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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

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Abstract Apolipoprotein[a] (apo[a]) is a highly polymorphic glycoprotein that forms a covalent complex with apolipoprotein B-100 (apoB-100), producing a lipoprotein species referred to as lipoprotein[a] (Lp[a]). We have studied the effects of alterations in glycosylation of apo[a] on its intracellular processing and secretion as well as its ability to associate with low density lipoprotein (LDL) apoB-100. HepG2 cells transfected with a 6 kringle IV (6 K-IV) apo[a] minigene were treated with tunicamycin, an inhibitor of N-linked glycosylation, which eliminated apo[a]-B-100 complexes from the media. Tunicamycin treatment also reduced secretion of the 6 K-IV apo[a] protein from transfected McA-RH7777 cells by ~50%, but completely eliminated secretion of apo[a] species containing 9 and 17 K-IV repeats. Mixing experiments, performed with radiolabeled media (±tunicamycin) from transfected McA-RH7777 cells, demonstrated no alteration in the extent of association of apo[a] with human LDL. Similar mixing experiments using culture media from glycosylation-defective mutant chinese hamster ovary (CHO) cells transfected with the same apo[a] minigene showed identical results. Apo[a] secretion was demonstrated in all mutant cell lines in the absence of either N- or O-linked (or both) glycosylation. The mechanisms underlying the reduced secretion of apo[a] from transfected hepatoma cells were examined by pulse-chase radiolabeling and apo[a] immunoprecipitation. Tunicamycin treatment altered the efficiency of precursor apo[a] processing from the ER by increasing its ER retention time. The increased accumulation of precursor apo[a] in the ER was associated with alterations in the kinetics of association with two resident endoplasmic reticulum (ER) chaperone proteins, calnexin and BiP. These findings suggest that the glycosylation state and size of apo[a] appear to play a role in regulating its efficient exit from the endoplasmic reticulum. However, neither N- nor O-linked glycosylation of apo[a] exerts a major regulatory role in its covalent association with apoB-100.—Bonen, D. K., F. Nassir, A. M. L. Hausman, and N. O. Davidson. **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.*** **1998.** 39: **1629–1640.**

Supplementary key words apo[a] • lipoprotein[a] • apoB-100 • glycosylation • hepatoma cells • endoplasmic reticulum • Golgi apparatus

Lipoprotein[a], Lp[a], is a hybrid lipoprotein particle composed of low density lipoprotein (LDL) to which is attached another large glycoprotein referred to as apolipoprotein[a], (apo[a]) (1, 2). This attachment is believed to be mediated by covalent and possibly noncovalent mechanisms between the apo[a] glycoprotein and residues within the carboxyl terminus of apoB-100 (3–5). Circulating concentrations of Lp[a] vary widely both within individuals and among different ethnic populations (6–9), and elevated plasma concentrations of Lp[a] are associated with premature coronary artery disease (10). These observations, coupled with accumulating information suggesting that differences in plasma Lp[a] concentration in humans are attributable to heterogeneity in the hepatic production of Lp[a] (11), have focused attention on the cellular mechanisms which may regulate synthesis of this lipoprotein particle.

Apo[a] is a highly polymorphic glycoprotein which varies greatly in size and whose cDNA sequence demonstrates extensive homology to that of the precursor zymogen, plasminogen (12–14). This size heterogeneity is linked to polymorphisms within the *Apo[a]* gene and quantitative

Abbreviations: apo[a], apolipoprotein[a]; r-apo[a], recombinant apo[a]; apoB-100, apolipoprotein B-100; LDL, low density lipoprotein; Lp[a], lipoprotein[a]; TCN, tunicamycin; BFA, brefeldin A; Gal, galactose; Ga1NAc, N-acetylgalactosamine; CHO cells, Chinese hamster ovary cells.

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estimates have suggested that at least 90% of the variability in plasma Lp[a] concentrations can be accounted for by variations at the *Apo[a]* gene locus which result in different numbers of kringle IV (K-IV) repeats (15, 16). Based on the observation that the human *Apo[a]* gene is transcribed virtually exclusively in the liver (12, 17, 18), several laboratories have reported the development of cell culture systems which might recapitulate elements of the synthesis and assembly of Lp[a] (19–23). A major advance in this area has been the development of hepatic apo[a] expression systems in which the processing and secretion of this glycoprotein can be examined (19–23).

One of the most important post-translational modifications to the nascent apo[a] peptide is glycosylation, as up to 28% of the weight of the protein is carbohydrate (24). Similar values (23%) were obtained for a recombinant apo[a] expressed in human embryonic kidney 293 cells (19). The results of both carbohydrate analysis and peptide sequence information suggest the existence of both N-linked and O-linked glycosylation sites (12, 25), together with a high sialic acid content which may be a component of either glycan (24, 26). Because the addition of N-linked sugars begins cotranslationally in the ER and is proposed to play an important role in the conformational properties of secretory proteins, it is likely that alterations in glycosylation might also participate in modulating the rate at which apo[a] is processed through the secretory pathway. Currently, there is limited information concerning the possible functional importance of glycosylation of apo[a] in the context of Lp[a] assembly and metabolism. Earlier studies suggested that certain defects in glycosylation increased, while others decreased, secretion of a 15 K-IV apo[a] isoform from mutant CHO cells, although the ability of the secreted material to associate with apoB-100 was not diminished (5). More recent information regarding the influence of glycosylation of apo[a] demonstrated that both the presence of N-linked glycans and also glucose trimming is required for apo[a] secretion in baboon hepatocytes (27). Indeed, these latter studies demonstrated complete inhibition of apo[a] secretion from baboon hepatocytes after treatment with tunicamycin (27). Other studies, however, have demonstrated that O-linked glycosylation of another glycoprotein, apoA-II, alters the association properties and eventual distribution of this protein among human high density lipoprotein fractions (28), raising the possibility that glycosylation of apolipoproteins plays an important role in lipoprotein metabolism. In addition, alterations in sialylation and/or N-linked glycosylation of recombinant human tissue plasminogen activator alter its metabolic activity and its binding affinity for lysine residues (29).

The present studies were undertaken to address the question of whether glycosylation of apo[a] is a potentially important modification for its secretion from liver cells, particularly in relation to the size of the apo[a] glycoprotein. This may be of particular relevance in light of studies demonstrating an inverse relationship between the number of K-IV repeats and serum apo[a] concentration (13, 30–32). More recently, this relationship has been extended to an understanding of the likely mechanisms involved through the demonstration, in baboon hepatocytes, that larger isoforms of apo[a] are less readily processed from the endoplasmic reticulum to the Golgi (33, 34). In the present study, we have used established lines of both human and rat hepatoma cells expressing a 6 K-IV apo[a] isoform as a model system for the analysis of Lp[a] and apo[a] biosynthesis. Prior experience with this model indicated robust secretion of apo[a] and established the ability of the 6 K-IV isoform to recapitulate important elements of the larger isoforms, including its pattern of intracellular processing and also its ability to associate in a covalent complex with apoB-100 (23).

In the present studies, we demonstrate that tunicamycin treatment reduces the rate and extent of processing and secretion of apo[a] from transfected hepatoma cells in association with a diminished efficiency of apo[a] exit from the ER. Nevertheless, the association of the secreted apo[a] with apoB-100 was unaffected by the addition of either N- or O-linked sugars. Tunicamycin treatment produced a virtually complete inhibition of the maturation and secretion, from stably transfected hepatoma cells, of larger-sized apo[a] proteins containing 9 or 17 K-IV repeats. In investigating the factors that may contribute to the increased ER retention time of apo[a] after tunicamycin treatment, altered binding kinetics were discovered of apo[a] to both calnexin and BiP, suggesting that chaperone proteins may play an important role in the normal processing of apo[a].

EXPERIMENTAL PROCEDURES

Materials

Dulbecco's modified Eagle's medium (DMEM), Eagle's minimum essential medium (MEM), α-modified minimum essential medium (α-MEM), methionine- and cysteine-free DMEM, fetal bovine serum, and G418 were all obtained from Life Technologies, Inc. Reagents for gel electrophoresis and immunoblotting were purchased from Sigma and Life Technologies, Inc. Human LDL and rabbit polyclonal antibodies against human Lp[a] were generously supplied by G. Fless (University of Chicago). Goat anti-Lp[a] (human LDL adsorbed) was purchased from Biodesign International (Kennebunk, ME). This antibody exhibits no reactivity towards human apoB-100 (23, 24). Antibodies against calnexin and BiP were purchased from StressGen Biotechnologies Corp. (Victoria, B.C., Canada). Proteins A and G agarose, tunicamycin, as well as N-glycosidase F (peptide-N^4-(acetyl-β-glucosaminyl)asparagine-amidase, O-glycosidase (O-glycopeptide endo-d-galactosyl-N-acetyl-α-galactosamino hydrolase), neuraminidase (sialidase), and Endoglycosidase H (endo-β-N-acetyl-glucosaminidase H) were obtained from Boehringer-Mannheim. Brefeldin A was obtained from Epicentre Technologies (Madison, WI). Tran^{35}S-label (1000 Ci/mmol) was purchased from ICN Biomedicals, Inc. (Costa Mesa, CA). Unless otherwise noted, all other chemicals were purchased from Sigma.

Cell culture and transfections

HepG2 and McA-RH7777 cells transfected with a recombinant form of apo[a] containing 6 K-IV-like domains, as well as the kringle V and protease-like domains, described recently (23), were used in these studies. McA-RH7777 cells were also stably

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transfected with apo[a] expression constructs encoding 9 and 17 K-IV repeats. The pCMV-A10-neo plasmid (generously supplied by H-J. Müller, Boehringer Mannheim) encodes for K IV-repeats 1, 30–37 and kringle V, while the pRK5ha17 expression plasmid (19) encodes for K-IV-repeats 1–5, a fusion kringle consisting of repeats 6 and 26, followed by K-IV repeats 27–37 and kringle V. The CHO cell lines Pro-5 (36), Lec 1 (36), Lec 2 (36), Lec 8 (37), and ldlD-14 (provided by M. Krieger, MIT, and obtained through American Type Culture Collection) were grown in α-modified MEM (α-MEM) supplemented with 10% fetal calf serum, 2 mm glutamine, 100 IU/ml of penicillin, and 100 μg/ml of streptomycin. The CHO cell lines were transiently transfected with the same 6 K-IV apo[a] isoform described previously (23) using the calcium phosphate precipitation method described by Krieger et al. (37).

Tunicamycin treatment of cells

Transfected HepG2 and McA-RH7777 cells were preincubated for 2 h in methionine- and cysteine-free DMEM containing tunicamycin (1–10 μg/ml), followed by labeling for 3 h with the same medium containing Tran^{35}S-label (200 μCi/ml) and tunicamycin. In pulse-chase experiments, transfected McA-RH7777 cells were preincubated for 2 h, pulsed for 10 min (250 μCi/ml Tran^{35}S-label), and chased in medium containing 10 mm methionine and 3 mm cysteine. Tunicamycin (10 μg/ml) was added to all preincubation, pulse and chase medium. After labeling, culture medium was collected on ice, protease inhibitors were added (100 μm leupeptin, 450 μm aprotinin, 2 μm pepstatin, 1 mm phenylmethylsulfonyl fluoride, and 1 mm benzamidine), and the medium was centrifuged for 5 min at 10,000 rpm at 4°C to pellet cell debris. Cells were washed 3 times with cold PBS and scraped into cold lysis buffer (50 mm Tris, pH 7.4, 150 mm NaCl, 5 mm EDTA, 0.5% Triton X-100, 0.5% sodium deoxycholate) containing protease inhibitors. For analysis of transfected HepG2 cells, 100 mm ε-aminocaproic acid (ε-ACA) was included in all buffers. Cell lysates were clarified by centrifugation to remove insoluble material, and aliquots of both culture medium and lysates were immunoprecipitated with an anti-apo[a] antibody, or, where applicable, a combination of anti-apoB monoclonal antibodies (1D1, 4G3, Bsol 6, and Bsol 17) (a generous gift from Dr. R. Milne, Ottowa Heart Institute), followed by SDS-PAGE.

Brefeldin A and monensin treatment of cells

Transfected McA-RH7777 cells were preincubated for 2 h in the presence or absence of tunicamycin (10 μg/ml) along with brefeldin A (5 μg/ml) or monensin (10 μm), followed by a 10-min pulse with 250 μCi/ml Tran^{35}S-label, and a 10–240-min chase. Tunicamycin, brefeldin A, and monensin were included in the pulse and chase medium. After each chase time point, cell lysates were harvested in the presence of protease inhibitors as described above, and aliquots were immunoprecipitated using an anti-apo[a] antibody, followed by SDS-PAGE.

Incubation of human LDL with r-apo[a] from tunicamycin-treated cells or CHO mutant cell lines

Human (h)-LDL was prepared from normolipidemic subjects by preparative ultracentrifugation, dialyzed against 0.15 m NaCl, pH 7.4, containing 0.01% EDTA and 0.01% sodium azide, and the protein concentration was determined by the method of Lowry et al. (38). For tunicamycin experiments, stably transfected McA-RH7777 cells were preincubated for 2 h with 10 μg/ml tunicamycin, followed by radiolabeling for 3 h in the same media as described above. For mixing experiments with culture medium from transfected CHO cell lines, the intracellular pools of galactose (Gal) and N-acetylgalactosamine (GalNAc) were depleted as previously described (28). Briefly, cells were grown for 36–48 h without serum in a 1:1 mix of α-modified minimum essential medium and Ham's F-12 medium containing 2 mm glutamine, 100 IU/ml of penicillin, and 100 μg/ml of streptomycin and supplemented with 6.25 μg/ml each of insulin and transferrin, 6.25 ng/ml selenous acid, 5.35 μg/ml linoleic acid, and 1.25 mg/ml bovine serum albumin. Cells were radiolabeled for 6 h with 150 μCi/ml Tran^{35}S-label in methionine- and cysteine-free DMEM, containing 0.4 mg/ml proline, 2 mm glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 50 μm $ZnCl_2$. Where indicated in the figure legend, stably transfected ldlD cells were supplemented with either 20 μm galactose (Gal), 200 μm of N-acetylgalactosamine (GalNAc) or both Gal and GalNAc during the labeling period. For all mixing experiments, radiolabeled culture medium containing equal amounts of trichloroaceticacid-precipitable radioactivity were incubated with h-LDL (1.5 μg) at 37°C for 1.5 h, followed by immunoprecipitation using anti-apo[a] antiserum and SDS-PAGE.

Immunoprecipitations

For single immunoprecipitations, cells were lysed with denaturing lysis buffer (100 mm NaCl, 100 mm Tris, pH 7.5, 10 mm EDTA, 1% Triton-X-100, 0.1% SDS) and protease inhibitors leupeptin (100 μm), aprotinin (450 μm), pepstatin (2 μm), EDTA (5 μM), phenylmethylsulfonyl fluoride (1 mM), and benzamidine (1 mm). Both culture medium and lysates were precleared by incubating with protein G agarose for 2–3 h at 4°C. For immunoprecipitation of culture medium, 5 × IP buffer was added to a final concentration of 150 mm NaCl, 5 mm EDTA, 50 mm Tris, pH 7.4, 1% Triton-X-100, 1 mg/ml BSA, 20 mm methionine, and 0.02% sodium azide. Antiserum was added to precleared cell lysates and medium samples and incubated overnight at 4°C. Protein G agarose, preadsorbed with wild-type cell lysates and washed 5 times in IP buffer, was added and incubated for another 2–3 h at 4°C. The protein G agarose pellet was washed four times in immunoprecipitation wash buffer (50 mm Tris, pH 7.4, 0.65 m NaCl, 10 mm EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS), two times in water, and boiled for 5 min in SDS sample buffer (4% SDS, 20% glycerol, 0.001% bromophenol blue, 125 mm Tris, pH 6.8). Where indicated, 100 mM DTT was included in the sample buffer for reducing SDS-PAGE.

Analysis of chaperone protein binding to apo[a]

For the examination of apo[a] precursor binding to calnexin and BiP, confluent monolayers of transfected McA-RH7777 cells were preincubated for 2 h ± tunicamycin, followed by pulse-chase analysis. At the end of each chase, cells were quickly chilled to 4°C and treated with 50 mm iodoacetamide (IAA) in PBS for 10 min to alkylate intracellular sulfhydryls (39). Before lysis, the cells were washed three times in ice-cold PBS, and then in nondenaturing buffer containing 0.1 m NaCl, 25 mm Tris, pH 7.5, 1% Triton-X-100, 5 mm EDTA, and a cocktail of protease inhibitors as described above. When samples were to be used for immunoprecipitation of BiP, cells were alkylated in situ, followed by the addition of 10 U/ml apyrase (to enzymatically deplete ATP). After exposure to apyrase for 60 min at 4°C, 10 mm IAA was added. Cell lysates were first immunoprecipitated with a rabbit polyclonal antibody to calnexin or BiP in lysis buffer. The first immunoprecipitation complex was retrieved by incubation with protein A agarose as above. After washing three times in TS wash buffer (10 mm Tris, pH 7.4, 150 mm NaCl), the protein A agarose was boiled 5 min in TS buffer containing 1% SDS. After centrifugation, the supernatant was diluted with 1 ml of IP buffer, and used for recapture–immunoprecipitation with goat anti-human apo[a] antisera and protein G agarose, as above. Normal preimmune goat serum was used as a control for the second immunoprecipitation. All immunoprecipitates were analyzed by SDS-PAGE and fluorography. The bands corresponding to precursor and ma-

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ture apo[a] were cut from dried gels, digested with Solvable (Packard Instrument, Inc., Meriden, CT), and radioactivity was determined by scintillation counting.

Endoglycosidase digestions

Culture medium and cell lysates from tunicamycin-treated and control cells were immunoprecipitated for apo[a] as described above. For digestion with Endo H and N-glycosidase F, the protein G agarose beads were resuspended in 13 μl of 100 mm sodium acetate, pH 5.6, containing 0.3% SDS, 0.3% β-mercaptoethanol and boiled for 5 min. The samples were then diluted with 27 μl of 100 mm sodium acetate, pH 5.6, and allowed to cool to room temperature. For Endo H digestion, 5 mU Endo H and PMSF (1 mm) were added to the samples. For digestion with N-glycosidase F, the samples were adjusted to final concentrations of PMSF (1 mm), NP-40 (1%) and N-glycosidase F (20 U/ml). Samples were incubated at 37°C for 16 h, at which time the digestions were terminated by the addition of concentrated SDS-PAGE sample buffer. For those samples to be digested with neuraminidase and O-glycosidase, the protein G agarose beads were resuspended in 13 μl of 10 mm calcium acetate, 20 mm sodium cacodylate, pH 6 containing 0.3% SDS, 0.3% β-mercaptoethanol and boiled for 5 min. The samples were diluted with 27 μl 10 mm calcium acetate, 20 mm sodium cacodylate, pH 6, and cooled to room temperature. After the addition of PMSF (1 mm), NP-40 (1%), and neuraminidase (40 mU), samples were incubated at 37°C for 16 h. Those samples to be digested with O-glycosidase were first digested with neuraminidase at 37°C for 1 h, followed by the addition of O-glycosidase (2 mU) and further incubation at 37°C for 16 h, followed by the addition of concentrated SDS-PAGE sample buffer.

Statistical comparisons

Where indicated in the text, comparisons were made using Student's *t*-test for paired or unpaired samples and undertaken using the InStat statistical package, version 2.0 (GraphPad Software, San Diego, CA).

RESULTS

Tunicamycin treatment of transfected HepG2 cells inhibits apo[a] and apoB-100 secretion

HepG2 cells, stably transfected with a 6 K-IV apo[a] isoform (23), were radiolabeled in the presence of increasing amounts of tunicamycin in order to examine the effects of alterations in N-linked glycosylation on the secretion of apo[a] and its association with apoB-100. Analysis of apo[a] (**Fig. 1A**) immunoprecipitated from the media of these cells reveals a dose-dependent increase in the mobility of recombinant apo[a] with increasing doses of tunicamycin, consistent with the expected inhibition of N-linked glycosylation. In addition, a dose-dependent decrease in apo[a] accumulation is evident (Fig. 1A), doses of tunicamycin greater than 5 μg/ml essentially eliminating Lp[a] from the media (Fig. 1A). Further studies demonstrated that tunicamycin treatment (>5 μg/ml) of wild-type HepG2 cells also reduced the secretion of apoB-100 by over 90% (Fig. 1B).

In order to examine whether the ability of apo[a] to associate with apoB-100 is influenced by the absence of N-linked oligosaccharides in apo[a], stably transfected McA-RH7777 cells were pretreated for 2 h and labeled for 3 h

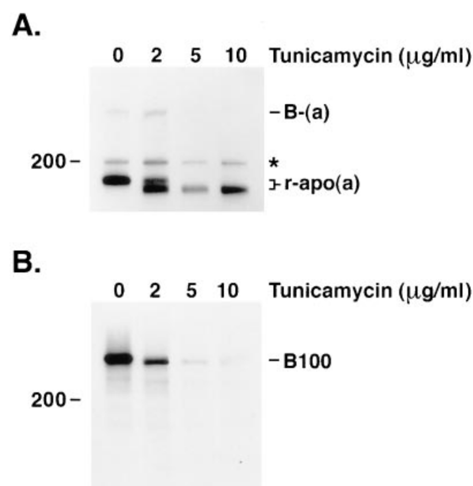

Fig. 1. Tunicamycin treatment of transfected HepG2 cells inhibits Lp[a] secretion. HepG2 cells transfected with the 6 K-IV apo[a] minigene (A) or wild type cells (B) were labeled for 2 h with Tran^{35}S-label in medium containing 0–10 μg/ml tunicamycin. A: Culture media containing equal TCA-precipitable counts was immunoprecipitated with polyclonal goat anti-apo[a] IgG (αa) and analyzed on 4–12% SDS-PAGE without reduction. B: Media from labeled wild type HepG2 cells was immunoprecipitated with a panel of mouse monoclonal anti-apoB (αB) antibodies and analyzed by 4–12% SDS-PAGE under reducing conditions. Control immunoprecipitations used preimmune goat (NGS) or normal mouse serum (NMS). The migration of both apo[a] and apoB-100 is indicated to the right of the panel. The fluorograms are a representative of three separate experiments. The asterisk (*) represents a non-specific protein that is recovered in the immunoprecipitates.

in the presence of tunicamycin. Aliquots of the media were then mixed with human LDL prior to immunoprecipitation. Immunoprecipitable apo[a] was demonstrated to be complexed with apoB-100 in comparable proportions (~30% complexed) either with or without tunicamycin treatment (**Fig. 2**, minus DTT, left panel). These values are comparable to our previous findings where only 25–30% of the 6 K-IV apo[a] isoform from transfected HepG2 cells is capable of associating with apoB-100, or in similar mixing experiments when conditioned medium from transfected McA-RH7777 cells was mixed with human LDL (23). This is also consistent with reports from Koschinsky et al. (19, 20), that have been interpreted to suggest that uncoupled recombinant apo[a] represents a population of misfolded protein which is unable to complex with LDL (20). The decrease in apo[a] secretion with tunicamycin treatment is again evident in the reduced gels (Fig. 2, right panel); studies addressing the mechanism(s) for this decreased secretion are presented in a later section.

Endoglycosidase digestion of precursor and mature forms of apo[a]

To ascertain whether tunicamycin treatment (10 μg/ml) of transfected McA-RH7777 cells resulted in the complete inhibition of N-linked glycosylation, apo[a] from both media and lysates were subjected to both endogly-

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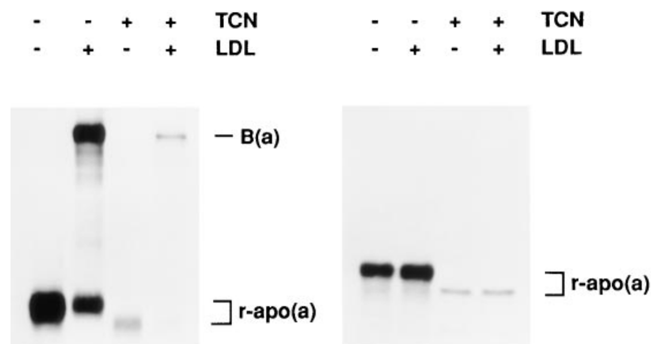

Fig. 2. Inhibition of N-linked glycosylation does not alter the association of apo[a] with human LDL. McA-RH7777 cells transfected with the 6 K-IV apo[a] isoform were preincubated for 2 h with 10 μg/ml tunicamycin and radiolabeled for 3 h with 200 μCi/ml Tran^{35}S-label in the presence or absence of tunicamycin (±TCN). Culture media (equal TCA-precipitable radioactivity) were incubated with human LDL (+LDL) for 1.5 h at 37°C, immunoprecipitated with a polyclonal anti-apo[a] antibody and analyzed on 4–12% SDS-PAGE gels under non-reducing (left panel) and reducing (right panel) conditions. The mobility of free (r-apo[a]) and complexed (B-[a]) forms are indicated. This is a representative of three separate experiments.

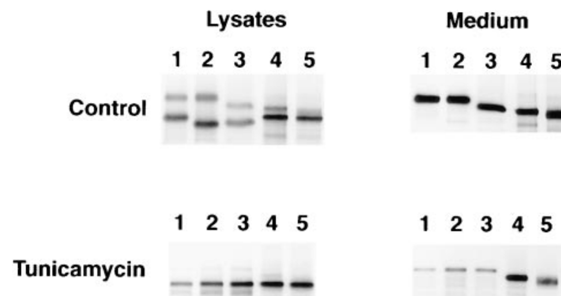

Fig. 3. Sensitivity of apo[a] to endoglycosidases. McA-RH7777 cells transfected with the 6 K-IV apo[a] minigene were incubated for 2 h in the presence or absence of tunicamycin (10 μg/ml) and radiolabeled for 3 h with 200 μCi/ml Tran^{35}S-label in the same media. Aliquots of culture medium and cell lysates were immunoprecipitated with a polyclonal anti-apo[a] antibody, followed by digestion with PBS (control, lane 1), endoglycosidase H (lane 2), N-glycosidase F (lane 3), neuraminidase (lane 4), or neuraminidase and O-glycosidase (lane 5) and analyzed by SDS-PAGE and fluorography. This is a representative of triplicate experiments.

cosidase H (Endo H) and N-glycosidase F digestion. As each K-IV repeat contains one potential N-linked and three potential O-linked carbohydrate sites (12), apo[a] has a high content of both of these glycans (24). Endo H cleaves high mannose N-linked oligosaccharides, but not complex N-linked carbohydrates (40, 41). Digestion with N-glycosidase F, which removes both complex and high mannose N-linked sugars but not O-linked oligosaccharides (42, 43), resulted in a large increase in the mobility of both the precursor and mature forms of apo[a], demonstrating the presence of N-linked glycans (**Fig. 3**, lane 3, top panel). However, both intracellular and secreted forms of apo[a] from tunicamycin-treated cells demonstrated the absence of N-linked carbohydrates upon digestion with N-glycosidase F (Fig. 3, lane 3, bottom panel). Endo H did not affect the mobility of mature apo[a], but increased the mobility of the precursor in control cells (Fig 3. lane 2, top panel). However, as no high mannose N-linked sugars are present with tunicamycin treatment, the precursor apo[a] is not sensitive to Endo H digestion (Fig. 3, lane 2, bottom panel). Neuramindase removes terminal sialic acid residues from both N-linked and O-linked sugars (44), while O-glycosidase removes O-linked sugars after sialic acid residues have been removed (45, 46). Treatment with neuraminidase alone (Fig. 3, lane 4) or neuraminidase plus O-glycosidase (Fig. 3, lane 5) resulted in an increase in mobility of intracellular and secreted apo[a] in both control and tunicamycin-treated cells, confirming the presence of both sialic acid and O-linked sugars in the fully processed protein. Consistent with the findings in baboon hepatocytes (22), neither neuraminidase nor O-glycosidase changed the mobility of the precursor, demonstrating the pre-Golgi location of this form of apo[a]. Therefore, tunicamycin treatment of transfected hepatoma cells resulted in the complete removal of N-linked glycans from apo[a], but did not affect the addition of O-linked carbohydrates or sialic acid, which occurs as a late Golgi event.

Alterations in the glycosylation of apo[a] secreted from glycosylation-defective CHO cells does not influence its extracellular association with apoB-100

In order to pursue further the question of whether N- and/or O-linked glycosylation of apo[a] influences its eventual association with apoB-100, the 6 K-IV apo[a] construct was transfected into several glycosylation-defective Chinese hamster ovary (CHO) cells (Lec 1, Lec 2, Lec 8, ldlD-14) and the wild-type, parental cell line for these mutants (Pro5). Lec 1 cells lack N-acetylglucosaminyltransferase so that N-linked carbohydrate addition is blocked at the $Man_5GlcNAc_2Asn$ intermediate (47). Lec 2 and the Lec 8 cell lines are defective in the addition of sialic acid and galactose, respectively (48, 49), and the ldlD cell line (37) is deficient in UDP-Glc/Gal 4-epimerase and cannot synthesize oligosaccharides containing galactose (Gal) or N-acetylgalactosamine (GalNAc). These transfected cell lines were radiolabeled and the labeled media was mixed with human LDL to determine whether the nonglycosylated apo[a] could associate with apoB-100. All of the cell lines secreted apo[a]. However, apo[a] secreted from the glycosylation-defective CHO cells demonstrated altered electrophoretic mobility, as predicted from the differing degrees of the glycosylation defect (**Fig. 4**). Additionally, although not formally examined, apo[a] secretion was reduced in the Lec 8 and particularly in the ldlD cells in comparison to the Pro5 parental line, which has no defects in glycosylation (compare lanes 5 and 6 respectively to lane 2 in Fig. 4B). Mixing experiments revealed that apo[a], secreted from all of the mutant cells, was competent to associate with LDL-apoB-100, creating reconstituted B-[a] complexes of varying sizes (Fig. 4A), which dissociated upon reduction (Fig. 4B). As determined by laser scanning densitometry, the complexed form of apo[a] ac-

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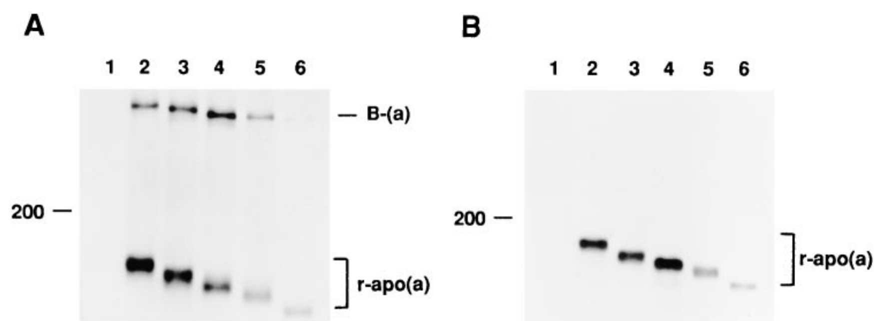

Fig. 4. Alterations in the glycosylation of apo[a] secreted from mutant CHO cells does not influence its extracellular association with apoB-100. Glycosylation- defective CHO mutant cell lines were transiently transfected with an empty, control vector (lane 1) or the 6 K-IV apo[a] expression plasmid (lanes 2–6). The cells were radiolabeled with Tran^{35}S-label, 48 h after transfection, and culture medium was incubated with LDL for 1.5 h at 37°C. The incubation mixtures were immunoprecipitated for apo[a] and analyzed by 4–12% SDS-PAGE under nonreducing (A) and reducing (B) conditions. Lanes 1 and 2, Pro 5; lane 3, Lec 1; lane 4, Lec 2; lane 5, Lec 8; and lane 6, ldlD-14 cells. The mobility of the free (r-apo[a]) and complexed (B-[a]) forms are shown. These results are a representative of 3–4 experiments.

counted for approximately 35–40% of the total apo[a] with slightly lower values for the ldlD cells, where only 17% of the apo[a] associated with apoB-100. As noted above, mixing experiments performed with culture medium from transfected McA-RH7777 cells yielded a similar range of values (~20–30%) for the complexed form of apo[a] (23). Taken together, these findings suggest that alterations in the glycosylation of apo[a] fail to modulate its association with apoB-100.

O-linked glycosylation of apo[a] is not necessary for extracellular association with LDL apoB-100

In order to examine whether O-linked glycosylation of apo[a] is necessary for its association with apoB-100, similar mixing experiments were performed with human LDL and radiolabeled culture medium from transfected ldlD cells grown in the presence or absence of galactose (Gal) and/or N-acetylgalactosamine (GalNAc). Stably transfected ldlD cells were radiolabeled in the presence of the indicated substrate and aliquots of the media were mixed with LDL. Immunoprecipitation of apo[a] from the medium of unsupplemented ldlD cells demonstrated ~18% to be in a covalent complex with apoB-100 (**Fig. 5**). Similar analyses, from cells supplemented with either Gal and/or GalNAc demonstrated that apo[a]–apoB-100 complexes accounted for ~19–22% of the total apo[a]. These results indicate that correction of the metabolic defects in the mutant ldlD cells produces no discernible effects upon the ability of apo[a] to associate with apoB-100, suggesting that neither N- nor O-linked glycosylation is required for apo[a]–B-100 association.

N-linked glycosylation of apo[a] is necessary for normal intracellular processing

Recent studies in baboon hepatocytes have demonstrated that tunicamycin treatment eliminates apo[a] secretion and results in its retention in the ER (27). The data presented above (Fig. 1 and Fig. 2) suggest that secretion of a 6 K-IV apo[a] from transfected hepatoma cells is diminished, but not eliminated. To pursue this observation, transfected McA-RH7777 cells expressing a 6 K-IV apo[a] isoform were treated with 10 μg/ml tunicamycin and then subjected to pulse-chase analysis to examine intracellular synthesis and secretion of apo[a]. Total protein synthesis, as inferred from TCA-precipitable counts/mg protein, was comparable in both treated and control cells (data not shown). As shown in **Fig. 6** (top panel), apo[a] from both control and treated cells demonstrates the ex-

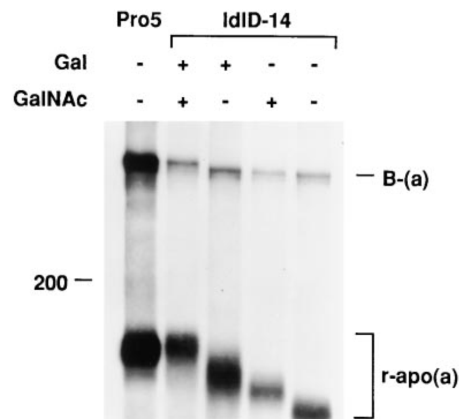

Fig. 5. Reversal of the glycosylation defects in transfected ldlD-14 cells fails to alter the association of secreted r-apo[a] with human apoB-100. The 4-epimerase-deficient CHO cell line, ldlD-14, was stably transfected with the 6 K-IV apo[a] plasmid and cells were radiolabeled in the presence or absence of 20 mm galactose (Gal) and/or 200 mm N-acetylgalactosamine (GalNAc). Aliquots of the culture medium were incubated for 1.5 h at 37°C with human LDL. The incubation mixtures were immunoprecipitated for apo[a] and analyzed under nonreducing conditions on 4% SDS-PAGE. Culture medium from transfected Pro5 cells grown without sugar supplements and incubated with LDL is shown in lane 1. Lanes 2–5 depict mixing experiments using LDL with radiolabeled culture medium from transfected ldlD-14 cells grown in the presence of the indicated sugar supplement. The mobility of the free (r-apo[a]) and complexed (B-[a]) forms are shown. The fluorograms are a representative of triplicate experiments.

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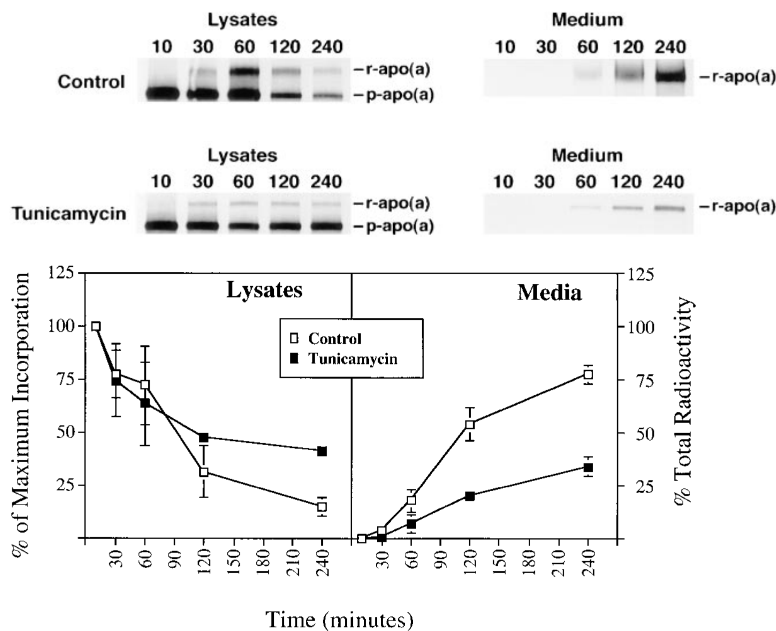

Fig. 6. N-linked glycosylation of apo[a] is necessary for normal intracellular processing. Top panel: transfected McA-RH7777 cells expressing a 6 K-IV apo[a] isoform were preincubated for 2 h with 10 μg/ml tunicamycin, followed by a 10-min pulse with Tran^{35}S-label and a 10–240 min chase. Tunicamycin was included in both pulse and chase media. Aliquots of culture medium and cell lysates from each time point were immunoprecipitated with goat anti-apo[a] antiserum and analyzed by SDS-PAGE and fluorography. Bottom panel: the bands corresponding to precursor and mature r-apo[a] were cut from dried gels, digested, and the radioactivity determined. This graphical representation is derived from the mean ± SD of 4–6 experiments and data is presented as percent of maximum incorporation of radioactivity into precursor apo[a] (Lysates) or as the amount of apo[a] secreted into the medium (Medium) at each time as a percent of total apo[a] at each time point. Secreted apo[a] is presented as immunoprecipitable apo[a] secreted in the culture medium as a percentage of the total apo[a] (i.e., lysates plus media) at each time point.

pected precursor–product distribution after pulse-chase, with the mature form of apo[a] appearing in lysates at 30 min, followed by its secretion into the medium beginning at 60 min. Quantitation of immunoprecipitable apo[a] showed that 15.3 ± 1.6% (n = 6, control) and 17.5 ± 1.9% (n = 4, tunicamycin) of total apo[a] appeared in the fully processed form at 30 min. However, at doses of 10 μg/ml tunicamycin, there was a decrease in the rate and extent of processing of apo[a] into the mature form at 60 min. Specifically, 42 ± 3% (n = 6) apo[a] was in the mature form in control cells compared to 28 ± 2% (n = 4) in the tunicamycin-treated cells, $P < 0.0001$. In addition, there was a decrease in the secretion of apo[a] with 18 ± 5% (n = 6) of total apo[a] secreted by 60 min from control cells compared to 7 ± 4% (n = 4) from tunicamycin-treated cells, $P < 0.0001$ (Fig. 6, bottom panel). A similar 2-fold difference was found at 240 min of chase with 77 ± 4% (n = 6) of total apo[a] secreted in control cells compared to only 34 ± 5% (n = 4) secreted in tunicamycin-treated cells, $P < 0.0001$ (Fig. 6, bottom panel).

The influence of size and glycosylation state on apo[a] maturation and secretion

To address the issue of apo[a] size and the role of N-linked glycans in apo[a] processing and secretion, McA-RH7777 cells, stably transfected with apo[a] constructs containing either 9 (35) or 17 (19) K-IV repeats, were treated with tunicamycin as detailed above. Control experiments demonstrated a similar pattern of intracellular processing for the 17 K-IV isoform from these cells as previously noted for the 6 K-IV isoform, with a temporally defined precursor–product relationship and mature, processed apo[a] detectable in the media by 60 min of chase (**Fig. 7A**). Maximal secretion of the mature 17 K-IV isoform was apparent by 2–4 h of chase. Cells transfected with either the 6, 9, or 17 K-IV isoform were preincubated for 2 h with 10 μg/ml tunicamycin and then labeled for another 3 h in the presence of tunicamycin. Tunicamycin treatment reduced secretion of the 6 K-IV apo[a], as demonstrated above, but appeared to eliminate secretion of apo[a] containing either 9 or 17 K-IV repeats (Fig. 7B). In addition, inhibition of N-linked glycosylation was associated with increased migration, on SDS-PAGE, of the apo[a] precursor from cell lysates of all three isoforms (Fig. 7C). Tunicamycin treatment reduced the levels of mature apo[a] in lysates of cells expressing the 6 and 9 K-IV-repeat apo[a], while eliminating the mature form from cells expressing the 17 K-IV isoform (Fig. 7C). Additionally, even though a band corresponding to the mature 9 K-IV apo[a] was detected in tunicamyin-treated cell lysates (Fig. 7C), none was detected in the culture media. Additionally, examination of a longer chase period of 6 h failed to reveal a product corresponding to the mature form of the 17 K-IV apo[a] isoform in tunicamycin-treated cell lysates (Fig. 7C).

Decreased apo[a] secretion in tunicamycin treated cells is not a consequence of intracellular degradation

To confirm that the decreased secretion of apo[a] in tunicamycin treated cells was due to increased ER retention time and not to degradation of the precursor form of the protein, transfected McA-RH7777 cells expressing a 6 K-IV apo[a] isoform were treated with tunicamycin in the presence of either brefeldin A plus nocodazole or monensin. Brefeldin A, a fungal metabolite, blocks the transport of secretory proteins from the ER to the Golgi by causing the disassembly of the Golgi apparatus (50–52). Nocodazole inhibits microtubule assembly, thereby blocking any retrograde transport of Golgi proteases to the ER (53). Therefore, treatment with both brefeldin A and nocodazole retains secretory proteins in the ER without any mixing of ER and Golgi components. On the other hand, monensin, a sodium ionophore, inhibits the transport of proteins from medial to *trans*-Golgi (54, 55). As shown in **Fig. 8**,

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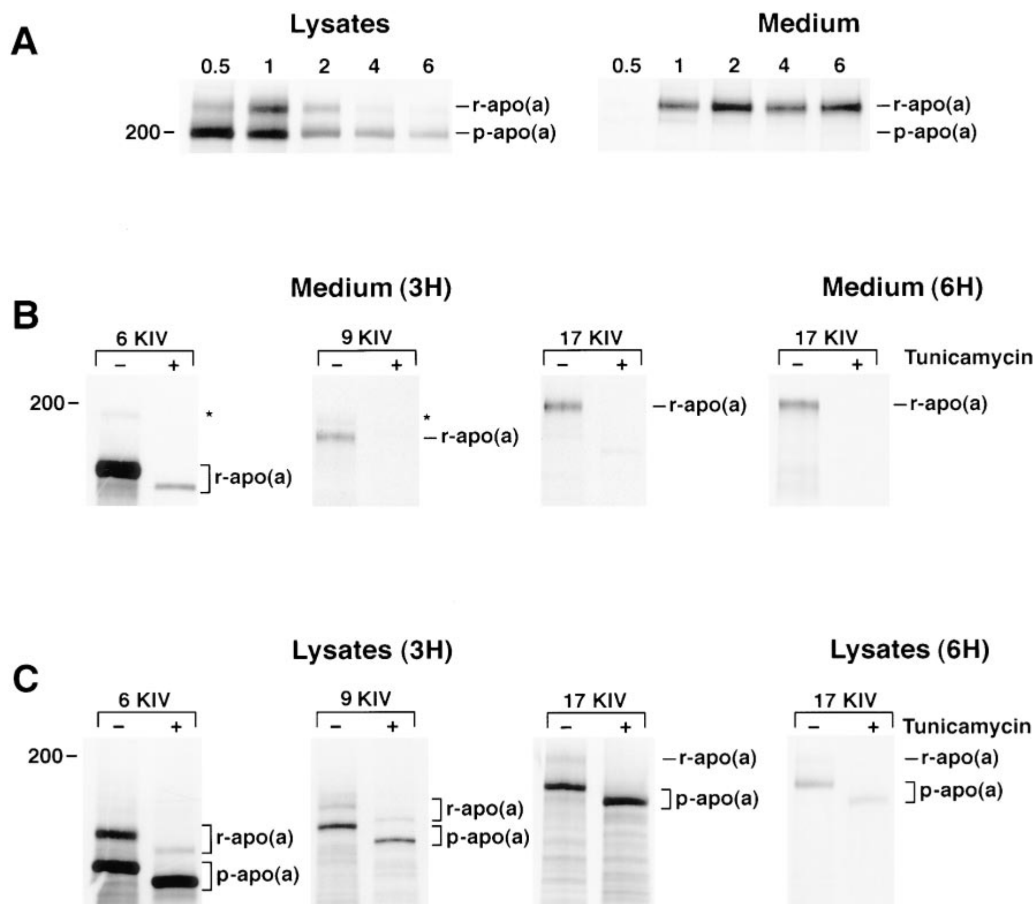

Fig. 7. The presence of N-linked glycans is necessary for the maturation and secretion of larger-sized apo[a]. A: McA-RH7777 cells stably transfected with the 17 K-IV apo[a] isoform were pulsed for 1 h with Tran^{35}S-label, followed by a 0.5–6 h chase. Aliquots of culture medium and lysates were immunoprecipitated with anti-apo[a] antiserum and analyzed by SDS-PAGE and fluorography. This is a representative of duplicate experiments. B: McA-RH7777 cells expressing the 6, 9, or 17 K-IV isoforms of apo[a] were preincubated for 2 h with tunicamycin (10 μg/ml) and labeled for 3 h in the same medium (3H) or pulsed for 1 h, followed by a 6 h chase in the same medium (6H). Aliquots of culture medium (B) and cell lysates (C) were immunoprecipitated with anti-apo[a] antiserum, followed by SDS-PAGE and fluorography. The migration of the precursor (p-apo[a]) and mature (r-apo[a]) forms of apo[a] are indicated. *, a nonspecific protein recovered in the immunoprecipitates. This is a representative of triplicate experiments.

tunicamycin-treated cells showed progressive accumulation of the precursor form of apo[a] when maintained either in the presence of brefeldin A plus nocodazole or monensin. In both cases, apo[a]-immunoprecipitable radioactivity was maintained throughout the chase at levels between 75 and 90% of maximum, suggesting that there is minimal degradation of nonglycosylated apo[a] retained in the ER or Golgi compartments. In contrast, when incubated with brefeldin A plus nocodazole, but without tunicamycin (Fig. 8, top panel), cells show a decline in immunoprecipitable intracellular apo[a], with approximately 34% recovery of the precursor at 240 min, suggesting that ER degradation may play a role in the regulation of the normal processing of apo[a]. Nevertheless, our current data suggest that the decrease in apo[a] secretion noted in the absence of N-linked glycosylation is not the result of its intracellular degradation, but rather a decrease in the processing events that facilitate migration through the secretory apparatus.

Presence of N-linked carbohydrates on apo[a] alters its association with ER chaperones calnexin and BiP

To begin to understand the factors that may contribute to the increased ER retention time of nonglycosylated apo[a], sequential immunoprecipitations were undertaken to detect apo[a] in association with chaperone proteins. As shown in control cells (**Fig. 9**, top panel), calnexin interacts with the precursor form of apo[a] early in the chase, with maximum association noted at 10 min and decreased binding at 30 min. Only trace amounts of the precursor can be detected in association with calnexin at later time points. By contrast, the association of apo[a] with BiP was not detectable until 60 min of chase, with decreased association evident at 120 min, suggesting that apo[a] may associate with these two chaperones in a sequential manner, first with calnexin and then with BiP. To confirm the specificity of the apo[a] capture, preimmune normal goat serum (NGS) was included in the second round of immunoprecipitation as a control (Fig. 9). Addi-

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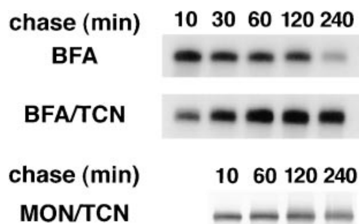

Fig. 8. Tunicamycin treatment results in retention, but not degradation of apo[a]. McA-RH7777 cells transfected with the 6 K-IV apo[a] isoform were pretreated for 2 h with 10 μg/ml tunicamycin (TCN) and either 5 μg/ml brefeldin A (BFA) plus nocodazole (20 μg/ml) or 10 mm monensin (MON), followed by a 10-min pulse and 10–240-min chase in the same medium. Cell lysates were immunoprecipitated using a polyclonal goat anti-apo[a] antibody and analyzed by reducing SDS-PAGE. This is a representative of three experiments.

tionally, no apo[a] product was obtained when NGS was used in the first round of immunoprecipitation in place of anti-calnexin or anti-BiP (data not shown).

The pattern of association of apo[a] with these two chaperones was altered in tunicamycin-treated cells. As shown in Fig 9, bottom panel, calnexin interacts with the precursor form of apo[a] for an extended period, starting at 10 min, and in a manner that was sustained throughout the 240-min chase. In addition, the interaction with BiP reveals important differences between the control and tunicamycin-treated cells, with an association evident at 10 min of chase in tunicamycin-treated cells (Fig. 9, lower panel). In addition, there is a later reappearance of this association with BiP at 120 and 240 min. The data suggest that the incompletely or abnormally folded apo[a], synthesized in the presence of tunicamycin, is held in prolonged association with calnexin and with altered binding kinetics to BiP.

DISCUSSION

The current studies were undertaken to explore the response of transfected hepatoma cells, expressing a 6 K-IV apo[a] minigene, to the effects of alterations in glycosylation. These potential effects were explored in relation to two distinct aspects of apo[a] behavior, namely its ability to associate with apoB-100 and its pattern of intracellular processing and secretion. These particular aspects were selected as, first, the ability of apo[a] to associate with apoB-100 is considered indispensable to the formation of Lp[a] and, second, studies have demonstrated that the major factor(s) responsible for regulating the plasma levels of Lp[a] in humans are related to hepatic secretion (56, 57). Accordingly, these two properties of apo[a] assume considerable importance in terms of potential therapeutic strategies to control elevated plasma levels of this protein.

Studies undertaken with HepG2 cells expressing a 6 K-IV isoform demonstrated a decrease in secretion of both apo[a] and also in the secretion of apoB-100, the latter confirmed in wild-type HepG2 cells, also. The most plausible explanation for the virtual absence of Lp[a] in the media of these transfected HepG2 cells, after tunicamycin treatment, thus presumably reflects a combination of decreases in both apo[a] and apoB-100 secretion. Previous work by other investigators has also demonstrated a decrease in the synthesis and secretion of apoB-100 in

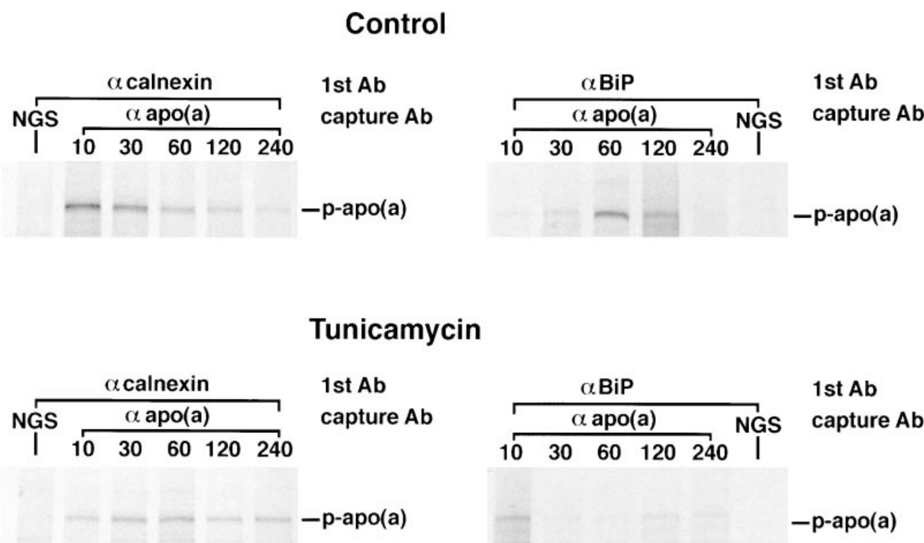

Fig. 9. Sequential immunoprecipitation reveals the association of apo[a] with calnexin and BiP. McA-RH7777 cells expressing the 6 K-IV apo[a] isoform were pulsed for 10 min with Tran^{35}S-label and chased for 10–240 min in the presence (lower panel) or absence (upper panel) of 10 μg/ml tunicamycin. Cell lysates were immunoprecipitated first with either anti-calnexin or anti-BiP polyclonal antibodies, followed by reimmunoprecipitation using anti-apo[a] as a capture antibody. Immunoprecipitates were analyzed by 4–12% SDS-PAGE. Preimmune goat serum (NGS) was used as a control for the apo[a] capture. The migration of the precursor apo[a] (p-apo[a]) is shown. This is a representative of three experiments.

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HepG2 cells after treatment with tunicamycin (58, 59), which has been suggested to result from increased degradation of apoB-100 (59). This suggestion is consistent with earlier studies in chicken hepatocytes that demonstrated that tunicamycin had no effect on the ability of apoB-100 to associate with triglyceride or on the secretion of very low density lipoprotein, but decreased synthesis and secretion of apoB-100 by ~40% (60). It is noteworthy, however, that studies in COS cells demonstrated no effects of tunicamycin treatment on apoB-41 synthesis or secretion (61). Nevertheless, it should be emphasized that 11 of the 16 potential sites for N-glycosylation reside in the carboxyl terminus of apoB-100, suggesting that the full length form of apoB may be more susceptible to the effects of tunicamycin treatment than amino-terminal fragments (61). The mechanisms underlying the apparent decrease in apoB-100 secretion after tunicamycin treatment of HepG2 cells are thus likely to involve alterations in degradation. The findings of the current study, taken together with previous studies in baboon hepatocytes (27), indicate that the decrease in apo[a] secretion noted after tunicamycin treatment most plausibly involves alterations in ER processing and not augmented intracellular degradation. This point is worthy of emphasis as it illustrates a clear distinction in processing of the two components of Lp[a] within hepatocytes.

Previous studies of both human plasma apo[a] (24) and apo[a] secreted from baboon hepatocytes (22) have shown that, along with N-linked oligosaccharides, apo[a] also contains O-linked glycans as well as sialic acid residues. As apo[a] contains such an abundance of carbohydrate residues, we undertook an examination of whether alterations in the extent and pattern of glycosylation resulted in alterations in the ability of the secreted protein to associate with apoB-100. Previous studies by Frank and colleagues (5) had demonstrated that an apo[a] isoform with 15 K-IV repeats, secreted from mutant, glycosylation-defective CHO cells (Lec 1, Lec 2 and Lec 8), demonstrated comparable association with apoB-100. Nevertheless, as the expression construct used in the current studies contains only 5 of the unique K-IV repeats (K-IV, 6–10; see reference (23)) and none of the identical repeats of K-IV, type 2, it was formally possible that alterations in the post-translational modification of the 6 K-IV isoform could alter its conformation and consequently impair the ability of this minigene product to associate with apoB-100. The results with the first panel of CHO mutants demonstrate that alterations in N- and/or O-linked glycosylation were without effect on the ability of the 6 K-IV isoform to associate with apoB-100. In order to more formally exclude the involvement of O-linked glycosylation in this process, the mutant ldlD cells were utilized. In transfected ldlD cells, which lack the 4-epimerase enzyme, depletion of Gal and GalNAc results in the synthesis of apo[a] with no O-linked sugars. Under normal conditions, glucose is the sole source of hexose in the culture medium, and its uptake and metabolic conversion provides all of the requisite nucleotide sugars (37). Under these growth conditions, the 4-epimerase enzyme is required for the synthesis of UDP-Gal and UDP-GalNAc from their corresponding glucose isomers. The addition of Gal to the culture medium permits normal N-linked glycosylation but not O-linked (37). By contrast, the addition of GalNAc permits the synthesis of truncated O-linked chains but without their complement of galactose and sialic acid residues, while addition of both substrates effectively restores glycosylation (37). As the ability of apo[a] to associate with apoB-100 was comparable under all conditions of media supplementation, we conclude that neither N- nor O-linked glycosylation of apo[a] is required for the covalent association of these proteins.

Several important findings emerge from the studies concerning apo[a] processing and secretion after tunicamycin treatment. First, in regard to the 6 K-IV isoform, despite the elimination of N-linked glycosylation, a portion of apo[a] is still able to exit the ER and undergo processing to a more "mature" form of the protein, albeit at a slower rate. The decay curve for the intracellular precursor form of apo[a] (shown in Fig. 6, bottom panel) demonstrates a markedly increased ER retention time in tunicamycin-treated over control cells, with a $t_{1/2}$ of approximately 150 min in treated cells compared to a $t_{1/2}$ of 75 min in control cells. Second, despite the absence of N-linked sugars, a portion of intracellular apo[a] is still directed into the secretory pathway and eventually leaves the cell. It should be emphasized that both of these findings, specifically the presence of both a precursor and mature form of intracellular apo[a] in tunicamycin-treated cells, as well as the ability of a portion of the intracellular apo[a] to be secreted, are in marked contrast to the results observed in baboon hepatocytes (27). In these studies, processing of the precursor form of apo[a] was completely blocked in primary hepatocytes treated with tunicamycin, and consequently apo[a] secretion was eliminated (27). As White and coworkers (27) examined only the naturally available isoforms of baboon apo[a], which are much larger than the 6 K-IV isoform used in our studies, these findings raise the interesting possibility that inhibition of N-linked sugar addition produces an effect on ER processing and secretion of apo[a] which is related to the number of K-IV repeats.

In order to examine this latter possibility, further studies were undertaken using McA-RH7777 cells stably transfected with apo[a] isoforms containing 9 or 17 K-IV repeats. As it is known that large apo[a] isoforms take longer to exit the ER than smaller forms of apo[a], pulse-chase studies were performed with an increased pulse time (1 h), followed by longer chase time points (0.5–6 h) to insure that both intracellular and secreted 17 K-IV apo[a] could be detected. Once the optimal time point for the detection of apo[a] secretion was established (i.e., 2–4 h of chase, Fig. 7), further studies were undertaken using a single (3 h) time point to evaluate secretion efficiency. The findings indicate that addition of N-linked carbohydrates to apo[a] is indeed required for larger-sized isoforms to leave the ER, as tunicamycin treatment retarded apo[a] maturation (17 K-IV >> 9 K-IV) and eliminated secretion of both the 9 and 17 K-IV isoforms, while the 6 K-IV isoform was relatively spared.

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In attempting to understand the mechanisms that may contribute to the delayed exit of apo[a] from the ER after tunicamycin treatment, the interaction between apo[a] and known chaperone proteins was examined. It has been proposed that quality control mechanisms made up of molecular chaperones and folding enzymes exist in the ER which ensure the functional integrity of secretory proteins and regulate their transport (62, 63). Two chaperone proteins were selected for initial examination, one a transmembrane protein (calnexin) and the other a soluble ER protein (BiP), both known to be abundantly expressed in liver cells (64–67). The data suggest that the incompletely or abnormally folded 6 K-IV apo[a] isoform found after tunicamycin treatment is held in prolonged association with calnexin and with altered binding kinetics to BiP. These results contrast with other studies where tunicamycin treatment was found to impair association of several secretory and viral glycoproteins with calnexin, implying that the N-linked carbohydrate moieties on these glycoproteins are required for their interaction with calnexin (67, 68). The current findings suggest that the presence of N-linked glycans on apo[a] is necessary for efficient secretion of apo[a], but not required for apo[a]/calnexin interaction. It will be important to extend these findings to other isoforms of apo[a], as it cannot be immediately concluded from these studies that the same paradigm applies to the larger isoforms found in human populations. These and other studies concerning the relationship of K-IV number to secretion efficiency from liver cells will be the focus of future reports.

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