Efficient glycosylation site utilization by intracellular apolipoprotein B: implications for proteasomal degradation

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Abstract **The balance between the hepatic assembly of apolipoprotein B (apoB) and its presecretory degradation at the level of the endoplasmic reticulum (ER) may control the secretion of apoB-containing lipoproteins. In one model, apoB that fails to assemble with lipid undergoes translocation arrest, exposing the protein to the cytosolic proteasome. To examine apoB's translocation behavior under various metabolic conditions, glycosylation site utilization studies were performed. A 70-amino acid peptide containing three sites for N-linked glycosylation was appended to the C-terminus of apoB-50 (amino-terminal 50% of apoB) and expressed in both hepatic and nonhepatic cell lines. When the C-terminal reporter peptide was released by cyanogen bromide cleavage, all of the sites were glycosylated irrespective of cell type, labeling time, or assembly status. Similar peptide mapping of endogenous apoB-100 expressed in HepG2 cells was performed to monitor glycosylation at Asn residues 2752 (apoB-61), 2955 (apoB-65), and 3074 (apoB-68). N-linked glycosylation occurred at a minimum of two of the three sites, a frequency identical to that observed in apoB-100 recovered from cell media. Treatment of cells with proteasome inhibitors produced a 2.5-fold increase in intracellular apoB but failed to cause accumulation of an unglycosylated form. These results indicate that *1*) the efficient translocation of apoB into the ER occurs independently of microsomal triglyceride transfer protein and its assembly with lipid and *2*) despite its large size and affinity for lipid, delivery of misassembled apoB to the proteasome requires retrograde translocation from the ER lumen to cytosol.**—Huang, X. F., and G. S. Shelness. **Efficient glycosylation site utilization by intracellular apolipoprotein B: implications for proteasomal degradation.** ***J. Lipid Res.*** **1999.** 40: **2212–2222.**

Supplementary key words endoplasmic reticulum • protein turnover • proteolysis • quality control • translocation • retrograde translocation • dislocation • lipoprotein assembly • very low density lipoprotein • HepG2 cells

The translocation of secretory precursor proteins across the endoplasmic reticulum (ER) membrane is believed to occur cotranslationally via a tight junction between the ribosome and the ER membrane. Although the ribosome–membrane junction may be dynamic (1, 2), it appears by most criteria that once initiated, polypeptides chain translocation progresses to completion (3, 4). More recently, this paradigm has been challenged in part to account for the delivery of misfolded secretory precursor proteins to the cytosol where they undergo multiubiquitination and proteasomal degradation (5). The existence of such a pathway is based primarily on the behavior of apolipoprotein B (apoB), a 4536 amino acid secretory glycoprotein that promotes the hepatic assembly and secretion of very low density lipoproteins (6).

The cotranslational assembly of apoB into lipoprotein particles requires a pool of secretion-coupled lipid and the ER-localized cofactor microsomal triglyceride transfer protein (MTP) (7, 8). It has been proposed that under conditions of limiting lipid or the absence of MTP, as occurs in the genetic disorder abetalipoproteinemia, the translation and translocation of apoB becomes uncoupled (9–12). This generates a transmembrane form in which the bulk of the protein mass is situated on the cytosolic side of the ER membrane. Should lipid become available, full-length transmembrane apoB has been reported to undergo a process of posttranslational "lipid facilitated translocation" into the ER lumen (13). However, if the translocation-arrested form of apoB persists, it is targeted for degradation by the ubiquitin/proteasome-mediated pathway in the cytosol (14, 15). The balance between the assembly and proteasomal degradation of apoB may represent the predominant control point for the regulation of hepatic triglyceride-rich lipoprotein production (16).

The translocation arrest model is based primarily on apoB's accessibility to exogenously added proteases and antibodies in microsomes and permeabilized cells (11,

Abbreviations: apoB, apolipoprotein B; CNBr, cyanogen bromide; ER, endoplasmic reticulum; MTP, microsomal triglyceride transfer protein; PAGE, polyacrylamide gel electrophoresis; PNGase F, peptide-N-glycosidase F, SDS, sodium dodecyl sulfate.

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17–20). In HepG2 cells, approximately 75% of apoB-100 is reported to display cytosolic domains at steady state (10, 11, 19). However, efforts to identify the domains that specify apoB's reduced translocation efficiency have not yielded a consensus nor has a mechanism been proposed to account for how cotranslational assembly events in the ER lumen regulates apoB's translocation efficiency. Pause transfer domains, many of which are positioned in the amino terminal (α_1) domain of apoB (see Fig. 1A), have the capacity to transiently uncouple translation from translocation in cell-free systems (2, 21–24). These studies raised the possibility that the transient pausing of translocation observed in vitro may be related to the apparent existence of transmembrane apoB in cultured cells. However, in transfected cells, pause transfer sequences failed to detectably arrest protein translocation into the ER (25, 26). More recently, sequences farther downstream in apoB including the β_1 and β_2 domains (Fig. 1A) have been implicated in the inefficient translocation of apoB (27, 28). However, other reports have questioned the validity of the translocation arrest model altogether (29, 30).

In the present study, N-linked glycosylation was explored as marker for domain-specific translocation of apoB across the ER membrane. N-linked glycosylation represents a reliable translocation marker as this modification occurs in the lumen of the ER prior to cell homogenization or permeabilization (31). These studies revealed that a recombinant form of apoB-50 transfected into both hepatic and nonhepatic cells and apoB-100 expressed endogenously by HepG2 cells were efficiently glycosylated. Furthermore, inhibition of the proteasome in HepG2 cells, which resulted in a dramatic accumulation of intracellular apoB, failed to cause accumulation of unglycosylated protein as would be predicted by the translocation arrest model. These studies indicate that once initiated, apoB's forward translocation proceeds independently of MTP and its assembly with lipid. Hence, despite its large size and lipophilic properties, the proteasomal degradation of apoB most likely requires processive retrograde movement from the ER lumen to the cytosol.

EXPERIMENTAL PROCEDURES

Materials

The protease inhibitor, N-acetyl-leucyl-leucyl-norleucinal (ALLN), sheep polyclonal anti-human apoB, and rabbit anti-α_2-macroglobulin were from Roche Molecular Biochemicals (Indianapolis, IN). Lactacystin was obtained from E.J. Corey, Harvard University. Monoclonal antibodies 3F5 and 4G3 were provided by R.W. Milne and Y.L. Marcel, Lipoprotein and Atherosclerosis Group, University of Ottawa Heart Institute. The 3F5 epitope has been mapped to amino acids 2835–2922 of apoB-100 and 4G3 to amino acids 2980–3084 (32). Anti-FLAG monoclonal antibody M2 and M2-agarose were from Eastman Kodak Scientific Imaging Systems (New Haven, CT) and Sigma Chemical (St. Louis, MO). BCA protein assay reagent was from Pierce Chemical Co. (Rockford, IL).

Construction of apoB-50GF

PCR was used to fuse a 9-amino acid spacer sequence (VGGGIEGRG), amino acids, 20–83 of yeast pro-α-factor (33), and the 8-amino acid FLAG epitope (DYKDDDDK) to the C-terminus of apoB-50 (Fig. 1A). The modified sequence was cloned into the expression vector pCMV5 (34) and verified by DNA sequencing. The glycosylation reporter sequence appended to the C-terminus of apoB-50 lacks Met residues allowing it to be released intact by CNBr cleavage.

Analysis of apoB-50GF C-terminal peptide glycosylation

COS-1 and HepG2 cells were cultured and transiently transfected as described (25, 35). Forty-eight hours post-transfection (25) cells were labeled with [^{3}H]Leu (NEN, Boston, MA) in Leu-deficient MEM for the indicated times. Cell pellets were pooled and extracted with lysis buffer (1% Triton X-100, 25 mm Tris HCl, pH 7.5, 300 mm NaCl, 50 μg/ml ALLN, 10 μg/ml pepstatin, 10 μg/ml leupeptin, and 1 mm PMSF). Control experiments showed nearly quantitative extraction of intracellular apoB under these lysis conditions (see Fig. 5 and data not shown). After centrifugation for 10 min at 14,000 *g*, the extract was subjected to immunoaffinity isolation with anti-FLAG–agarose. After washing the beads 3 times with lysis buffer, antigen was released by boiling for 5 min in 0.5% SDS. After centrifugation, supernatants were adjusted to 80 mg/ml CNBr, 70% formic acid and incubated in darkness for 16 h at room temperature. Samples were dried to completion using a Speed Vac (Savant Instruments), dissolved in 200 μl water, dried again, and dissolved by addition of 100 μl water and incubation at 100°C for 5 min (1% final concentration of SDS). After addition of 900 μl of lysis buffer, samples were subjected to immunoprecipitation with anti-FLAG monoclonal antibody M2 and protein G-Sepharose (25). Immunoprecipitated protein and peptides were eluted by boiling in 80 μl 0.5% SDS, 1% β-mercaptoethanol. After adjusting samples to 1% NP-40, 50 mm sodium phosphate, pH 7.5, equal aliquots were incubated with or without 2,500 units PNGase F (New England Biolabs, Beverly, MA) at 37°C for 2 h. Samples were mixed with 1/3 volume of SDS-PAGE sample buffer concentrated 3× and resolved by 4–20% gradient SDS-PAGE. Each gel lane in Figs. 2 and 3 shows products derived from the equivalent of one 150-mm dish of transfected cells. Control experiments revealed that the reactivity of anti-FLAG monoclonal antibody M2 towards both apoB50GF and its C-terminal peptides produced by CNBr was virtually identical irrespective of their glycosylation status (data not shown).

Analysis of apoB-100 glycosylation in HepG2 cells

HepG2 cells in 150-mm dishes were extracted with lysis buffer as described above and the apoB was immunoprecipitated with anti-apoB polyclonal antibody and protein G-Sepharose (36). Samples were eluted and treated with CNBr and PNGase F as described above for apoB-50GF. After electrophoresis, samples were electrophoretically transferred to nitrocellulose and probed with the indicated monoclonal antibody. Immunoreactive products were detected by incubation with anti-mouse IgG conjugated to horseradish peroxidase (Sigma Chemical Co.) followed by incubation with chemiluminescence reagent (Renaissance; NEN). To examine the effects of proteasome inhibition on apoB-100 glycosylation, HepG2 cells were incubated for 4 h with 40 μg/ml of ALLN and processed as described above. The effects of proteasome inhibitors (40 μg/ml ALLN and 1 μm lactacystin) were monitored in parallel dishes labeled for 4 h with [^{35}S]Met/Cys (Easytag, NEN). For this analysis, the labeled cells were lysed under the same conditions described above and the apoB was subjected to immunoprecipitation and 4–20% gradient SDS-PAGE. Radioactive bands were quantitated by phosphorimager analysis.

RESULTS

Construction and characterization of an apoB-50-glycosylation reporter

A cDNA was constructed encoding the amino-terminal 50% of apoB followed by a spacer sequence, the propeptide of yeast prepro-α-factor (33), and the 8-amino acid FLAG epitope tag (DYKDDDDK) (37). The apoB portion of the construct (amino acids 1–2268) contains the entire α_1 and β_1 domain and a small portion of the α_2 domain (38) (**Fig. 1A**). The propeptide contains three potential sites for N-linked glycosylation. To characterize the apoB-50GF construct, its behavior was compared to a previously characterized form of apoB-50 containing only the 8-amino acid FLAG sequence at its C-terminus (apoB-50F) (25). Both plasmids were transiently expressed in HepG2 cells, an MTP positive, lipoprotein-producing cell line. After labeling cells for 3 h, a post-nuclear membrane fraction was prepared and treated with sodium carbonate, pH 11.5, to remove lumenal contents (25). The pellet (P) fraction was solubilized with SDS. The supernatant fraction (S) was further fractionated by density gradient centrifugation into a $d > 1.25$ g/ml lipid-poor bottom (B) fraction and a lipidated $d < 1.25$ g/ml top (T) fraction, as described (39). Each fraction was subjected to immunoprecipitation with anti-FLAG monoclonal antibody and protein G-Sepharose (25).

Only a small percentage of apoB-50F and apoB-50GF was extracted from HepG2 cell microsomes by sodium carbonate (Fig. 1B, lanes 1–6). This behavior has been observed previously and could possibly result from apoB's transmembrane integration with the lipid bilayer (9, 17). Alternatively, it has been reported that carbonate resistance may be caused by the tendency of unlipidated or underlipidated apoB to aggregate and/or interact with the inner leaflet of the ER membrane (40). In any case, most of the apoB-50F and apoB-50GF that was extracted from HepG2 cell membranes was present in the $d < 1.25$ g/ml (T) lipoprotein fraction (lanes 2 and 3; 5 and 6). To confirm that the association of both apoB-50 constructs with lipid was specific, the MTP dependence of the assembly process was monitored. As observed in lanes 8, 9, 14, and 15, neither apoB-50 construct recovered from transfected COS cells (an MTP negative cell line) (41) was present in the $d < 1.25$ g/ml top fraction. Upon coexpression with the large subunit of MTP, approximately 60% of the carbonate-extractable protein underwent assembly with lipid (lanes 11 and 12; 17 and 18). The results in Fig. 1B indicate that both apoB-50F and apoB-50GF undergo similar extents of either MTP-

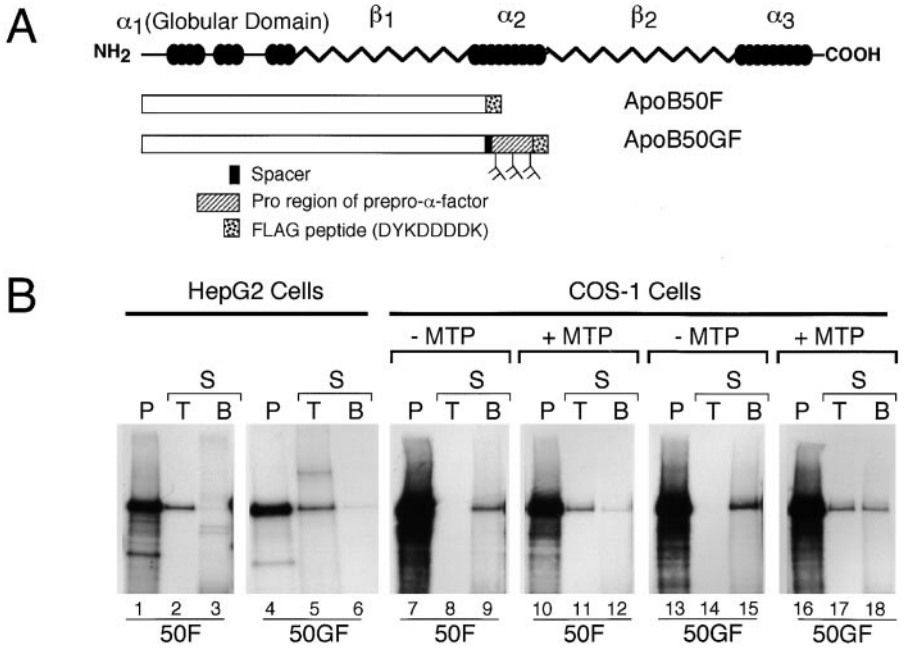

Fig. 1. Characterization of an apoB-50 glycosylation reporter construct. A: A domain map indicates the relative position of the α_1, β_1, α_2, β_2, and α_3 domains of apoB (38). Aligned underneath are the two constructs (apoB-50F and apoB-50GF) used in glycosylation studies. Branched symbols projecting down from the pro region of prepro-α-factor indicate potential sites for N-linked glycosylation. B: HepG2 cells (lanes 1–6) or COS cells (lanes 7–18) were transfected with either apoB-50F (50F) or apoB-50GF (50GF) in the absence (−) or presence (+) of MTP large subunit as indicated (39). Forty-eight h post-transfection cells were labeled for 3 h with [^{35}S]Met/Cys. A post-nuclear membrane fraction was isolated and extracted with 0.1 m sodium carbonate, pH 11.5. Pellets (P) were solubilized directly. Supernatant fractions were subjected to density gradient centrifugation to obtain a $d < 1.25$ g/ml top (T) fraction and a $d > 1.25$ g/ml bottom (B). ApoB was recovered from each sample by immunoprecipitation, fractionated by 6% SDS-PAGE, and visualized by fluorography.

dependent assembly with lipid or aggregation/membrane association.

C-terminal glycosylation sites in apoB-50GF are efficiently utilized in both hepatic and nonhepatic cells

Utilizing C-terminal glycosylation as a reporter, the translocation behavior of intracellular apoB-50GF was monitored. Both HepG2 and COS-1 cells were transfected with apoB-50GF and labeled for 6 h with [^{3}H]Leu. The apoB-50GF was affinity-purified by batch binding to anti-FLAG-agarose and analyzed by SDS-PAGE directly (**Fig. 2**, lanes 1 and 4) or subjected to digestion with CNBr. FLAG-containing products of the CNBr digestion were recovered by immunoprecipitation with anti-FLAG monoclonal antibody and treated with or without PNGase F to remove N-linked carbohydrate (lanes 2, 3, 5, and 6). Protein and C-terminal peptides were analyzed by SDS-PAGE and fluorography.

CNBr digestion was efficient giving rise to one major product. Upon deglycosylation with PNGase F, the major product migrated as a ~23 kDa peptide, consistent with cleavage at the last Met residue in the protein (apoB amino acid residue 2152) (Fig. 2, curved solid arrow). A minor product, corresponding to cleavage at the penultimate Met (residue 2015), was also generated, giving raise to a peptide of ~39 kDa (Fig. 2, open curved arrow). To examine glycosylation site utilization within the peptides, the patterns obtained with and without PNGase F cleavage were compared. This comparison revealed that under the steady-state labeling conditions used, virtually no unglycosylated peptide was generated in either cell line. Hence, apoB-50GF appears to undergo complete translocation in both a liver-derived cell that expresses MTP (HepG2) and a nonhepatic, MTP-negative cell line (COS-1).

The results obtained in COS-1 cells were surprising as it has been reported that apoB translocation is fully blocked in cell lines that do not express MTP. We therefore explored the behavior of apoB-50GF in CHO cells, another MTP-negative cell line that was reported to be incapable of translocating apoB-53 across the ER membrane (9, 42). In addition, we used shorter labeling times to explore whether transient transmembrane forms of apoB are generated early after the completion of translation. CHO cells were stably transfected with apoB-50GF as described (39), and three positive clones were pooled and labeled with [^{3}H]Leu for 20 or 60 min. As a comparison, transiently transfected COS cells were labeled under identical conditions. When the C-terminal CNBr-generated peptides were analyzed as described above, no unglycosylated products were detected in CHO cells after either 20 or 60 min of labeling (**Fig. 3**, compare lanes 1 and 2 to lane 3). In COS cells, only a small amount (~5% of total) of unglycosylated peptide was generated (lanes 4 and 5, lower arrow).

Intracellular and secreted forms of HepG2 cell apoB-100 are glycosylated to the same extent

Unstimulated HepG2 cells degrade ~75–90% of newly synthesized apoB-100 (43). This percentage is similar to the amount of apoB reported to be accessible to exogenous proteases (10, 11, 19, 44). We sought to confirm the existence of untranslocated apoB by monitoring glycosylation site utilization at positions downstream of putative sites of translocation arrest. For these studies, we utilized two monoclonal antibodies whose epitopes contained nearby glycosylation consensus sites.

ApoB-100 was affinity-purified from HepG2 cell lysate and media using anti-apoB polyclonal antibodies. This

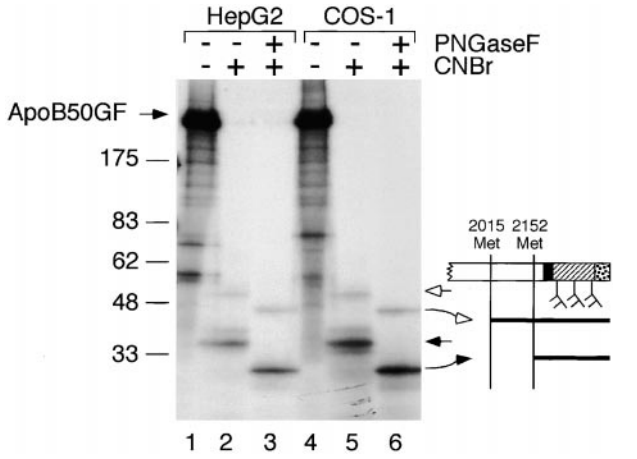

Fig. 2. C-terminal glycosylation sites in apoB-50GF are efficiently utilized. ApoB-50GF was transiently transfected into HepG2 and COS cells as indicated. Cells were labeled with [^{3}H]Leu for 6 h and lysed with nonionic detergent. ApoB-50GF was affinity purified with anti-FLAG-agarose and treated with (+) or without (−) CNBr. After immunoprecipitation, CNBr-treated samples were incubated in the presence or absence of PNGase F as indicated. Samples were fractionated by 4–20% gradient SDS-PAGE and visualized by fluorography. The diagram to the right shows the region of apoB-50GF present in the major (solid curved arrow) and minor (hollow curved arrow) deglycosylated cleavage products. The small, left facing arrows indicate the relative mobilities of the corresponding glycosylated peptides.

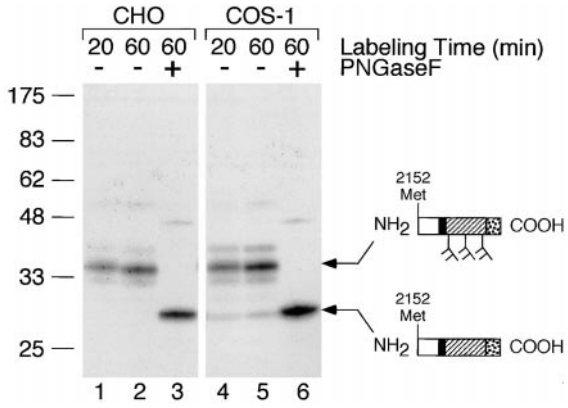

Fig. 3. Efficient C-terminal glycosylation of apoB-50GF occurs independently of MTP and labeling time. Stably transfected CHO cells or transiently transfected COS-1 cells were labeled for 20 or 60 min with [^{3}H]Leu as indicated. The apoB-50GF was affinity purified, treated with CNBr, and incubated in the absence (−) or presence (+) of PNGase F, as described in Fig. 2. The diagram to the right indicates the region of apoB-50GF present in the glycosylated and deglycosylated major cleavage product.

material was digested with CNBr and equal aliquots treated with or without PNGase F. The peptides were resolved by 15% SDS-PAGE, transferred to nitrocellulose, and probed with monoclonal antibody 3F5 (**Fig. 4A**) or 4G3 (Fig. 4B). Each antibody revealed the presence of two predominant cleavage products. Based on their size and the presence of one or two glycosylation sites, we concluded that the lower bands in each case corresponded to the complete digestion product and the upper band corresponded to cleavage at the upstream Met residue (Fig. 4, arrows). No immunoreactive products were detected elsewhere in the blots (data not shown).

By comparing samples treated with and without PNGase F, the glycosylation site utilization for each peptide could be established. The 18 kDa peptide was predominantly glycosylated once; however, a small amount of unglycosylated peptide (~10%) was also detected (Fig. 4A, compare lower bands in lanes 3 and 4). The 36 kDa peptide contains two potential sites for glycosylation. Comparison of the upper bands in lanes 3 and 4 revealed that approximately 80% of these peptides are glycosylated twice and 20% once. However, no completely unglycosylated 36 kDa peptide was detected. The minor under-glycosylation of these peptides is not due to a defect in translocation as identical glycosylation profiles were observed in apoB secreted by HepG2 cells (Fig. 4A, lanes 1 and 2).

Results obtained with monoclonal antibody 4G3 were similar; however, only a small amount of the complete 11,440 Da cleavage product was produced (Fig. 4B, lower arrow). The more abundant 29.5 kDa incomplete cleavage product contained two glycosylation sites that were both efficiently utilized. More importantly, the glycosylation profiles were identical in cells and media, confirming

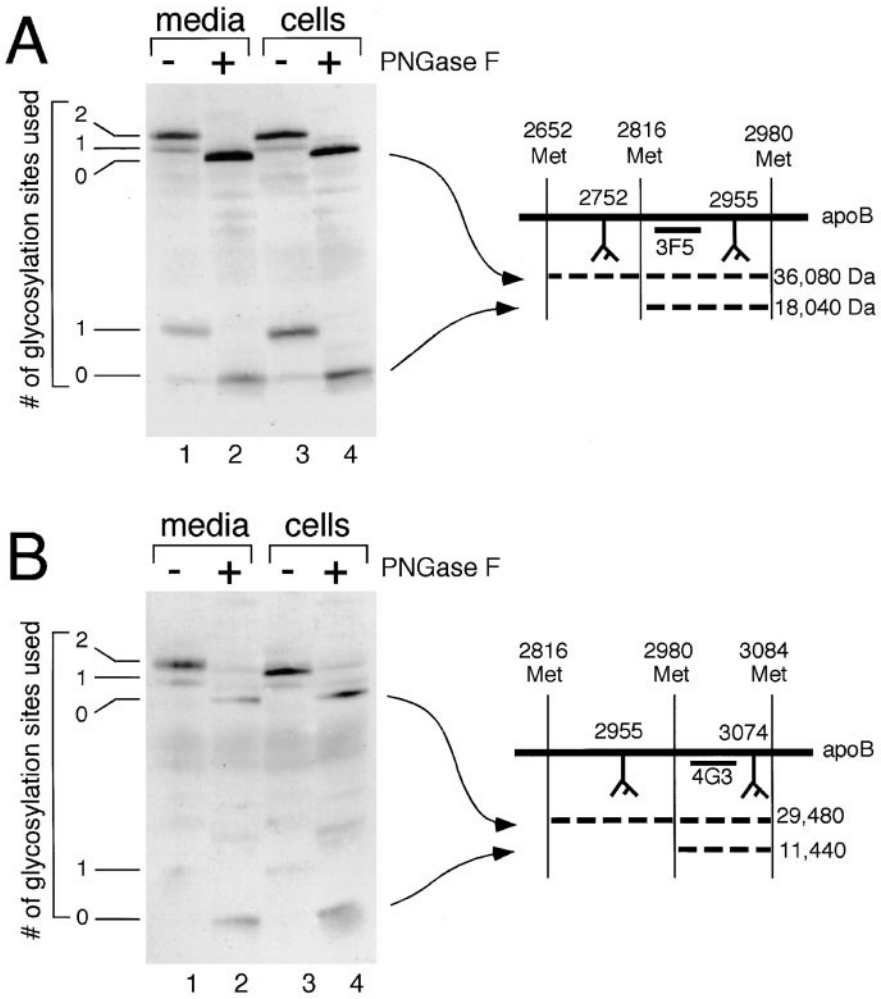

Fig. 4. Intracellular and secreted forms of HepG2 cell apoB-100 are similarly glycosylated. ApoB-100 from HepG2 cells and conditioned media was recovered by immunoprecipitation with anti-apoB polyclonal antibodies. Immunoprecipitates were treated with CNBr and PNGase F, as indicated. Samples were fractionated by 15% SDS-PAGE and transferred to nitrocellulose membranes. Nitrocellulose was probed with monoclonal antibodies 3F5 (A) or 4G3 (B). Diagrams to the right of the Western blots indicate the location of the apoB glycosylation sites, Met residues, and 3F5 and 4G3 epitopes (numbers indicate amino acid coordinates of mature apoB polypeptide). Arrows to the dotted lines show the location of each peptide generated by CNBr, followed by the calculated molecular mass of the deglycosylated form.

that the small amount of underglycosylation observed is unrelated to translocation status.

Intracellular apoB-100 remains soluble in nonionic detergent

In some cases, retrotranslocated proteins accumulate in the cytosol as detergent-insoluble aggregates (45, 46). While apoB does not appear to display a cytosolic localization, we specifically tested whether our detergent lysis procedure effectively extracted all intracellular apoB. HepG2 cells were extracted with lysis buffer containing 1% Triton X-100 as described under Experimental Procedures. After centrifugation for 10 min at 14,000 *g*, pellets were incubated in 1% SDS for 10 min followed by addition of four volumes of lysis buffer and probe sonication (46). The second supernatant was subjected to centrifugation and the pellet was boiled in 1% SDS for 5 min. After addition of 4 volumes of lysis buffer, the supernatant was subjected to a final round of centrifugation. Aliquots of the cleared supernatants from each extraction step were subjected to immunoprecipitation with antibodies to apoB or α_2-macroglobulin. Analysis of the gel (**Fig. 5**) indicated that, as with α_2-macroglobulin, a control secretory protein, >95% of the intracellular apoB-100 was present in the initial detergent extract. This result confirms that despite apoB's resistance to extraction by sodium carbonate, it is readily soluble in nonionic detergent. Hence, the glycosylation studies monitor almost the entire intracellular population of apoB.

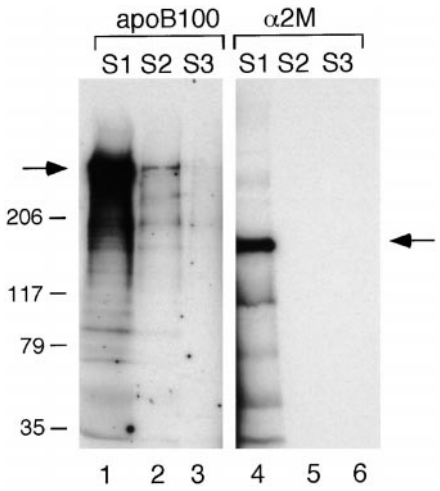

Fig. 5. Intracellular apoB-100 is soluble in nonionic detergent. HepG2 cells were labeled with [^{35}S]Met/Cys for 3 h and extracted with lysis buffer as described under Experimental Procedures. The extract was centrifuged to obtain the S1 supernatant. The pellet was incubated and vortexed for 10 min with 1% SDS followed by addition of 4 volumes of lysis buffer and probe sonication. After centrifugation to obtain the S2 supernatant, the final pellet was boiled in 1% SDS for 5 min followed by addition of 4 volumes of lysis buffer. The cleared supernatants from each centrifugation step were subjected to immunoprecipitation with antibodies to apoB-100 or α_2-macroglobulin (α2M) as indicated. The mobility of full length apoB-100 and α2M are indicated by arrows.

Inhibition of proteasome function has no effect on the glycosylation status of intracellular apoB-100

Results obtained above are clearly inconsistent with protease protection data in HepG2 cells indicating that at steady state, 75–100% of apoB has been arrested in its translocation. Nonetheless, we sought additional means of identifying the putative translocation arrested form of apoB by incubating cells with the proteasome inhibitors ALLN and lactacystin (47). When HepG2 cells were incubated with these compounds and then labeled for 4 h, a 2- to 3-fold increase in intracellular apoB was observed (**Fig. 6**). This increase in the steady-state population of apoB represents protein that would normally undergo turnover by the proteasome and according to the translocation arrest model should be transmembrane and, therefore, unglycosylated.

To determine whether proteasome inhibition affected the ratio of glycosylated to unglycosylated apoB, dishes of unlabeled HepG2 cells incubated with ALLN in parallel to the radiolabeled cells in Fig. 6 were subjected to the peptide mapping protocol used to analyze glycosylation in Fig. 4. These blots were intentionally overexposed to film in an attempt to detect unglycosylated peptides. However, despite the 2.5-fold increase in apoB-100 caused by proteasome inhibition (Fig. 6), the relative amount of unglycosylated peptide was not increased compared to the control cells (compare **Fig. 7** with Fig. 4). Finally, the glycosylation site utilization patterns observed in apoB peptides derived from media (lanes 1 and 2) and cells (lanes 3 and 4) were virtually identical, even after proteasome inhibition.

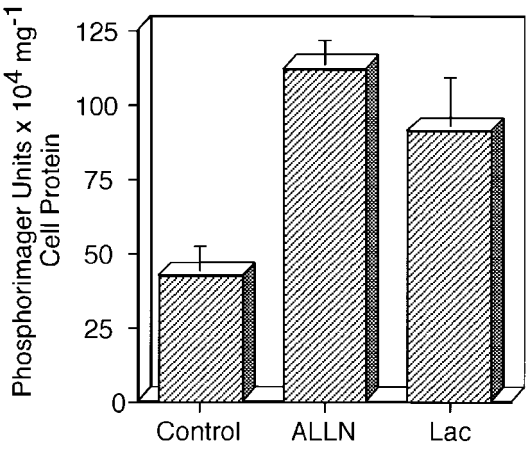

Fig. 6. Proteasome Inhibition causes a steady-state accumulation of cellular apoB-100. HepG2 cells in 6-well dishes were preincubated for 20 min with and without 40 μg/ml ALLN or 1 μm lactacystin, as indicated. Media were removed and replaced with Met/Cys-deficient labeling media containing [^{35}S]Met/Cys and the indicated inhibitor. After 4 h of labeling, cells were harvested and subjected to detergent lysis. Lysates were assayed for protein mass by the BCA method and apoB content was measured by immunoprecipitation and 4–20% gradient SDS-PAGE. Radiolabeled band intensity was quantitated by phosphorimager analysis. Values are expressed as mean ± SD (n = 3).

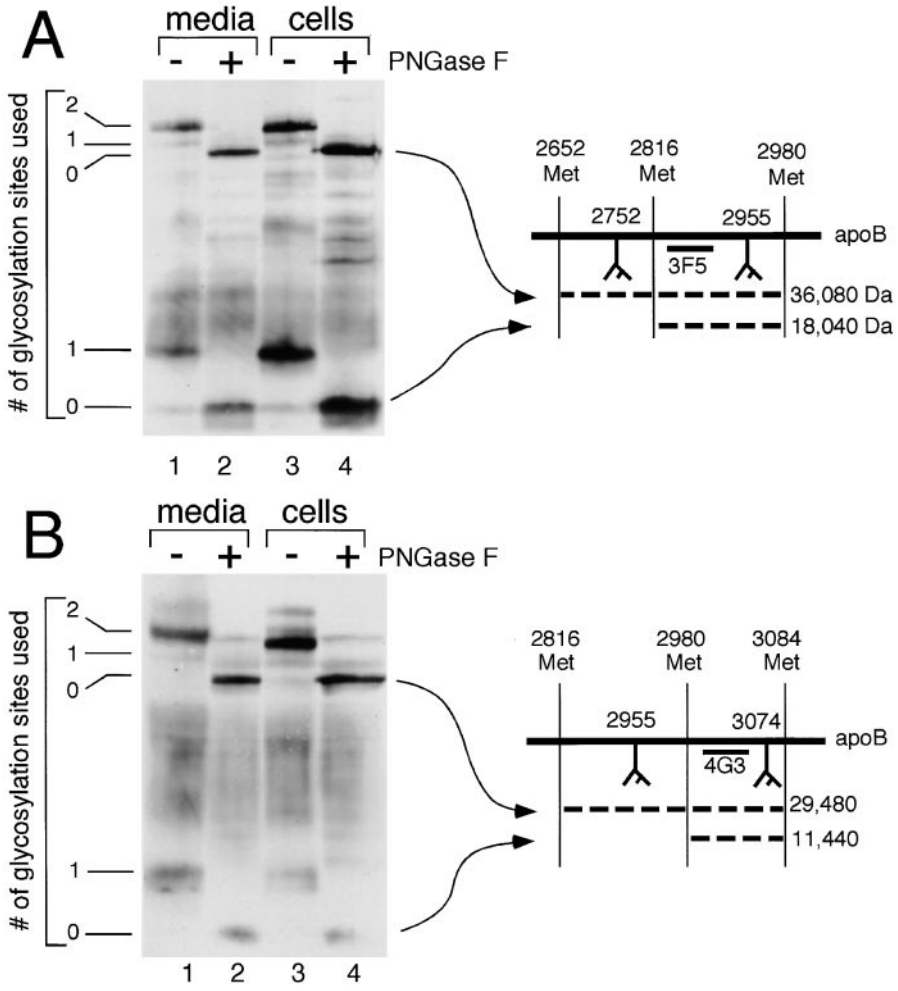

Fig. 7. ALLN-mediated accumulation of intracellular apoB has no effect on glycosylation site utilization. ApoB-100 from ALLN-treated HepG2 cells and conditioned media was recovered by immunoprecipitation with anti-apoB polyclonal antibodies. Immunoprecipitates were treated with CNBr and PNGase F, as indicated. Samples were fractionated by 15% SDS-PAGE and transferred to nitrocellulose membranes. Nitrocellulose was probed with monoclonal antibodies 3F5 (A) or 4G3 (B). Diagrams to the right of the Western blots indicate the location of the apoB glycosylation sites, Met residues, and 3F5 and 4G3 epitopes (numbers indicate amino acid coordinates of mature apoB polypeptide). Arrows to the dotted lines show the location of each peptide generated by CNBr, followed by the calculated molecular mass of the deglycosylated form.

DISCUSSION

The predominant mechanism for the disposal of misfolded secretory precursor proteins may involve retrograde movement via the Sec61p complex from the ER lumen to the cytosol, where the protein undergoes multiubiquitination and proteasomal degradation (5, 48–51). It has also been suggested that the proteasome may receive secretory protein substrates via a process of cotranslational translocation arrest (42). This model is based primarily on the behavior of apoB, which, under conditions of limiting lipid or MTP, undergoes misfolding and subsequent degradation by the ubiquitin/proteasome-mediated pathway (15, 52). The results presented here, however, demonstrate efficient N-linked glycosylation at sites downstream of all domains reported to mediate apoB's inefficient translocation (10, 27, 28). As the active site for oligosaccharyltransferase resides in the lumen of the ER (31, 53), these results suggest that the forward translocation of apoB into the ER may be an efficient and essentially unregulated process.

The glycosylation results reported here are inconsistent with many previous reports, indicating that the majority of apoB undergoes translocation arrest. These studies were based on apoB's accessibility to proteases and antibodies in microsomes or permeabilized cells. However, there is little consensus as to the site(s) in apoB responsible for this novel behavior. Sites of translocation arrest have been reported to exist within the α_1 (10), β_1 (27), and β_2 (28) domains of apoB (Fig. 1A). The experiments described in the current report utilized a recombinant form of apoB50 to directly test whether the α_1 or β_1 region of apoB can modulate translocation efficiency. The results displayed in Figs. 2 and 3 clearly indicate that the C-terminus of the

apoB-50GF construct is efficiently glycosylated in HepG2, COS, and CHO cells. As the latter two cell lines lack MTP and are incapable of lipidating or secreting apoB (9, 39, 41), it is clear that neither MTP nor assembly with lipid is required for the delivery of the C-terminus of apoB-50 into the ER lumen. While the protease accessibility of endogenous and transfected forms of apoB-48 and apoB-50 is variable, others have observed complete protection of the amino terminal 48–50% of apoB in both hepatic and nonhepatic cells (25, 28, 54). Hence, while apoB-50 may contain domains that transiently pause translocation in cell free systems, as demonstrated by Chuck and Lingappa (22), it appears to contain no domain or combination of domains capable of disrupting its translocation in cells.

In another recent study, the β_2 domain of apoB was reported to be responsible for the translocation arrest of apoB. In stably transfected rat hepatoma cells, the percentage of total apoB-64 in protease-accessible form (translocation arrested) at steady-state was 65% (28). However, in the current study, the glycosylation sites located at amino acids 2752 (apoB-61), 2955 (apoB-65), and 3074 (apoB-68) were all efficiently utilized and displayed a nearly identical pattern observed in secreted apoB-100. These results indicate that the amino terminal 68% of apoB undergoes efficient translocation into the ER and raises doubts regarding the use of protease protection assays as a quantitative measure of apoB translocation status.

An underlying assumption in the interpretation of glycosylation site utilization studies performed here is that, as with other secretory proteins, the translocation of apoB is a vectoral (N to C-terminal) process. Under this assumption, glycosylation at any site along the polypeptide chain indicates successful translocation of all upstream domains. However, for transmembrane proteins, the stopping and starting of translocation gives rise to alternating cytosolic and lumen domains. Although apoB ultimately becomes a soluble secretory protein, a recent study in HepG2 cells reported that apoB undergoes essentially quantitative assembly with the ER membrane as a 4-membrane spanning domain protein (19). However, the percentage of apoB detected in this putatively transmembrane configuration by proteinase K digestion of digitonin permeabilized cells remained fixed during a pulse chase experiment and was unaffected by oleate, a treatment that stimulates the assembly of apoB with lipid and presumably its release into the ER lumen as a soluble lipoprotein particle. There are many aspects of this model that are difficult to reconcile with the biology of apoB assembly and secretion.

If apoB remains associated in transmembrane configuration throughout the secretory pathway, it is not clear how it is finally released in soluble form from the cell. Second, if it is released from a post-ER compartment (as the pulse-chase analysis suggests), it could not be glycosylated by oligosaccharyltransferase, as this enzyme is exclusively localized in the rough ER. However, one of the epitopes judged to be on the cytosolic side of the membrane is only 12 amino acids downstream of a glycosylation site that is utilized in human LDL (55) and 147 amino acids downstream of a site shown to be utilized in this study. Hydropathy analysis of the region between these known glycosylation sites and the putative cytosolic domain reveals predominantly hydrophilic/amphipathic sequences that would not be predicted to serve as a membrane spanning domain (**Fig. 8**). While it is conceivable that apoB remains associated with the Sec61p translocation channel in the rough ER (52, 56), the pulse-chase analyses indicate that apoB maintains its transmembrane orientation throughout the secretory pathway (19). We conclude that the glycosylation profile of apoB, as well as its resistance to proteolysis in intact microsomes observed in some studies (29, 30), is inconsistent with its existence as a transmembrane protein in the secretory pathway.

One possible way to reconcile our glycosylation data with literature reporting large amounts of transmembrane apoB is to invoke a model in which forward translocation (and, therefore, glycosylation) proceeds to completion but retrograde transport is discontinuous. The glycosylation studies presented here, however, support the notion that retrograde translocation of apoB must be highly processive, generating very few full-length transmembrane inter-

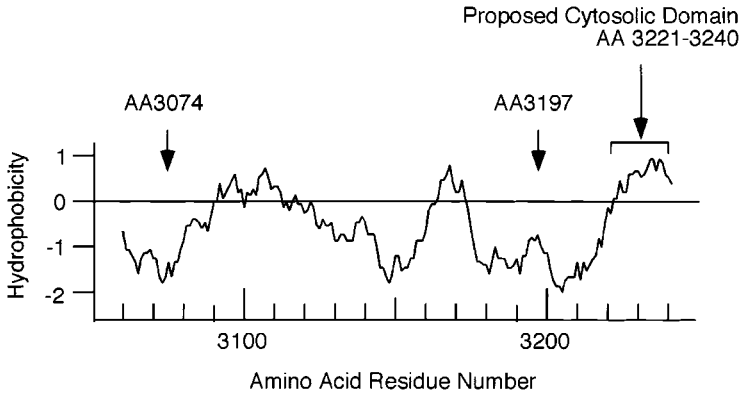

Fig. 8. Hydropathy profile of apoB in region of N-glycosylation sites and proposed cytosolic domain. Hydropathy values (62) were averaged for each 19 amino acid window between amino acid 3051 and 3250. Increasing numerical values correspond to increasing hydrophobicity. The position of a proposed cytosolic epitope of apoB is indicated (56). Amino acid (AA) 3074 was shown in the current study to be efficiently N-glycosylated in the intracellular population of HepG2 cell apoB. Both amino acids 3074 and 3197 were shown previously to be N-glycosylated in human LDL apoB (55). For these sites to be utilized, a membrane-spanning domain must exist between the glycosylation sites and the proposed cytosolic domain. On average membrane-spanning domains are 19–21 amino acids in length, display a minimum hydrophobicity of 1.6 (62), and are generally uninterrupted by charged amino acid residues. The peak hydrophobicity in this region is <1 and contains no more than 11 contiguous uncharged amino acids.

mediates. In other systems, retrotranslocated proteins are deglycosylated by a cytosolic N-glycanase (57, 58). Under normal circumstances it is difficult to detect deglycosylated cytosolic intermediates, presumably because dislocation and not proteasomal degradation is rate limiting. However, under conditions of proteasome inhibition, large amounts of mutant CFTR and other membrane proteins have been observed to undergo deglycosylation and accumulation in the cytosol as insoluble aggregates (45, 46). The studies here, however, indicate that even when the proteasome is inhibited by ALLN, apoB does not accumulate in deglycosylated form (Fig. 7). The glycosylation data presented above are also consistent with immunofluorescence and subcellular fractionation studies demonstrating that apoB retains its ER-localization, even during prolonged incubation with ALLN (29, 52). In contrast, substrates such as CFTR accumulate in ALLN-treated cells with a characteristic juxtanuclear, cytosolic localization (46).

While the glycosylation data may be inconsistent with many protease protection studies indicating the existence of large amounts of transmembrane apoB, others have concluded that apoB-100 in HepG2 cell microsomes is not particularly sensitive to exogenous trypsin (29, 30). Furthermore, the protease susceptibility of apoB is unaffected by proteasome inhibitors (29). The fact that there is no change in the glycosylation status, protease sensitivity, or subcellular localization of apoB in response to proteasome inhibition indicates that retrotranslocation of apoB may depend on proteasome activity. Other data support the notion that, for some protein substrates, little dislocation can occur in the absence of a functional proteasome. A mutated ATP-binding cassette transporter, Pdr5, which normally undergoes dislocation and proteasomal degradation, was observed to remain in the ER when expressed in yeast cells expressing a functionally impaired proteasome. Perhaps different protein substrates have different requirements for dislocation. For apoB, it appears that bulk retrograde movement from the ER lumen to the cytosol does not occur to any significant extent in the presence of proteasome inhibitors.

Although proteasome inhibition fails to cause the accumulation of cytosolic apoB, it has been reported that inhibition of the proteasome causes a several-fold increase in multiubiquitinated apoB (14, 15). At face value, this would appear to contradict our conclusions that almost all apoB is lumenally disposed and that retrotranslocation of apoB requires a functional proteasome. However, the steady-state percentage of intracellular apoB that is multiubiquitinated appears to be extremely small. This is apparent from the fact that much of the multiubiquitinated apoB runs with slower gel mobility than the nonubiquitinated form. Yet when apoB is detected by Western blotting or immunoprecipitation of total labeled protein, even under conditions of proteasome inhibition, one sees virtually no signal that runs in this multiubiquitinated electrophoretic position (14, 15, 59). This indicates that while some multiubiquitinated apoB does exist at steady state and its relative abundance increases as a function of proteasome inhibition, it represents an extremely small pool. Again, the extremely low steady-state accumulation of multiubiquitinated apoB is consistent with a processive degradation mechanism in which the bulk movement of misfolded apoB from the ER lumen to the cytosol is dependent upon proteasome function.

Recently, it has been observed that in addition to the full-length protein, nascent chains of apoB are multiubiquitinated and degraded by the proteasome (42, 59, 60). This fact has been used to support the case for translocation arrest, as it would appear unlikely that a protein could be recruited for retrotranslocation even before its initial translation and forward translocation is completed. However, proteasomal degradation is observed only for those forms of apoB that are ⩾65% complete (60). As it takes ~9 min to translate 65% of apoB-100 in HepG2 cells (61), it is possible that some misfolded apoB is recruited for retrotranslocation even before translation is complete. In support of this, protease protection experiments have failed to observe a selective loss of nascent polypeptide chains of apoB, suggesting that they also undergo cotranslational translocation into the ER lumen prior to their degradation (29, 30). Nonetheless, the studies here focus only on the processing of full-length apoB, the form that has been most systematically studied in terms of topology and proteasomal degradation.

In conclusion, the glycosylation studies presented here indicate that the translocation of apoB is efficient and is not dependent upon MTP or assembly with lipid. While apoB undergoes a unique assembly process in the ER, its proteasomal degradation appears to follow a commonly observed process of Sec61p-dependent dislocation from the ER lumen to the cytosol. While some proteins appear to undergo dislocation independently of proteasome function, apoB dislocation appears to require an active proteasome. As with its assembly, there may be specialized factors required for apoB dislocation, perhaps involving lipolytic enzymes to separate apoB from lipoprotein lipids in the ER lumen. Hence, many important aspects of the retrotranslocation and proteasomal degradation of apoB require further exploration.

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