closed (**Table 5**).

TABLE 5. Availability of albumin in subcellular fractions for interaction with anti-albumin in ELISA

Fraction	Inhibition Binding	Albumin Detected
	%	*ng/mg protein*
Total microsomes		
+ Taurocholate	50–60	15.49 ± 3.55 (8)
− Taurocholate	0	nd
trans-Golgi		
+ Taurocholate	30–40	19.27 ± 0.06 (3)
− Taurocholate	0	nd

Albumin was assayed by ELISA as described in Methods in total microsomes (1–2 mg protein) and *trans*-Golgi fractions (0.7 mg protein) in the presence and absence of 0.4% taurocholate to permeabilize the vesicular fractions; nd, not detectable.

Association of lumenal lipids with apoB

In order to determine whether apoB in the cisternal contents of all subcellular fractions is associated with lipids, the protein was immunoprecipitated after radiolabeling the cisternal lipids with [^{3}H]palmitic acid. More than 95% of the ^{3}H in the lumenal lipid was in triacylglycerol with the remainder in the phospholipids. The apoB immunoprecipitate contained 85.1% ± 1.62%, 76.5% ± 3.44, 79.8% ± 9.52, 90.1% ± 2.41, and 78.8% ± 6.6% of the radiolabeled lipids of the *trans*-Golgi, *cis*-Golgi, RER, and SER, and total microsomes, respectively. Cisternal apoB is therefore associated with VLDL precursor lipids.

DISCUSSION

The ELISA probing experiments were aimed to determine whether epitopes of apoB in subcellular fractions are expressed as in LDL-apoB. Thus, if all of the membrane-bound pool of apoB is at the cytosolic surface of the vesicles, then competition should be the same as that for the same amount of apoB in LDL determined from the competition curves. In the case of the *trans*-Golgi fractions, the measured competition in closed vesicles was close to that calculated for all epitopes. The membrane-bound form of apoB in Golgi is therefore completely expressed at the surface of the vesicles.

In contrast to the results from studies of *trans*-Golgi membranes, all epitopes are expressed between 55 and 70% in SER, in RER (with the exception of that recognized by MAC 22), and *cis*-Golgi (with the exception of that recognized by MAC 31). RER contains nascent apoB that may contribute to the pool of apoB expressed at the cytosolic side of RER vesicles; such a pool would not exist in other fractions. There are two explanations for our observations. ApoB may be integrated into the membrane so that epitopes are partially shielded compared with

LDL-apoB or there may be two pools of apoB, one of which is expressed at the cytosolic side of the membrane and the second at the lumenal surface. Although our present results cannot differentiate between these two possibilities, a number of points support the former interpretation. In the *trans*-Golgi fraction the membrane-bound apoB is fully recognized and therefore no epitopes are shielded; in the presence or absence of taurocholate, the ELISA competition curves for all subcellular fractions are parallel with those for LDL-apoB; in rat liver and HepG2 cells, the membrane-bound apoB in the endoplasmic reticulum does not appear to be a precursor of that secreted (5, 6, 27); and, as triglyceride synthesis takes place in SER in addition to RER (18), it is necessary that a pool of apoB be available. It is thermodynamically unlikely that completely synthesized apoB is translocated across the membrane for packaging with lipid in the lumen.

Interpretation of results from these experiments is affected by the degree of cross-contamination between the Golgi and endoplasmic reticulum fractions. From the data in Table 1 and Table 3 it can be calculated that the maximum contribution of endoplasmic reticulum membrane apoB to the *trans*-Golgi fraction is 0.13 μg/mg of protein. This represents only 4.5% of the apoB in the Golgi membrane fraction and is therefore not significant. The contribution of contaminating Golgi membrane apoB to the RER is also insignificant at 0.053 μg/mg of protein or 5.1% of the membrane-bound apoB. The contribution of Golgi apoB to the SER membrane-bound fractions was 0.43 μg/mg protein or 38.7% of the SER membrane apoB. However, as all epitopes of contaminating apoB from the *trans*-Golgi membrane are completely expressed, this does not affect our conclusion but rather indicates that the pool of apoB at the lumenal surface of the SER may be larger than that calculated from the raw data. The *cis*-Golgi contains a maximum of 0.23 μg apoB/mg protein derived from endoplasmic reticulum contamination. This is 15.5% of the total membrane apoB. The identification of the *cis*-Golgi fraction is based on morphological appearance and function (21, 28–31); however, this fraction has characteristics intermediate between those of endoplasmic reticulum and *trans*-Golgi.

Davis et al. (5) have probed the accessibility of apoB in subcellular fractions from rat liver to trypsin. They detected loss of apoB by Western blotting followed by densitometry. Those methods are at best semi-quantitative and the results given have very large standard deviations. Despite the limitations, however, these studies yielded results consistent with ours and suggested that a large fraction of apoB (about 56% of the apoB-48 and about 70% of the apoB-100) is at the cytosolic side of the endoplasmic reticulum membranes. ApoB in Golgi preparations was not accessible to external trypsin. However, membrane-bound and lumenal apoB were not separated. As the lumenal apoB of *trans*-Golgi from rat liver accounts for more than 85% of the total (7), the methods used by Davis et al. (5) may not have been sufficiently sensitive to detect loss of the membrane-bound form.

An attractive experimental approach to study the translocation and topography of apoB is in vitro translation in the presence of microsomal vesicles. This is technically difficult because of the large size of apoB. However, there have been two studies in which a truncated form of apoB consisting of up to 15% of the N-terminal of the mRNA has been translated in vitro in the presence of canine microsomal membranes (32–34). Both studies showed that apoB is translocated across the microsomal membrane and remains membrane-bound. However, when the topography of newly synthesized apoB was probed with proteases, one investigation concluded that truncated apoB has several transmembrane domains although these are not integrated into the membrane (32, 33), while the second concluded that apoB is associated with the inner (cisternal) surface of the microsomal vesicle (34). Although this approach is elegant, interpretation of these studies is complicated by the fact that canine pancreas microsomes lack the ability to synthesize lipids for secretion. The proper transfer of apoB to the lumen of the endoplasmic reticulum/Golgi compartment must be intimately linked to synthesis and transfer of VLDL lipids.

Regulation of apoB secretion is post-translational in HepG2 cells and McArdle RH7777 cells (12, 35). Newly synthesized apoB is either incorporated into the endoplasmic reticulum membrane and probably degraded or translocated into the lumen of the secretory compartment (1–10, 27). A model for the translocation of apoB through the secretory compartment that takes into account these observations and the findings reported in this paper is shown in **Fig. 4.** In this model, apoB either remains associated with the membrane so that epitopes are exposed at the cytosolic surface or is translocated to the lumenal surface from which site it is packaged with lipid in the lumen. Both forms of membrane-associated apoB move within the two-dimensional plane of the membrane to the SER where further triacylglycerol synthesis takes place and the apoB at the lumenal surface of the membrane is

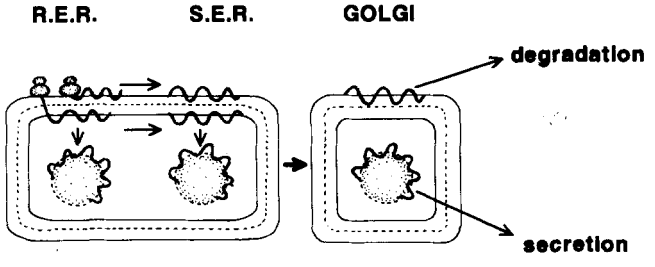

Fig. 4. Model for the intracellular transport of apoB. See text for details.

transferred to the lumen. The apoB at the cytosolic surface of the membrane is transferred to the Golgi region in a membrane-bound form and the VLDL precursor particles move to the Golgi lumen where further maturation, including addition of phospholipid and cholesterol, takes place (15, 16, 18). The mature VLDL particles are secreted. This model suggests that assembly of apoB with lipid is post-translational. Initially, this appears to conflict with the observations of Borén et al. (27) which suggest that these events occur co-translationally in HepG2 cells. However, RER and SER form a functional continuum and the temporal events in VLDL assembly may differ in HepG2 cells and hepatocytes. The pool of apoB associated with the SER lumenal surface in our model may be partly associated with VLDL lipid but not yet released into the lumen. Further work is needed to determine the molecular details of apoB association with membranes in both cell systems.

The endoplasmic reticulum has been suggested as the site of degradation of apoB (9, 10). This is based largely on the observation that Brefeldin A does not block degradation of apoB in HepG2 cells under conditions in which protein (alpha 1-antitrypsin) transport from endoplasmic reticulum to Golgi is inhibited (9). However, the action of Brefeldin A may be to redistribute Golgi components back into the endoplasmic reticulum (36). The endoplasmic reticulum/Golgi compartment is a functionally continuous system in which membrane flow occurs in both directions and Brefeldin A perturbs this equilibrium. Our results show clearly that in normal liver the membrane form of apoB reaches the Golgi region. It may be degraded at this site or retrieved to the endoplasmic reticulum for degradation or reutilization.

One of the most intriguing questions arising from this model is what determines whether newly synthesized apoB is translocated across the endoplasmic reticulum membrane or remains at the cytosolic side of the membrane. This is a key regulatory step in the assembly of VLDL and may be the site of the defect of abetalipoproteinemia (37). In hepatoma cells oleate stimulates triacylglycerol synthesis and secretion and either directly or indirectly reduces apoB degradation (10, 12), while in rat hepatocytes insulin stimulates apoB degradation (11). Concomitant synthesis and translocation of triacylglycerol to the endoplasmic reticulum lumen may thus be the factor that determines whether apoB is secreted or remains membrane-bound and enters a futile pathway of synthesis followed by degradation.

In these studies we have not directly investigated the nature of association of apoB with membranes; however, it has been observed recently that charged amino acids in the transmembrane region may be the structural motif necessary for targeting membrane proteins for degradation (38). Similar motifs may determine the fate of the membrane-bound pool of apoB.

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