The N-linked oligosaccharides at the amino terminus of human apoB are important for the assembly and secretion of VLDL

Jelena Vukmirica,* Tomoko Nishimaki-Mogami,† Khai Tran,* Jing Shan,* Roger S. McLeod,[1,]* Jane Yuan,* and Zemin Yao[2,]*

Lipoprotein and Atherosclerosis Group,* Departments of Pathology and Laboratory Medicine and Biochemistry, Microbiology, and Immunology, University of Ottawa Heart Institute, Ottawa, ON, Canada, K1Y 4W7; and the National Institute of Health Sciences,† Kamiyoga 1-18-1, Setagaya-ku, Tokyo 158-8501, Japan

Abstract We determined the role of N-linked glycosylation of apolipoprotein B (apoB) in the assembly and secretion of lipoproteins using transfected rat hepatoma McA-RH7777 cells expressing human apoB-17, apoB-37, and apoB-50, three apoB variants with different ability to recruit neutral lipids. Substituting Asn residue with Gln at the single glycosylation site within apoB-17 (N^{158}) decreased its secretion efficiency to a level equivalent to that of wild-type apoB-17 treated with tunicamycin, but had little effect on its synthesis or intracellular distribution. When selective N-to-Q substitution was introduced at one or more of the five N-linked glycosylation sites within apoB-37 (N^{158}, N^{956}, N^{1341}, N^{1350}, and N^{1496}), secretion efficiency of apoB-37 from transiently transfected cells was variably affected. When all five N-linked glycosylation sites were mutated within apoB-37, the secretion efficiency and association with lipoproteins were decreased by >50% as compared with wild-type apoB-37. Similarly, mutant apoB-50 with all of its N-linked glycosylation sites mutagenized showed decreased secretion efficiency and decreased lipoprotein association in both d < 1.02 and d > 1.02 g/ml fractions. The inability of mutant apoB-37 and apoB-50 to associate with very low-density lipoproteins was attributable to impaired assembly and was not due to the limitation of lipid availability. The decreased secretion of mutant apoB-17 and apoB-37 was not accompanied by accumulation within the cells, suggesting that the proportion of mutant apoB not secreted was rapidly degraded. However unlike apoB-17 or apoB-37, accumulation of mutant apoB-50 was observed within the endoplasmic reticulum and Golgi compartments. These data imply that the N-glycans at the amino terminus of apoB play an important role in the assembly and secretion of lipoproteins containing the carboxyl terminally truncated apoB.— Vukmirica, J., T. Nishimaki-Mogami, K. Tran, J. Shan, R. S. McLeod, J. Yuan, and Z. Yao. **The N-linked oligosaccharides at the amino terminus of human apoB are important for the assembly and secretion of VLDL.** ***J. Lipid Res.*** **2002.** 43: **1496–1507.**

Supplementary key words apoB-17 • apoB-37 • apoB-50 • McA-RH7777 cell • subcellular fractionation

The asparagine (N)-linked oligosaccharides of proteins are an important component of the quality control mechanisms of eucaryotic cells. Multiple roles have been assigned to N-linked oligosaccharides, including folding of nascent polypeptides, protection from proteolytic degradation, intracellular trafficking, secretion, cell surface expression, maintenance of protein conformation, and enzymatic activity (1–3). Human apolipoprotein (apo) B-100, a major structural protein of VLDL synthesized in the liver, is a 4,536 amino acid glycoprotein. There are 20 potential N-linked glycosylation sites within apoB-100, of which 16 Asn residues are conjugated with oligosaccharides on plasma LDL. Each mole of apoB-100 contains 5–6 mol of high-mannose type, and 8–10 mol of complex type oligosaccharides (4). As a member of the vitellogenin family of lipid transport and storage proteins (5–7), apoB-100 possesses numerous amphipathic α-helices and β-strands that constitute the major structural framework for the assembly and integrity of triglyceride-rich lipoproteins (8, 9). The precise amino acid sequences within apoB-100 that are involved in lipid binding have not yet been identified. The regions enriched in amphipathic β-strands are thought to interact directly and irreversibly

Manuscript received 15 February 2002 and in revised form 22 May 2002.
DOI 10.1194/jlr.M200041-JLR200

Abbreviations: apo, apolipoprotein; ER, endoplasmic reticulum; GFP, green fluorescent protein; MTP, microsomal triglyceride transfer protein; TG, triacylglycerol.

[1] Current address: Department of Biochemistry, Dalhousie University, Halifax, Nova Scotia, Canada B3H 4H7.

[2] To whom correspondence should be addressed.
e-mail: zyao@ottawaheart.ca

with the VLDL neutral lipid core, whereas the regions rich in amphipathic α-helices are less tightly associated with lipid (10). The minimum length of apoB that is required for the assembly of a neutral lipid core is between 23–28% of the N-terminal portion of apoB-100 (11–13).

Post-translational modifications of apoB, such as disulfide bond formation (14, 15) and palmitoylation (16–18), have been shown to play a role in the assembly and secretion of apoB-containing lipoproteins. However, clear biochemical evidence for a role of N-linked oligosaccharides in these processes has not been obtained. Early studies with rat or chicken hepatocytes suggested that inhibition of N-linked glycosylation of apoB with the chemical inhibitor tunicamycin had no major impact on VLDL secretion (19, 20, 21). However, experiments with human hepatoblastoma HepG2 cells showed that secretion of apoB-100 was reduced by tunicamycin treatment (22), and the decreased apoB-100 secretion in tunicamycin-treated HepG2 cells was associated with increased apoB degradation (23). Recently, working with transfected McA-RH7777 cells that express various C-terminally truncated forms of human apoB, we also observed that tunicamycin treatment impaired apoB secretion in all cell lines examined (**Fig. 1B**). These seemingly conflicting results on the effect of tunicamycin may highlight pleiotropic effects that may limit the use of this inhibitor (e.g., the dose and duration of tunicamycin used for the treatment with different cells).

In the current study, we tested the role of amino-terminal apoB N-linked glycosylation using site-specific mutagenesis (N-to-Q) within three C-terminally truncated forms of human apoB, namely apoB-17, apoB-37, and apoB-50, that have different abilities to assemble a neutral lipid core. Our results provide new evidence that the N-linked oligosaccharides are important for efficient secretion of apoB and apoB-containing lipoproteins.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were obtained from Life Technologies Inc. ProMix^{TM} (a mixture of [^{35}S]methionine and [^{35}S]cysteine, 1,000 Ci/mmol), peroxidase-conjugated anti-mouse immunoglobulin G antibody, and protein A-Sepharose CL-4B beads were obtained from Amersham Pharmacia Biotech. The chemiluminescent blotting substrate and tunicamycin was obtained from Roche. Monoclonal

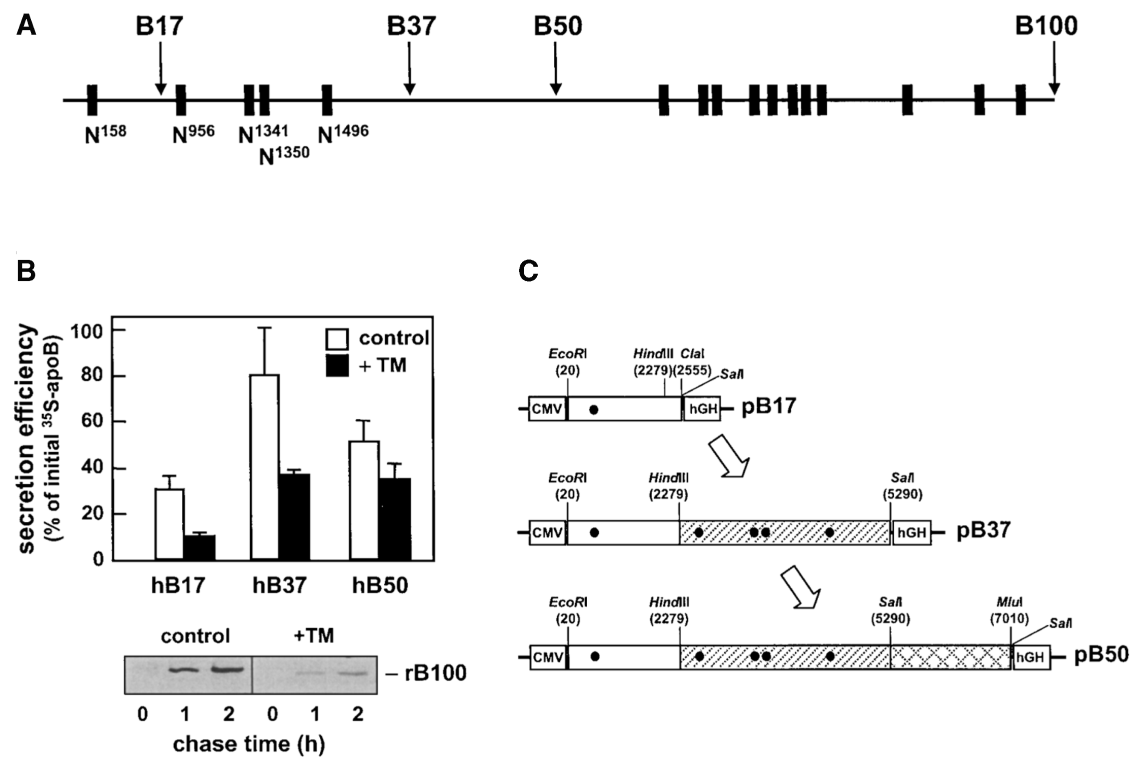


Fig. 1. Tunicamycin treatment decreases apoplipoprotein B (apoB) secretion and generation of apoB N-glycan mutants. A: Position of the N-linked oligosaccharides within human apoB-100. Closed rectangles represent the utilized glycosylation sites. B: McA-RH7777 cells stably transfected with human apoB-17, apoB-37, and apoB-50 variants (60 mm dish) were pretreated for 3 h in DMEM (20% serum) ± 5 μg/ml tunicamycin, pulse-labeled with [^{35}S]amino acids (200 μCi/ml) in methionine-deficient DMEM (20% serum) for 1 h, and chased up to 2 h (± tunicamycin). The medium-associated [^{35}S]apoB-17, [^{35}S]apoB-37, and [^{35}S]apoB-50 during chase were recovered by immunoprecipitation. Secretion efficiency of each apoB variant is presented as (medium apoB at 2 h chase) / (initial apoB at 60 min pulse) × 100%. Data are the mean of three experiments with mixed cultures of cells except for apoB-50 where individual G418-resistant colonies were used. Bottom, endogenous rat [^{35}S]apoB-100 secreted during chase. C: Construction of expression plasmids encoding apoB-17, apoB-37, or apoB-50 containing N-to-Q substitution at Asn residues 158, 956, 1341, 1350, and 1496 (closed ovals). Restriction sites and the number of nucleotides of the apoB cDNA sequences are indicated. CMV, cytomegalovirus promoter-enhancer sequences; hGH, human growth hormone transcription termination and polyadenylation signals. The *Mlu*I restriction site in pB50 was engineered as described previously (11).

antibodies 1D1, 3E11, 374, 2D8, and 1C4 that were raised against human apoB were a gift of R. Milne and Y. Marcel (University of Ottawa Heart Institute). The anti-apoA-I antiserum was a gift of J. E. Vance (University of Alberta). Antibody against α-mannosidase II was a gift of M. G. Farquhar (University of California, San Diego). The anti-calnexin antibody was obtained from StressGen (Victoria, BC).

ApoB expression plasmids

The expression plasmid pB17wt that contained nucleotides 20–2555 of the human apoB cDNA was prepared by inserting the coding sequence between *Eco*RI and *Cla*I sites (open boxes in Fig. 1C) at the polylinker region of pCMV5 vector . The N-to-Q substitution was introduced into pB17wt at Asn^{158} using the QuikChange™ site-directed mutagenesis kit (Stratagene Inc., La Jolla, CA) according to the manufacturer's instructions. The plasmids pB17wt and $pB17N^{158}$ were used to create pB37wt and $pB37N^{158}$, respectively, by inserting a *Hind*III-*Sal*I fragment (nucleotides 2279–5290 of the apoB cDNA, striped boxes in Fig. 1C) that was derived from pB48 (24). Subsequently, $pB37N^{956}$, $-N^{1341}$, $-N^{1350}$, $-N^{1496}$, $-N^{158–956}$, $-N^{158–1341}$, $-N^{158–1350}$, and $-N^{158–1496}$ that contained N-to-Q substitutions at selected or combined Asn^{956}, Asn^{1341}, Asn^{1350}, and Asn^{1496} positions were generated by multiple rounds of mutagenesis using $pB37N^{158}$ as a template. To create plasmid $pB50N^{158–1496}$, a *Sal*I-*Sal*I fragment (from nucleotide 5290 of the apoB cDNA to the polylinker of pCMV5, crosshatched box in Fig. 1C) was excised from $pB37N^{158–1350}$ and inserted into pB50 (15) that had been digested with *Sal*I. The resulting $pB50N^{158–1350}$ was used as a template to introduce N^{1496}-to-Q to create $pB50N^{158–1496}$. Sequences of all primers used for mutagenesis are available from the authors upon request. The resulting plasmids were purified by centrifugation twice in a CsCl gradient, and the apoB coding regions were authenticated by sequencing.

Cell culture and transfection

McA-RH7777 cells were cultured in DMEM containing 10% fetal bovine serum and 10% horse serum. Transfection of the cells with expression plasmids encoding wild-type or mutant forms of apoB-17, apoB-37, or apoB-50 was achieved using the previously described calcium phosphate precipitation method (25). Expression of the recombinant human apoB was verified by immunoblotting of the conditioned media using monoclonal antibodies 1D1, 3E11, 374, 2D8, and 1C4 (26, 27). Experiments described below were performed using transiently transfected McA-RH7777 cells or mixed culture of G418-resistant transfectants, except with apoB-50-expressing cells where individual G418-resistant colonies were used.

Metabolic labeling

Cells were pretreated with tunicamycin and subjected to pulse-chase experiments (with $[^{35}S]$methionine/cysteine) to determine the synthesis and secretion of apoB and apoA-I (experimental details are described in the figure legends). At different time points during the chase, the cell and medium apoB and apoA-I were immunoprecipitated and analyzed by polyacrylamide gel electrophoresis (3–15% gel) in the presence of 0.1% SDS (SDS-PAGE) as described previously (12). In the case of apoB-50, the chase media were separated into $d < 1.02$ and $d > 1.02$ g/ml fractions by ultracentrifugation prior to immunoprecipitation.

Density ultracentrifugation

Cells were pulse-labeled with $[^{35}S]$methionine/cysteine for 1 h and chased for 2 h in DMEM containing 20% fetal bovine serum and 0.4 mM oleate as described previously (28). At the end of chase, the media and microsomal contents were separated into 12 fractions in a sucrose density gradient as described previously (29). The apoB proteins in each fraction were immunoprecipitated (using an anti-apoB polyclonal antibody that recognized both human and rat apoB) and subjected to SDS-PAGE and fluorography. For apoB-50, the microsomal contents were fractioned into $VLDL_1$ ($S_f > 100$), $VLDL_2$ (S_f 20–100), and other density fractions by cumulative rate flotation (30). The lumenal content of the microsomes was isolated as described previously (30).

Subcellular fractionation

Cells were homogenized using a ball-bearing homogenizer as described previously (30). The postnuclear supernatant was prepared and fractionated on preformed Nycodenz gradients (31) by centrifugation in an SW 41 rotor (4°C, 90 min, 37,000 *g*) (32, 33). A total of 19 fractions (600 μl/fraction) were collected, and an aliquot of each fraction was subjected to SDS-PAGE (3–15% gel) and immunoblotted to detect α-mannosidase II, calnexin, and apoB, respectively.

Lipid assay. Metabolic labeling of lipid with $[^3H]$glycerol was performed as described previously (30).

Immunocytochemistry

Cells stably expressing apoB-50wt or apoB-$50N^{158-1496}$ were plated on coverslips for 24 h, fixed with 3% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min, and permeabilized with 0.1% Triton X-100 (in PBS) for 3 min. Paraformaldehyde was quenched with 50 mM ammonium chloride (in PBS) for 30 min, and the cells were incubated with 10% FBS (in PBS) for 20 min. Monoclonal antibody 1D1 was used to probe the recombinant human apoB (1 h) with goat anti-mouse IgG Alexa Fluro 488 conjugate (#A-6440; Molecular Probes) as secondary antibody. The endoplasmic reticulum (ER) and Golgi were probed with anti-calnexin and anti-α-mannosidase II antibodies, respectively, with Alexa Fluro 594 conjugated anti-rabbit IgG (#R-6394; Molecular Probes) as secondary antibody. Between all steps, cells were washed three times with PBS. All incubation and washing procedures were performed at room temperature. After staining, cells were mounted onto the glass slide using SlowFade Antifade kits (#S-7461; Molecular Probes). The images were captured using the MRC-1024 laser scanning confocal imaging system (Bio-Rad).

RESULTS

Tunicamycin inhibits secretion of ApoB

Human apoB-100 has 20 potential N-linked glycosylation sites, of which 16 are conjugated with oligosaccharides (Fig. 1A). In McA-RH7777 cells expressing recombinant human apoB-17, apoB-37, or apoB-50, tunicamycin treatment decreased secretion efficiency of the nonglycosylated apoBs by varied extents as compared with the controls (i.e., no tunicamycin treatment) (Fig. 1B). The secretion of endogenous apoB-100 in these cells was also decreased by tunicamycin treatment (Fig. 1B, bottom). Similar inhibitory effect of tunicamycin treatment on secretion of rat apoB-100 was observed in primary rat hepatocytes (data not shown). These results suggested that the lack of N-linked oligosaccharides is associated with impaired secretion of apoB. To test further the role of N-linked oligosaccharides on apoB secretion, we performed N-to-Q sub-

ASBMB JOURNAL OF LIPID RESEARCH

stitution at the glycosylation sites within apoB-17, apoB-37, and apoB-50 that contain different number of N-glycans (Fig. 1C).

The effect of N-to-Q mutation on apoB-17 synthesis and secretion

When the single glycosylation site N^{158} within apoB-17 was mutagenized, the secretion efficiency of the mutant apoB-17 (designated apoB-17N^{158}) was decreased by 50% as compared with the wild-type apoB-17 (apoB-17wt). The inhibitory effect of N^{158}-to-Q substitution on apoB-17 secretion was almost equivalent to that of tunicamycin treatment (**Fig. 2A**). The level of expression of apoB-17wt and apoB-17N^{158} was similar in the two cell cultures (as judged by ^{35}S incorporation in Fig. 2B). The amount of cell-associated [^{35}S]apoB-17 during chase was similar between the two transfectants (Fig. 2B).

Loss of N-linked oligosaccharides in proteins has been shown to associate with delayed ER exit (2). To gain an insight into the mechanisms for the impaired secretion of mutant apoB-17, we determined the effect of the loss of N-glycan on intracellular distribution of metabolically labeled apoB-17N^{158}. In these experiments, samples were taken 15 and 60 min after labeling with [^{35}S]amino acids and analyzed by subcellular fractionation using Nycodenz gradient centrifugation. Calnexin and α-mannosidase II were used as ER and medial Golgi markers. No discernible difference was observed in the relative distribution in ER or Golgi between [^{35}S]apoB-17wt and [^{35}S]apoB-17N^{158} at 15 or 60 min labeling time points (data not shown). These results suggest that the impaired secretion of apoB-17N^{158} was not associated with intracellular accumulation but with degradation.

Several mechanisms exist in the hepatic cells by which newly synthesized apoB can be degraded, either at the stage after apoB translation or else during apoB polypeptide chain elongation (34). Rapid degradation of apoB during or immediately after apoB translation is mediated by proteasome, which can be prevented by inhibitors ALLN or MG132 in McA-RH7777 cells (15). To test the possible role of proteasome in apoB-17N^{158} degradation, we included proteasomal inhibitors ALLN and MG132 during metabolic labeling (Fig. 2C). While inhibition of proteasome elevated the intracellular levels of endogenous [^{35}S]apoB-100 by 50% at the end of 30 min labeling, no significant change in the intracellular levels of apoB-17wt or apoB-17N^{158} was observed (Fig. 2C, bottom). These results imply that apoB-17N^{158} is unlikely degraded by proteasome during translation.

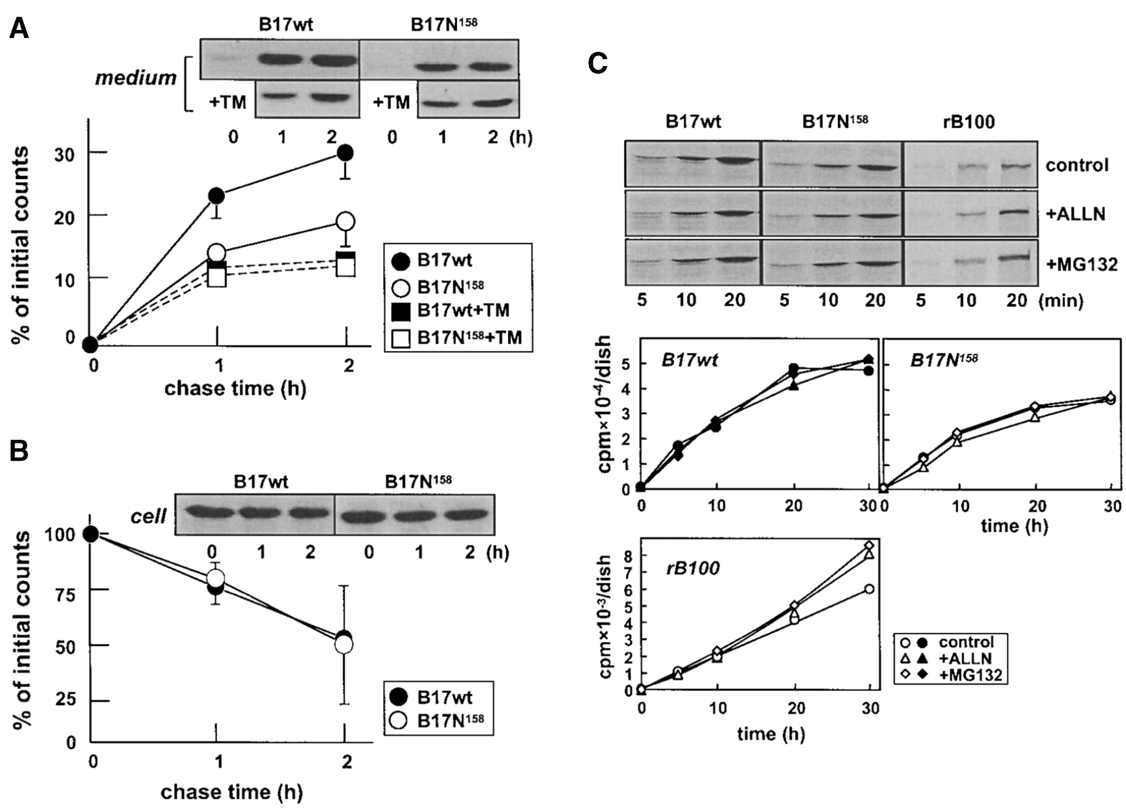
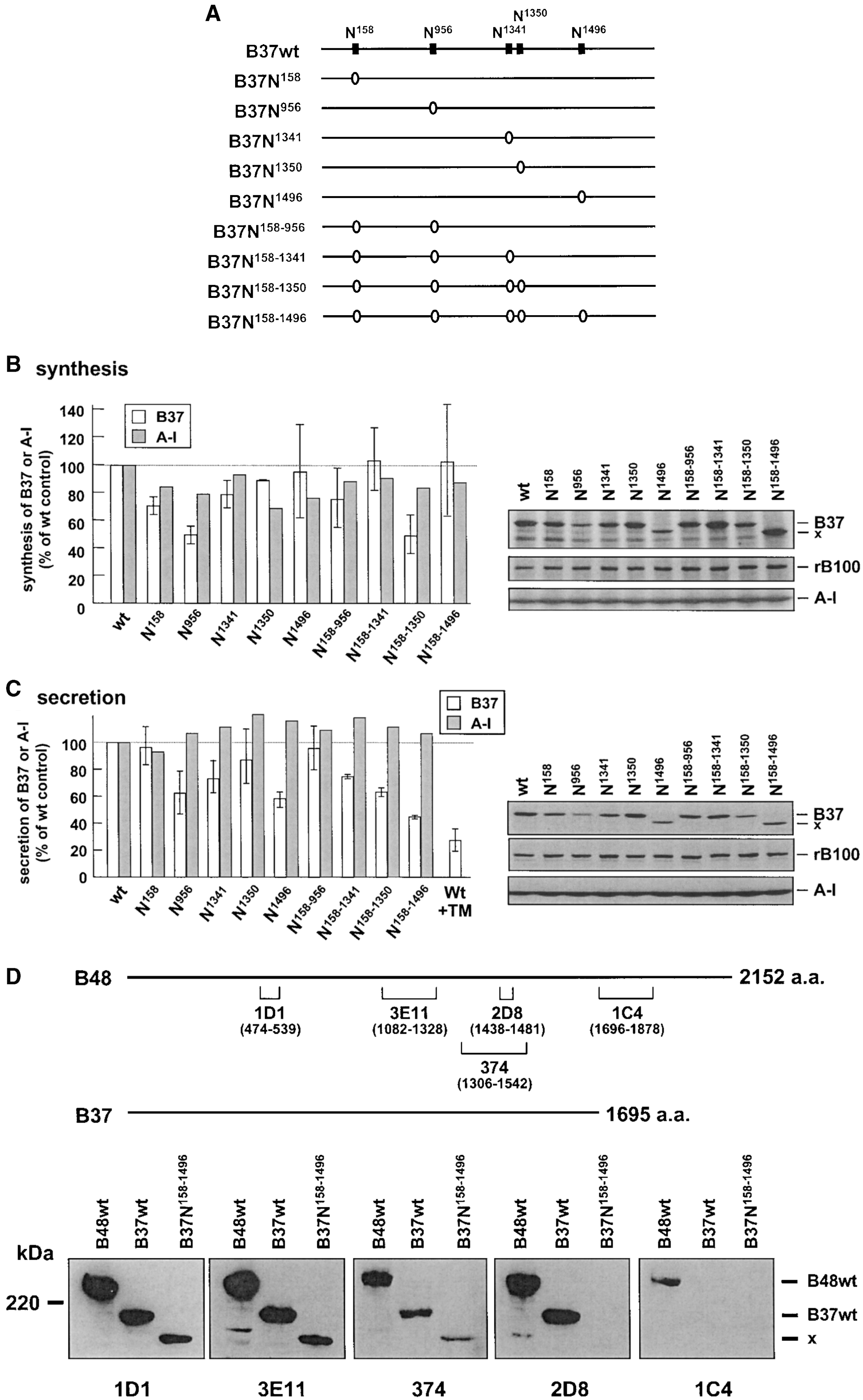


Fig. 2. Lack of N-glycan impairs apoB-17 secretion. Transiently transfected cells (60 mm dish) were pretreated for 3 h in DMEM (+ 20% serum) ± 5 μg/ml tunicamycin, pulse-labeled with [^{35}S]amino acids for 1 h, and chased up to 2 h (± tunicamycin). The medium (A) and cell-associated [^{35}S]apoB-17 (B) during chase were subjected to SDS-PAGE and visualized by fluorography. The recovery of medium and cell-associated [^{35}S]apoB-17 during chase was presented as "% of initial counts" that associated with cell apoB-17 at the end of pulse. Insets show representative fluorograms. Data are the mean of three experiments. C: Cells were continuously labeled with [^{35}S]amino acids (200 μCi/ml) in methionine-deficient DMEM (20% serum) for indicated times in the absence or presence of proteasomal inhibitors ALLN or MG132. Top, representative fluorograms; bottom, quantification of radioactivity associated with cell-associated [^{35}S]apoB-17wt, [^{35}S]apoB-17N^{158}, and endogenous rat [^{35}S]apoB-100 (*rB100*).



A
B37wt
N158 N956 N1341 N1350 N1496
B37N158
B37N956
B37N1341
B37N1350
B37N1496
B37N158-956
B37N158-1341
B37N158-1350
B37N158-1496
B synthesis
synthesis of B37 or A-I (% of wt control)
B37
A-I
wt N158 N956 N1341 N1350 N1496 N158-956 N158-1341 N158-1350 N158-1496
B37
x
rB100
A-I
C secretion
secretion of B37 or A-I (% of wt control)
Wt +TM
D
B48
2152 a.a.
1D1 (474-539)
3E11 (1082-1328)
2D8 (1438-1481)
1C4 (1696-1878)
374 (1306-1542)
B37
1695 a.a.
B48wt B37wt B37N158-1496
kDa
220
B48wt
B37wt
x
1D1 3E11 374 2D8 1C4

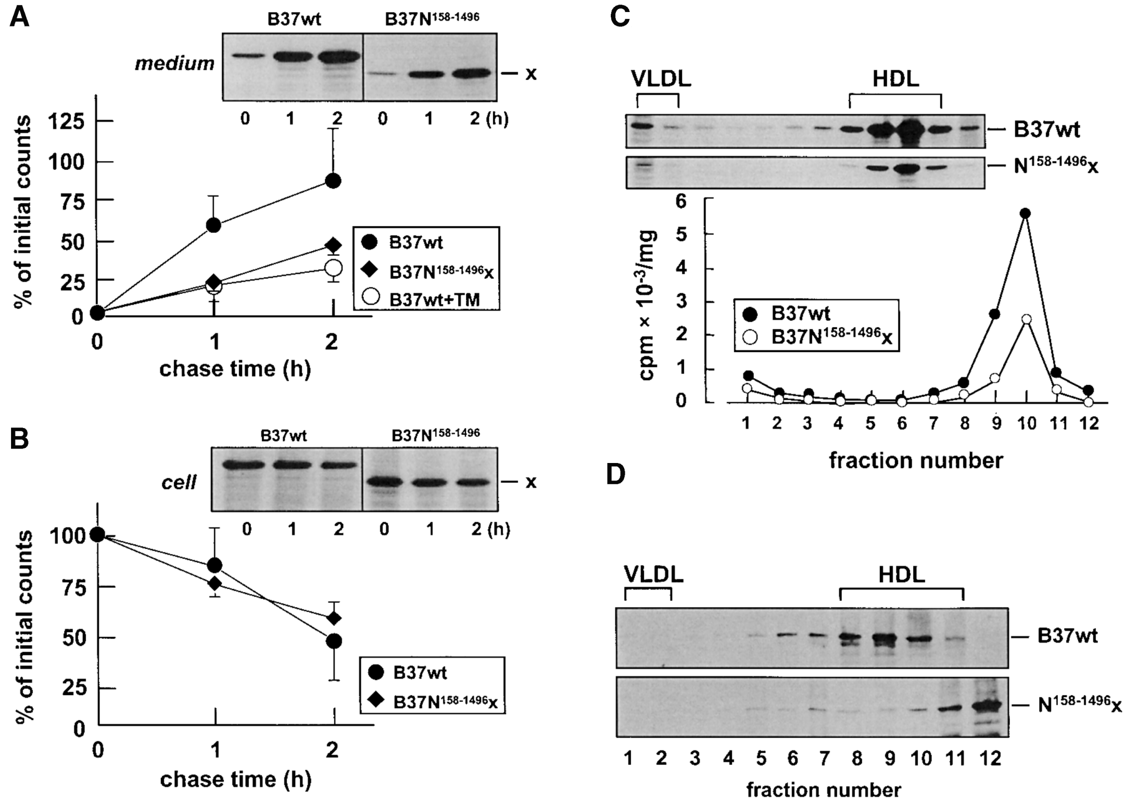


Fig. 4. Lack of N-glycans impairs apoB-37 secretion. Kinetics of apoB-37wt and apoB-37N$^{158-1496}$ in stably transfected cells determined by pulse-chase analysis as described in Figure 3. A: [^{35}S]apoB-37 secreted into medium. B: Cell-associated [^{35}S]apoB-37. C: Medium was collected at the end of 2 h chase and fractionated by ultracentrifugation in a sucrose density gradient. The [^{35}S]apoB-37 in each fraction was analyzed by SDS-PAGE and fluorography (top). The radioactivity associated with apoB-37wt and apoB-37N$^{158-1496}$ was quantified by scintillation counting (bottom). D: The lumenal content isolated from carbonate-treated microsomes was fractionated by sucrose density gradient. The apoB-37 proteins in each fraction were detected by immunoblotting.

The effect of selective or combined N-to-Q mutations on apoB-37 secretion

The above results demonstrated the importance of N-linked oligosaccharides in the secretion of apoB-17. However, apoB-17 lacks the ability to assemble neutral lipids (13) and thus is unsuitable as a model for lipoprotein studies. To determine the role of N-linked oligosaccharides in the secretion of lipoproteins, we used apoB-37 as a model that contains some lipid binding sequences (35) and four additional N-linked glycosylation sites (**Fig. 3A**). Introducing N-to-Q substitution at a single glycosylation site (i.e., N^{158}, N^{956}, N^{1341}, N^{1350}, and N^{1496}) allows us to determine if there is a critical N-linked glycan that is important for lipoprotein synthesis. Transient transfection with various apoB-37 constructs resulted in different levels of apoB-37 expression as shown by varied incorporation of

Fig. 3. Effect of selective or combined N-to-Q substitution on apoB-37 secretion efficiency. A: Schematic representation of the wild-type and nine mutant apoB-37s that contained single or more N-to-Q substitution at residues N^{158}, N^{956}, N^{1341}, N^{1350}, and N^{1496} (open ovals). B: Transiently transfected cells (2 days after transfection) were labeled with [^{35}S]amino acids for 60 min in the presence of exogenous oleate (0.4 mM). At the end of labeling, cells were lysed and cell-associated [^{35}S]apoB-37, endogenous rat [^{35}S]rapoB-100, and rat [^{35}S]apoA-I were immunoprecipitated and subjected to SDS-PAGE and fluorography (right). Radioactivity associated with [^{35}S]apoB-37, [^{35}S]rapoB-100, and [^{35}S]apoA-I was quantified. Incorporation of [^{35}S]amino acids into [^{35}S]apoB-37 and [^{35}S]apoA-I in mutant apoB-37-transfected cells is presented as the percent of apoB-37 or A-I in apoB-37wt-transfected cells. C: Cells were pulse labeled with [^{35}S]amino acids for 60 min and chased for 2 h. Exogenous oleate (0.4 mM) was included in pulse and chase media. The secreted [^{35}S]apoB-37, [^{35}S]rapoB-100, and [^{35}S]apoA-I at 2 h chase were subjected to SDS-PAGE and fluorography (right). Secretion of [^{35}S]apoB-37 and [^{35}S]apoA-I at the end of chase from mutant apoB-37-transfected cells is presented as % of apoB-37 or A-I secreted from apoB-37wt-transfected cells. Data for apoB-37 are the average of two to three independent transfection experiments. Secretion of A-I was determined once. D: Immunoblot analysis of apoB epitopes. At the top are linear maps of apoB48 and apoB-37 with the number of amino acids (a.a.) labeled at right. The epitope positions for monoclonal antibodies 1D1, 3E11, 374, 2D8, and 1C4 are indicated by square brackets below apoB-48. The numbers in parentheses represent the position of amino acids at the boundary of each antibody epitope. At the bottom are immunoblot analyses of apoB-37wt and apoB-37N$^{158-1496}$ (resolved on 5% polyacrylamide gel) with indicated monoclonal antibodies. Recombinant human apoB-48 (apoB-48wt) expressed in transfected McA-RH7777 cells (12) was used as a positive control for various antibodies.

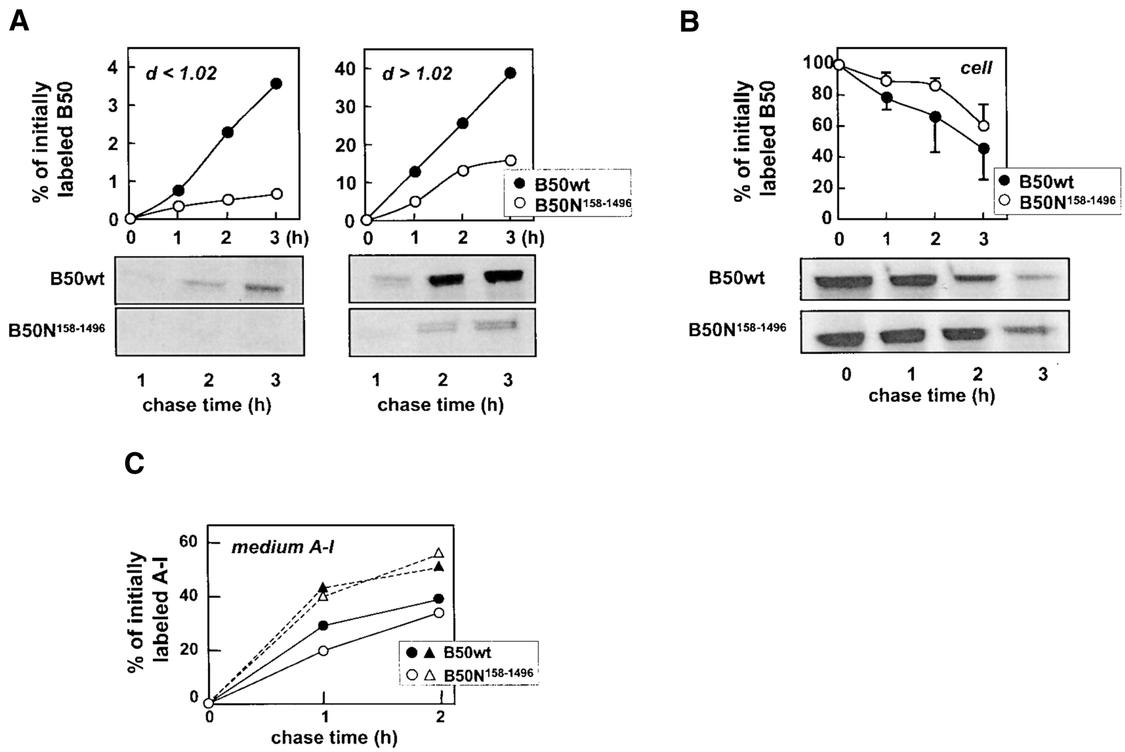


Fig. 5. Lack of N-glycans impairs apoB-50 secretion. A: Secretion of apoB-50wt and apoB-50N$^{158–1496}$ from stably transfected cells. Pulse-chase experiments were performed as described in Figure 3. Data are the mean of three independent experiments using different stable clones. Bottom, representative fluorograms of secreted [^{35}S]apoB-50wt and [^{35}S]apoB-50N$^{158–1496}$ in d < 1.02 (left) and d > 1.02 g/ml (right) fractions. B: Cell-associated [^{35}S]apoB-50wt and apoB-50N$^{158–1496}$ during chase. Data presented are average of two experiments with similar results. C: Secretion of endogenous [^{35}S]apoA-I. The experiments were performed twice. Data from both experiments (dashed and solid lines) are presented.

[^{35}S]amino acids (Fig. 3B). However, transient expression of these apoB-37 constructs had no marked effect on the synthesis of endogenous apoA-I (Fig. 3B) or apoB-100 (quantitative data not shown). The N-to-Q substitution at each single glycosylation site had a different effect on apoB-37 secretion efficiency. Thus, while secretion of apoB-37N^{158} and apoB-37N^{1350} was not different from apoB-37wt, secretion of apoB-37N^{956}, apoB-37N^{1341}, and apoB-37N^{1496} decreased by 30–40% compared with apoB-37wt (Fig. 3C). These data suggest that N-to-Q substitution at a single N-linked glycosylation site exerted no or partial inhibition on apoB-37 secretion. On the other hand, introducing N-to-Q substitution at multiple glycosylation sites (i.e., N$^{158–956}$, N$^{158-1341}$, N$^{158–1350}$, and N$^{158–1496}$) progressively and consistently decreased apoB-37 secretion efficiency. Thus, when all five glycosylation sites were mutated, the secretion efficiency of apoB-37N$^{158–1496}$ was decreased to 40% of apoB-37wt, a level almost equivalent to that of apoB-37wt treated with tunicamycin (Fig. 3C). The secretion efficiency of endogenous apoA-I (Fig. 3C) or apoB-100 (quantitative data not shown) was not altered among cells transiently transfected with various apoB-37 forms.

It was noted that N-to-Q substitution at N^{1496} resulted in increased electrophoretic mobility of apoB-37N^{1496} and apoB-37N$^{158-1496}$ (indicated by "x" in Fig. 3B and C). Sequencing analysis of the expression plasmid DNAs excluded the possibility of deletions within the apoB-37 coding sequence. To determine if the aberrant electrophoretic mobility was caused by proteolytic cleavage at the carboxyl terminus of the mutant apoB-37, we performed epitope mapping with a panel of monoclonal antibodies (Fig. 3D). As expected, apoB-37wt reacted with 1D1, 3E11, 374, and 2D8, but not with 1C4. The mutant apoB-37N$^{158–1496}$, however, reacted with 1D1, 3E11, and 374 normally, but lost its reactivity with 2D8 (there was no apoB fragments detectable by 2D8 on the 5% polyacrylamide gel). Thus, it is likely that mutation at residue N^{1496} rendered apoB-37 susceptible to proteolytic cleavage at amino acid residue approximately 1440, removing ~5% of apoB sequences. These results suggest an important role of glycosylation at residue N^{1496} for the stability of apoB-37. Loss of N-linked oligosaccharide resulting in proteolytic cleavage has been reported for other proteins (36).

The effect of N-to-Q substitution at all five N-linked glycosylation sites within apoB-37 was determined further using mixed cultures of stable transfectants. Again, the aberrant mobility of apoB-37N$^{158–1496}$ was observed (indicated by "x" in **Fig. 4**). Pulse-chase analysis showed that the secretion efficiency of apoB-37N$^{158–1496}$ was decreased by more than 2-fold as compared with that of apoB-37wt, which was similar to the effect of tunicamycin treatment on apoB-37wt secretion (Fig. 4A). However, there was no difference in the amount of intracellular apoB-37N$^{158–1496}$

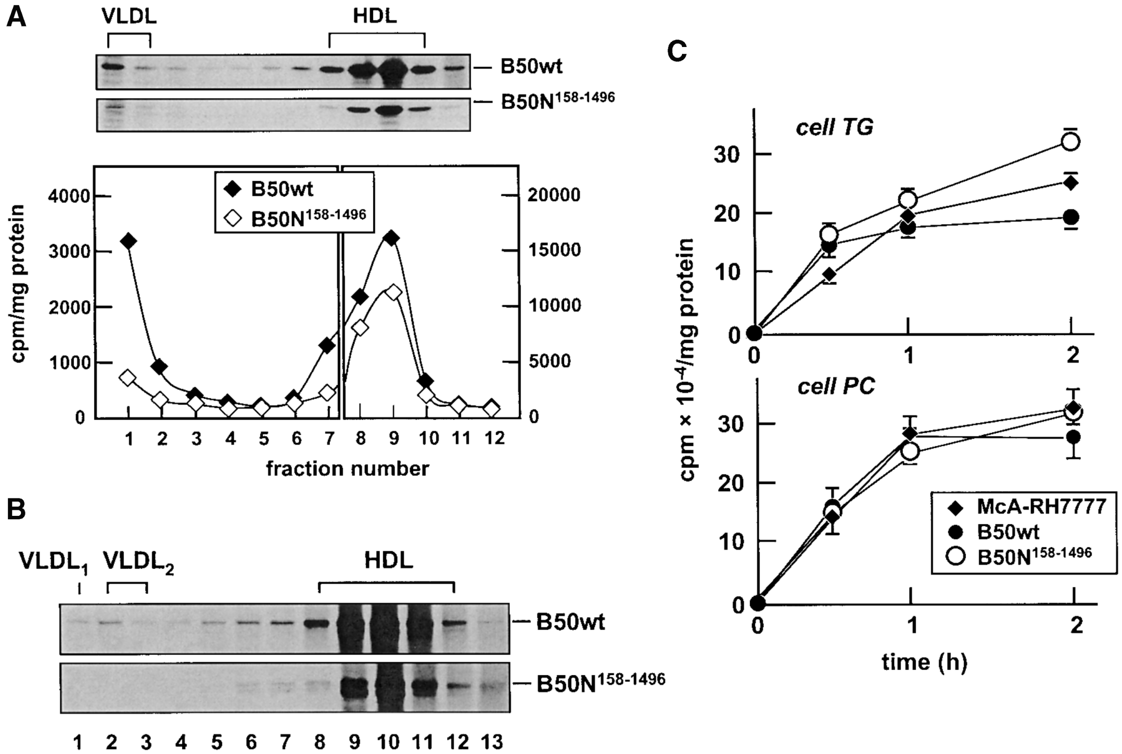


Fig. 6. Impaired apoB-50N$^{158-1496}$ secretion as VLDL and lipid synthesis in transfected cells. A: Medium collected at the end of 2 h chase was fractionated by ultracentrifugation in a sucrose density gradient, and [^{35}S]apoB-50 in each fraction was analyzed by SDS-PAGE and fluorography. Top, representative fluorograms of two experiments with similar results. Bottom, the radioactivity associated with [^{35}S]apoB-50wt and [^{35}S]apoB-50N$^{158-1496}$ quantified by scintillation counting. Note the scale of *y* axis for fractions 8–12 is different from that for fractions 1–7. B: Density distribution of apoB-50wt and apoB-50N$^{158-1496}$ in the microsomal lumen. One hour after labeling, the microsomes were prepared and the lumenal content was fractioned. [^{35}S]apoB in each fraction was immunoprecipitated and subjected to SDS-PAGE (3–15%) and fluorography. C: Cells (6-well plate) were labeled with [^{3}H]glycerol (5 μCi/well) in DMEM (20% serum and 0.4 mM oleate) for indicated times. Lipids were extracted from the cells and resolved by thin-layer chromatography. Radioactivity associated with TG and phosphatidylcholine (mean ± SD, n = 3) was quantified by scintillation counting.

during chase as compared with apoB-37wt (Fig. 4B), suggesting that apoB-37N$^{158-1496}$, like the apoB-17 mutant, was also rapidly degraded intracellularly. The effect of N-glycan mutation on intracellular distribution of apoB-37 was determined by subcellular fractionation using Nycodenz gradient ultracentrifugation. Immunoblot analysis showed that at steady state, there was no difference in the distribution in the ER or Golgi compartments between apoB-37wt and apoB-37N$^{158-1496}$ (data not shown).

The decreased secretion of apoB-37N$^{158-1496}$ was observed in both VLDL and lipoprotein fractions whose buoyancy resembling that of high-density lipoproteins (HDL) (Fig. 4C). Moreover, the lack of N-linked oligosaccharides resulted in a skewed distribution of apoB-37N$^{158-1496}$ toward denser fractions in the microsomal lumen (Fig. 4D). Thus, the lack of N-linked glycosylation in apoB-37 not only decreases its secretion as lipid-poor particles (i.e., the HDL-like species) but also impairs its ability to associate with neutral lipids in VLDL assembly.

N-to-Q substitution within apoB-50 results in decreased secretion

The studies with apoB-37 suggest strongly that appropriate glycosylation is required for efficient acquisition of lipids to form VLDL. However, the unexpected proteolytic cleavage of apoB-37N$^{158-1496}$ that removed ~5% apoB sequences from the carboxyl terminus of apoB-37 made the above data less conclusive. To ascertain that N-glycan of apoB indeed plays a role in VLDL assembly, we extended the mutagenesis studies to apoB-50 that contain the same number of N-glycan sites as apoB-37 (Fig. 1A) but contains more lipid binding sequences (35). Because the large size of the apoB-50 cDNA construct results in poor transfection efficiency, the experiments were conducted with selected stably transformants that expressed appreciable amount of apoB-50 proteins. Preliminary experiments showed that unlike what happened to mutant apoB-37, mutation at N^{1496} did not give aberrant electrophoretic mobility in apoB-50 (data not shown). This result suggests that amino acid sequences between the carboxyl termini of apoB-37 and apoB-50 can overcome the instability of the proteins introduced by N^{1496}-to-Q substitution.

We next performed pulse-chase experiments using two stable cell lines that expressed similar level of apoB-50wt or apoB-50N$^{158-1496}$ to determine the effect of lack of N-glycan on apoB-50 secretion efficiency. Results presented in **Fig. 5A** show that secretion efficiency of [^{35}S]apoB-50N$^{158-1496}$ was decreased in both d < 1.02 and d > 1.02

g/ml fractions as compared with that of apoB-50wt. The amount of cell-associated [^{35}S]apoB-50N$^{158–1496}$ during chase was greater than that of apoB-50wt (Fig. 5B), which suggested that, unlike apoB-17N^{158} or apoB-37N$^{158–1496}$, the mutant apoB-50 was not rapidly degraded intracellularly. Between the two stably transfected cells, the secretion efficiency of endogenous apoA-I was comparable as determined by pulse-chase analysis in two independent experiments (Fig. 5C). However, expression of apoB-50N$^{158–1496}$ resulted in 50% decrease in endogenous apoB-100 secretion efficiency, although synthesis of endogenous apoB-100 was not affected (data not shown). The inhibitory effect of apoB-50N$^{158–1496}$ expression on endogenous apoB-100 secretion was reminiscent of previous observation where expression of apoB-50 disulfide linkage mutants also inhibited endogenous apoB-100 secretion (15).

Examination of density distribution of the medium [^{35}S]apoB-50N$^{158–1496}$ confirms the above pulse-chase data, showing diminished secretion of [^{35}S]apoB-50N$^{158–1496}$ in both VLDL and HDL-like fractions (**Fig. 6A**). The decrease in secretion of apoB-50N$^{158–1496}$ as VLDL was also observed by immunoblot analysis to measure apoB-50N$^{158–1496}$ mass (data not shown). To determine if the decreased apoB-50N$^{158–1496}$-VLDL secretion was a consequence of impaired assembly, we examined the buoyant density of apoB-50N$^{158–1496}$-containing lipoproteins within the microsomal lumen. As shown in Figure 6B, at steady state the amount of ^{35}S-labeled apoB-50N$^{158–1496}$ associated with HDL-like fractions was reduced and that associated with VLDL fractions was absent as compared with apoB-50wt. Thus, the diminished apoB-50N$^{158–1496}$ secretion as VLDL was likely attributable to a defect in assembly of neutral lipid. The inability of apoB-50N$^{158–1496}$ to assemble VLDL, however, was not attributable to limited synthesis or availability of lipids in the transfected cells, as the synthesis of triacylglycerol (TG) and phosphatidylcholine (measured as [^{3}H]glycerol incorporation, Fig. 6C) and the mass of TG (data not shown) in apoB-50N$^{158–1496}$- and apoB-50wt-transfected cells were comparable. There was also no impairment in the activity of microsomal triglyceride transfer protein (MTP) in apoB-50N$^{158–1496}$-transfected cells (data not shown). These results suggest that the N-linked oligosaccharides play an important role during bulk TG incorporation in the assembly of VLDL containing apoB-50.

Effect of N-glycan mutation on intracellular distribution of apoB-37 and apoB-50

The effect of the loss of N-glycan on intracellular distribution of apoB-50 was determined by subcellular fractionation (Nycodenz gradient ultracentrifugation) followed by immunoblot analysis. At steady state, although the concentration of apoB-50N$^{158–1496}$ at steady state was increased in the ER and Golgi compartments, the relative distribution of apoB-50N$^{158–1496}$ was nearly identical to that of apoB-50wt (data not shown). Moreover, double immunofluorescence colocalization studies using confocal microscopy with cells expressing apoB-50wt or apoB-50N$^{158–1496}$ also showed no apparent difference in their distribution in the ER or Golgi (data not shown). In addition to indirect immunofluorescence studies, the effect of the loss of N-glycan on intracellular distribution was more directly determined using GFP (green fluorescent protein)-tagged apoB proteins (in this case we used apoB-46). The B46wt-GFP and B46N$^{158-1496}$-GFP fusion proteins contained the amino-terminal 2,099 amino acids of apoB plus GFP at their carboxyl termini. Including GFP at the carboxyl terminus of B46 did not affect its ability to form or secrete as lipoproteins in transiently transfected McA-RH7777 cells (Vukmirica and Yao, unpublished observation). Live cell imaging of apoB-46wt-GFP and apo-B46N$^{158–1496}$-GFP using video microscopy did not detect difference in their intracellular distribution (data not shown). These data together indicate that the loss of N-glycans is not associated with gross alterations in the intracellular distribution of apoB.

DISCUSSION

In this work, we have compared the role of N-linked oligosaccharides between three truncated apoB variants, namely apoB-17, apoB-37, and apoB-50, that have increasing lipid binding capabilities. The ability of human apoB polypeptide to associate with neutral lipids correlates positively with the number of amphipathic β-strands downstream of apoB-17 (35, 37), and proper lipidation confers post-translational stability of apoB and ensures its efficient secretion (34). The apoB-17 represents the amino-terminal β-α1 domain of apoB-100 (38), which is highly disulfide bonded and is secreted as lipid-poor forms (13). Although apoB-17 itself is unable to assemble neutral lipids to form lipoproteins, it has strong binding affinity toward MTP (39, 40) and is essential for lipid recruitment mediated by downstream lipid binding sequences (41). The in vivo studies of patients with hypobetalipoproteinemia (42) and cell culture studies with truncated forms of human apoB (12, 34) have suggested that the ability to assemble VLDL lies at the length transition between ~30% and ~40% of apoB, a region enriched with amphipathic β-strands (35). Our data have shown that the loss of N-glycans by site-directed mutagenesis results in decreased secretion efficiency of the apoB polypeptide regardless of their length, suggesting that the additional lipid binding sequences presented downstream of apoB-17 fail to offset the requirement for N-linked oligosaccharides. Furthermore, the current studies indicate that the lack of N-glycans not only impairs secretion of apoB as lipid-poor polypeptides (i.e., the HDL-like species containing apoB-37 or apoB-50), but also compromises the association of apoB-37 and apoB-50 with neutral lipids in forming VLDL. Thus, in addition to lipid binding sequences of apoB, the N-linked oligosaccharides conjugated to apoB also play a role conferring the post-translational stability and lipid assembly.

The observation that loss of a single N-glycan in apoB-17 results in impaired secretion of mutant apoB-17N^{158} (Fig. 2) is striking, which indicates that the β-α1 domain structure not only requires appropriate disulfide bonding (14, 15) but also N-linked oligosaccharides. The residue N^{158} in apoB is conserved between apoB and MTP, and is immediately adjacent to a disulfide bridge (C^{159}-C^{185} in apoB) that is also conserved between the two proteins. On the basis of the known crystal structure of lamprey lipovitellin, this disulfide bridge (C^{159}-C^{185}) is believed to connect two β-strands within the amino-terminal β-barrel structure of the β-α1 domain in human apoB (7). Although the current data suggest a significant role of N^{158}-glycan in apoB-17, previous mutagenesis studies showed that loss of the adjacent disulfide bond (C^{159}-C^{185}) had no impact on the secretion of apoB-29 (43) or apoB-50 (15). Insights into a functional role of the N^{158}-glycan in apoB-17 folding and stability has yet to await for structural information on the β-α1 domain.

It is noteworthy that although N^{158}-glycan is of particular importance to apoB-17, in the context of apoB-37 where multiple N-linked glycans exist, the single N^{158}-to-Q substitution exerts no effect on the mutant apoB-37N^{158} secretion. In fact, N-to-Q substitution at any single N-glycan site within apoB-37 had marginal effect on its secretion, except for N^{1496}. The loss of N^{1496} in apoB-37 not only resulted in decreased secretion (although not to the same extent as in apoB-37$N^{158-1496}$) but also affected electrophoretic mobility of apoB-37N^{1496} and apoB-37$N^{158-1496}$ (Fig. 3B and C). Epitope mapping experiments with apoB-37$N^{158-1496}$ implied that proteolytic cleavage might have occurred at the carboxyl terminus of the protein and removed ~5% of apoB sequences (Fig. 3D). Because the low molecular weight species of apoB-37$N^{158-1496}$ (designated apoB-37$N^{158-1496}$-x) was observed at the earliest time point of labeling within the ER, the proteolysis likely occurred in the ER. At the moment, the mechanism for this loss of N-glycan induced proteolysis is unknown; nor are we able to conclude definitively that N^{1496} is of particular significance to apoB structure, because proteolytic cleavage did not occur to mutant apoB-50$N^{158-1496}$. Induced proteolysis as a result of loss of N-glycans has been reported for mutant human transferrin receptor (36). When N-to-Q substitution performed at a single N-glycan site was contrasted with that performed at multiple sites, it became clear that secretion of apoB-37 was gradually impaired as the number of mutated N-glycan sites increased (Fig. 3C, right five bars marked B37). These data support the notion that cooperative action of multiple N-linked oligosaccharides takes place in the attainment of proper folding of proteins (2). The difference in the effect of $N^{158-1496}$ mutation on the susceptibility to the presumed proteolytic cleavage between apoB-37 and apoB-50 is intriguing. It suggests that whatever has happened to apoB-37$N^{158-1496}$ could be offset in the context of apoB-50, apparently by acquisition of lipid binding sequences downstream of apoB-37. The nature of this compensation is unclear. However, it suggests strongly that an interplay takes place between lipid binding sequences and N-linked oligosaccharides in achieving proper folding and lipidation of apoB.

Although the mechanism responsible for the impaired secretion of VLDL with mutants apoB-37 or apoB-50 is currently unclear, it is noted that the lack of N-glycans exerts different effects on the fate of these proteins. Pulse-chase experiments showed that while the impaired secretion of mutant apoB-37$N^{158-1496}$ (apoB-17N^{158} as well) was not accompanied with intracellular accumulation (Figs. 2A and 4B), there was a prolonged intracellular retention of nascent apoB-50$N^{158-1496}$ (Fig. 5B). The lack of an accumulation of apoB-17N^{158} or apoB-37$N^{158-1496}$ during chase is indicative of intracellular degradation of these mutant proteins. The degradation of apoB-17N^{158} and apoB-37$N^{158-1496}$ is unlikely mediated by proteasome that has been implicated in cotranslational degradation of apoB-100. Thus, while including proteasomal inhibitors during continuous labeling renders no increase in the incorporation of [^{35}S]amino acids into apoB-17N^{158}, even though the inhibitor treatment does prevent cotranslational degradation of endogenous apoB-100 in transfected cells (Fig. 2C). However, that no apoB-17N^{158} or apoB-37$N^{158-1496}$ was accumulated in the ER suggests that degradation, probably occurring in this compartment, preceded ER exit. In contrast, the effect of loss of N-glycan on cell apoB-50 was different from that on apoB-17 or apoB-37. Under no circumstances have we observed rapid degradation of apoB-50$N^{158-1496}$ during chase. The prolonged retention of apoB-50 within the secretary pathway suggests that lipid binding sequences between the carboxyl termini of apoB-37 and apoB-50 make the protein less susceptible to rapid degradation, even though these sequences cannot compensate for the requirement of N-glycans for efficient lipid recruitment. However, although the use of site-specific mutagenesis has allowed examination of the role of N-linked glycosylation of apoB more directly than the use of chemical inhibitor tunicamycin, it remains to be determined whether or not the Asn-to-Gln mutations may have caused gross structural changes that affect apoB function independent of N-glycans.

The mechanisms responsible for rapid degradation of mutant apoB-17 and apoB-37 and for the ultimate degradation of mutant apoB-50 retained within the cells also needs to be defined further. Recently, it has been reported the existence of a dual mechanism for ER-associated degradation of misfolded proteins. Thus, while misfolded membrane proteins are retained in the ER, misfolded soluble proteins are trafficked to the Golgi and then retrieved back to the ER for degradation (44). In the case of apoB, a secreted lipid binding protein, multiple degradation mechanisms have been described (34, 45). It is of interest to determine if apoB N-glycan mutants undergo ER exit and then retrograde retrieval prior to the ER degradation. It will also be of interest to examine if the loss of N-glycans in apoB will affect apoB translocation and retrograde translocation across the ER membrane. Recently, a group of molecular chaperones (such as immunoglobulin binding protein, protein disulfide isomerase, calcium binding protein 2, calreticulin, and glucose regulatory protein 94) were found in fractions containing VLDL particles within the lumen of microsomes (46). The effect of the loss of N-glycans in apoB on its interaction with molecular chaperones within the secretory compartments merits further investigation. The current study highlights that multiple factors, including lipids, molecular chaperones, and post-

ASBMB JOURNAL OF LIPID RESEARCH

translational modification of apoB (such as disulfide bonding, palmitoylation, and N-linked glycosylation) are involved in the complex VLDL assembly and secretion processes.

The authors thank R. Milne, Y. Marcel, M. G. Farquhar, and J. E. Vance for antibodies used in this work; and H. McBride and J. Ngsee for the guidance and assistance in fluorescence microscopy. We also thank Parke-Davis Pfizer for funds supporting primary hepatocyte facilities at University of Ottawa Heart Institute. This work was supported by Canadian Institutes of Health Research grant MT-15486 (Z.Y.); the Heart and Stroke Foundation of Ontario (Z.Y.); grants from the Ministry of Education, Science, and Culture of Japan and the Japan Health Science Foundation (T.N-M.); and a Canadian Institutes of Health Research Doctoral Research Award (J.V.).

REFERENCES

1. Opdenakker, G., P. M. Rudd, C. P. Ponting, and R. A. Dwek. 1993. Concepts and principles of glycobiology. *FASEB J.* **7:** 1330–1337.
2. Helenius, A. 1994. How N-linked oligosaccharides affect glycoprotein folding in the endoplasmic reticulum. *Mol. Biol. Cell.* **5:** 253–265.
3. Imperiali, B., and K. W. Rickert. 1995. Conformational implications of asparagine-linked glycosylation. *Proc. Natl. Acad. Sci. USA.* **92:** 97–101.
4. Taniguchi, T., Y. Ishikawa, M. Tsunemitsu, and H. Fukuzaki. 1989. The structures of the asparagine-linked sugar chains of human apolipoprotein B-100. *Arch. Biochem. Biophys.* **273:** 197–205.
5. Baker, M. E. 1988. Is vitellogenin an ancestor of apolipoprotein B-100 of human low-density lipoprotein and human lipoprotein lipase? *Biochem. J.* **255:** 1057–1060.
6. Anderson, T. A., D. G. Levitt, and L. J. Banaszak. 1998. The structural basis of lipid interactions in lipovitellin, a soluble lipoprotein. *Structure.* **6:** 895–909.
7. Mann, C. J., T. A. Anderson, J. Read, S. A. Chester, G. B. Harrison, S. Kochl, P. J. Ritchie, P. Bradbury, F. S. Hussain, J. Amey, B. Vanloo, M. Rosseneu, R. Infante, J. M. Hancock, D. G. Levitt, L. J. Banaszak, J. Scott, and C. C. Shoulders. 1999. The structure of vitellogenin provides a molecular model for the assembly and secretion of atherogenic lipoproteins. *J. Mol. Biol.* **285:** 391–408.
8. Chan, L. 1992. Apolipoprotein B, the major protein component of triglyceride-rich and low density lipoproteins. *J. Biol. Chem.* **267:** 25621–25624.
9. Yao, Z., and R. S. McLeod. 1994. Synthesis and secretion of hepatic apolipoprotein B-containing lipoproteins. *Biochim. Biophys. Acta.* **1212:** 152–166.
10. Shelness, G. S., and J. A. Sellers. 2001. Very-low-density lipoprotein assembly and secretion. *Curr. Opin. Lipidol.* **12:** 151–157.
11. McLeod, R. S., Y. Zhao, S. L. Selby, J. Westerlund, and Z. Yao. 1994. Carboxyl-terminal truncation impairs lipid recruitment by apolipoprotein B100 but does not affect secretion of the truncated apolipoprotein B-containing lipoproteins. *J. Biol. Chem.* **269:** 2852–2862.
12. McLeod, R. S., Y. Wang, S. Wang, A. Rusinol, P. Links, and Z. Yao. 1996. Apolipoprotein B sequence requirements for hepatic very low density lipoprotein assembly. Evidence that hydrophobic sequences within apolipoprotein B48 mediate lipid recruitment. *J. Biol. Chem.* **271:** 18445–18455.
13. Yao, Z. M., B. D. Blackhart, M. F. Linton, S. M. Taylor, S. G. Young, and B. J. McCarthy. 1991. Expression of carboxyl-terminally truncated forms of human apolipoprotein B in rat hepatoma cells. Evidence that the length of apolipoprotein B has a major effect on the buoyant density of the secreted lipoproteins. *J. Biol. Chem.* **266:** 3300–3308.
14. Shelness, G. S., and J. T. Thornburg. 1996. Role of intramolecular disulfide bond formation in the assembly and secretion of apolipoprotein B-100-containing lipoproteins. *J. Lipid Res.* **37:** 408–419.
15. Tran, K., J. Boren, J. Macri, Y. Wang, R. McLeod, R. K. Avramoglu, K. Adeli, and Z. Yao. 1998. Functional analysis of disulfide linkages clustered within the amino terminus of human apolipoprotein B. *J. Biol. Chem.* **273:** 7244–7251.
16. Zhao, Y., J. B. McCabe, J. Vance, and L. G. Berthiaume. 2000. Palmitoylation of apolipoprotein B is required for proper intracellular sorting and transport of cholesteroyl esters and triglycerides. *Mol. Biol. Cell.* **11:** 721–734.
17. Huang, G., D. M. Lee, and S. Singh. 1988. Identification of the thiol ester linked lipids in apolipoprotein B. *Biochemistry.* **27:** 1395–1400.
18. Kamanna, V. S., and D. M. Lee. 1989. Presence of covalently attached fatty acids in rat apolipoprotein B via thiolester linkages. *Biochem. Biophys. Res. Commun.* **162:** 1508–1514.
19. Struck, D. K., P. B. Siuta, M. D. Lane, and W. J. Lennarz. 1978. Effect of tunicamycin on the secretion of serum proteins by primary cultures of rat and chick hepatocytes. Studies on transferrin, very low density lipoprotein, and serum albumin. *J. Biol. Chem.* **253:** 5332–5337.
20. Bell-Quint, J., T. Forte, and P. Graham. 1981. Glycosylation of apolipoproteins by cultured rat hepatocytes. Effect of tunicamycin on lipoprotein secretion. *Biochem. J.* **200:** 409–414.
21. Siuta-Mangano, P., D. R. Janero, and M. D. Lane. 1982. Association and assembly of triglyceride and phospholipid with glycosylated and unglycosylated apoproteins of very low density lipoprotein in the intact liver cell. *J. Biol. Chem.* **257:** 11463–11467.
22. Bonen, D. K., F. Nassir, A. M. Hausman, and N. O. Davidson. 1998. Inhibition of N-linked glycosylation results in retention of intracellular apo[a] in hepatoma cells, although nonglycosylated and immature forms of apolipoprotein[a] are competent to associate with apolipoprotein B-100 in vitro. *J. Lipid Res.* **39:** 1629–1640.
23. Macri, J., and K. Adeli. 1997. Conformational changes in apolipoprotein B modulate intracellular assembly and degradation of ApoB-containing lipoprotein particles in HepG2 cells. *Arterioscler. Thromb. Vasc. Biol.* **17:** 2982–2994.
24. Hussain, M. M., Y. Zhao, R. K. Kancha, B. D. Blackhart, and Z. Yao. 1995. Characterization of recombinant human apoB-48-containing lipoproteins in rat hepatoma McA-RH7777 cells transfected with apoB-48 cDNA. Overexpression of apoB-48 decreases synthesis of endogenous apoB-100. *Arterioscler. Thromb. Vasc. Biol.* **15:** 485–494.
25. Blackhart, B. D., Z. M. Yao, and B. J. McCarthy. 1990. An expression system for human apolipoprotein B100 in a rat hepatoma cell line. *J. Biol. Chem.* **265:** 8358–8360.
26. Milne, R. W., R. Theolis, Jr., R. B. Verdery, and Y. L. Marcel. 1983. Characterization of monoclonal antibodies against human low density lipoprotein. *Arteriosclerosis.* **3:** 23–30.
27. Wang, X., R. Pease, J. Bertinato, and R. W. Milne. 2000. Well-defined regions of apolipoprotein B-100 undergo conformational change during its intravascular metabolism. *Arterioscler. Thromb. Vasc. Biol.* **20:** 1301–1308.
28. Tran, K., Y. Wang, C. J. DeLong, Z. Cui, and Z. Yao. 2000. The assembly of very low density lipoproteins in rat hepatoma McA-RH7777 cells is inhibited by phospholipase A2 antagonists. *J. Biol. Chem.* **275:** 25023–25030.
29. Boren, J., S. Rustaeus, and S. O. Olofsson. 1994. Studies on the assembly of apolipoprotein B-100- and B-48-containing very low density lipoproteins in McA-RH7777 cells. *J. Biol. Chem.* **269:** 25879–25888.
30. Wang, Y., K. Tran, and Z. Yao. 1999. The activity of microsomal triglyceride transfer protein is essential for accumulation of triglyceride within microsomes in McA-RH7777 cells. A unified model for the assembly of very low density lipoproteins. *J. Biol. Chem.* **274:** 27793–27800.
31. Rickwood, D., T. Ford, and J. Graham. 1982. Nycodenz: a new nonionic iodinated gradient medium. *Anal. Biochem.* **123:** 23–31.
32. Hammond, C., and A. Helenius. 1994. Quality control in the secretory pathway: retention of a misfolded viral membrane glycoprotein involves cycling between the ER, intermediate compartment, and Golgi apparatus. *J. Cell Biol.* **126:** 41–52.
33. Nohturfft, A., R. A. DeBose-Boyd, S. Scheek, J. L. Goldstein, and M. S. Brown. 1999. Sterols regulate cycling of SREBP cleavage-activating protein (SCAP) between endoplasmic reticulum and Golgi. *Proc. Natl. Acad. Sci. USA.* **96:** 11235–11240.
34. Yao, Z., K. Tran, and R. S. McLeod. 1997. Intracellular degradation of newly synthesized apolipoprotein B. *J. Lipid Res.* **38:** 1937–1953.
35. Segrest, J. P., M. K. Jones, V. K. Mishra, G. M. Anantharamaiah, and D. W. Garber. 1994. apoB-100 has a pentapartite structure composed of three amphipathic alpha-helical domains alternating with two amphipathic beta-strand domains. Detection by the computer program LOCATE. *Arterioscler. Thromb.* **14:** 1674–1685.
36. Hoe, M. H., and R. C. Hunt. 1992. Loss of one asparagine-linked

oligosaccharide from human transferrin receptors results in specific cleavage and association with the endoplasmic reticulum. *J. Biol. Chem.* **267:** 4916–4923.
37. Carraway, M., H. Herscovitz, V. Zannis, and D. M. Small. 2000. Specificity of lipid incorporation is determined by sequences in the N-terminal 37 of apoB. *Biochemistry.* **39:** 9737–9745.
38. Segrest, J. P., M. K. Jones, H. De Loof, and N. Dashti. 2001. Structure of apolipoprotein B-100 in low density lipoproteins. *J. Lipid Res.* **42:** 1346–1367.
39. Hussain, M. M., A. Bakillah, N. Nayak, and G. S. Shelness. 1998. Amino acids 430–570 in apolipoprotein B are critical for its binding to microsomal triglyceride transfer protein. *J. Biol. Chem.* **273:** 25612–25615.
40. Nicodeme, E., F. Benoist, R. McLeod, Z. Yao, J. Scott, C. C. Shoulders, and T. Grand-Perret. 1999. Identification of domains in apolipoprotein B100 that confer a high requirement for the microsomal triglyceride transfer protein. *J. Biol. Chem.* **274:** 1986–1993.
41. Gretch, D. G., S. L. Sturley, L. Wang, B. A. Lipton, A. Dunning, K. A. Grunwald, J. R. Wetterau, Z. Yao, P. Talmud, and A. D. Attie. 1996. The amino terminus of apolipoprotein B is necessary but not sufficient for microsomal triglyceride transfer protein responsiveness. *J. Biol. Chem.* **271:** 8682–8691.
42. Linton, M. F., R. V. Farese, Jr., and S. G. Young. 1993. Familial hypobetalipoproteinemia. *J. Lipid Res.* **34:** 521–541.
43. Huang, X. F., and G. S. Shelness. 1997. Identification of cysteine pairs within the amino-terminal 5% of apolipoprotein B essential for hepatic lipoprotein assembly and secretion. *J. Biol. Chem.* **272:** 31872–31876.
44. Vashist, S., W. Kim, W. J. Belden, E. D. Spear, C. Barlowe, and D. T. Ng. 2001. Distinct retrieval and retention mechanisms are required for the quality control of endoplasmic reticulum protein folding. *J. Cell Biol.* **155:** 355–368.
45. Fisher, E. A., M. Pan, X. Chen, X. Wu, H. Wang, H. Jamil, J. D. Sparks, and K. J. Williams. 2001. The triple threat to nascent apolipoprotein B. Evidence for multiple, distinct degradative pathways. *J. Biol. Chem.* **276:** 27855–27863.
46. Stillemark, P., J. Boren, M. Andersson, T. Larsson, S. Rustaeus, K. A. Karlsson, and S. O. Olofsson. 2000. The assembly and secretion of apolipoprotein B-48-containing very low density lipoproteins in McA-RH7777 cells. *J. Biol. Chem.* **275:** 10506–10513.