Presecretory degradation of apolipoprotein[a] is mediated by the proteasome pathway

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Abstract Plasma levels of atherogenic lipoprotein [a] (Lp[a]) vary over a 1000-fold range and are largely determined by the gene for its unique glycoprotein, apolipoprotein [a] (apo[a]). The apo[a] locus comprises more than 100 alleles, encoding proteins from <300 to >800 kDa. Using primary baboon hepatocyte cultures, we previously demonstrated that differences in the secretion efficiency of apo[a] allelic variants contribute to the variation in plasma Lp[a] levels. In the current study, we investigated the mechanism of apo[a] presecretory degradation. The proteasome inhibitors, acetyl-leucyl-leucyl-norleucinal and lactacystin, prevented apo[a] degradation and increased apo[a] secretion. Transfection with an HA-tagged ubiquitin construct demonstrated the accumulation of ubiquitinated apo[a] in the presence of lactacystin. These results suggest a role for the cytoplasmic proteasome in apo[a] proteolysis. Apo[a] that accumulated intracellularly in the presence of lactacystin remained sensitive to endo-B-N-glucosaminidase H, and apo[a] degradation was reversibly inhibited by brefeldin A, suggesting that transport to a post-endoplasmic reticulum (ER) pre-medial Golgi compartment is required for apo[a] degradation. Newly synthesized apo[a] bound to the ER chaperone calnexin and conditions that enhanced this interaction prevented apo[a] degradation, suggesting that calnexin can protect apo[a] from proteolysis. These studies provide further support for the role of the proteasome in endoplasmic reticulum quality control, and expand this role to one that influences plasma levels of the atherogenic lipoprotein Lp[a].—White, A. L., B. Guerra, J. Wang, and R. E. Lanford. **Presecretory degradation of apolipoprotein[a] is mediated by the proteasome pathway. *J. Lipid Res.* 1999.** 40: **275–286.**

Supplementary key words apolipoprotein[a] • calnexin • endoplasmic reticulum • proteasome • ubiquitin

Lipoprotein [a] (Lp[a]) is an unusual, cholesterol-rich lipoprotein found only in the plasma of humans, old world primates (1), and the hedgehog (2). Lp[a] consists of a modified form of plasma low density lipoprotein (LDL) in which apolipoprotein (apo) B-100, the sole protein component of LDL, is attached by disulfide linkage to an additional, high molecular weight glycoprotein, apo[a] (3). Apo[a] and apoB-100 are both synthesized by the liver (4–6); however, their association to produce Lp[a] takes place after secretion (7–9). Levels of Lp[a] in plasma vary tremendously among individuals (<1 to >100 mg/dl) (10), and are determined by the rate of Lp[a] production (11, 12). Plasma Lp[a] levels greater than 25–30 mg/dL are associated with an increased incidence of various atherosclerotic cardiovascular diseases (13). However, the molecular mechanisms that determine Lp[a] production rate remain largely unknown.

The gene for apo[a] is highly homologous to that of plasminogen (14). The apo[a] gene encodes multiple domains with homology to plasminogen kringle (K) 4, a single plasminogen K 5-like domain and a single copy of the plasminogen protease domain (14). The number of K4 domains encoded varies from approximately 12 to 51 (15). Other polymorphisms have also been identified in the apo[a] gene, and there are estimated to be greater than 100 apo[a] alleles (16). Inheritance at this highly polymorphic locus accounts for more than 90% of the inter-individual variation in plasma Lp[a] levels (17).

Differences in the number of K4 domains encoded in the apo[a] gene result in apo[a] glycoproteins of greatly varying size (18, 19). Thirty four different apo[a] isoforms have been identified in human plasma, from <300 to >800 kDa (15, 20). An inverse correlation exists between apo[a] size and plasma Lp[a] levels (18, 19, 21), such that small apo[a] isoforms tend to be associated with high plasma Lp[a] levels, while large isoforms are present at lower levels. However, there are many exceptions to this correlation and apo[a] alleles of identical size can give rise to very different Lp[a] levels (18, 21, 22). An extreme example is "null" apo[a] alleles, which do not give rise to

Abbreviations: apo, apolipoprotein; BFA, brefeldin A; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CST, castanospermine; endoH, endo-B-N-glucosaminidase H; Lp, lipoprotein; NEM, N-ethylmaleimide; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; TM, tunicamycin; ER, endoplasmic reticulum; SFM, serum free medium.

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detectable plasma apo[a] protein and which are distributed throughout the apo[a] size range (23–25).

Baboons show characteristics similar to humans in terms of their plasma Lp[a] levels and apo[a] isform sizes (26, 27). We have established primary baboon hepatocyte cultures as a model system to study Lp[a] biogenesis (6–8, 23, 28, 29). This system is unique in that it allows comparison of naturally occurring apo[a] allelic variants. Using this system, we recently demonstrated that post-translational mechanisms play an important role in determining Lp[a] production rate (7, 23). Specifically, differences in the efficiency with which newly synthesized apo[a] allelic variants escape the endoplasmic reticulum and undergo post-translational processing account, at least in part, for the inverse correlation between apo[a] size and plasma Lp[a] level. In addition, some null Lp[a] phenotypes are caused by the production of apo[a] proteins which are unable to undergo post-translational processing and are completely retained inside the liver and degraded (23).

To investigate the molecular mechanisms responsible for the post-translational regulation of Lp[a] production rate, we have begun to analyze the events in the ER which are responsible for the varying efficiencies with which apo[a] allelic variants are secreted (29). In the current study, we investigated the mechanisms responsible for the intracellular retention and degradation of apo[a], and the role of ER chaperone proteins in this process. Our data indicate a role in apo[a] degradation for the proteasome, a cytosolic multisubunit proteolytic complex, and suggest that apo[a] degradation can be prevented by enhancing its interaction with the ER chaperone, calnexin. Thus, differences in the susceptibility of apo[a] allelic variants to intracellular proteasome-mediated degradation may be one of the major factors determining plasma concentrations of the atherogenic lipoprotein, Lp[a].

EXPERIMENTAL PROCEDURES

Materials

[^{35}S]cysteine and Expre^{35}S^{35}S label were from DuPont NEN. Protein A agarose was from Repligen Corp. (Cambridge, MA) and goat anti-human Lp[a] was from Biodesign (Kennebunkport, ME). Rabbit anti-calnexin polyclonal and mouse anti-KDEL monoclonal antibodies were from StressGen (Victoria, British Columbia). Mouse anti-PDI was from Affinity BioReagents (Golden, CO). Methionine- and cysteine-free Williams medium E was purchased from Life Technologies, Inc. Tunicamycin, cycloheximide, puromycin, A23187, leupeptin, aprotinin, pepstatin, phenylmethylsulfonylfluoride (PMSF), N-acetyl-leu-leu-norleucinal (ALLN), and N-acetyl-leu-leu-methioninal (ALLM) were from Sigma. Castanospermine, jack bean α-mannosidase, DOTAP transfection reagent, mouse anti-HA (12CA5) monoclonal antibody, and sheep anti-human apoB were from Boehringer Mannheim. Rabbit anti-human albumin was from Dako. EndoH (endo–B–N-acetylglucosaminidase H) was from ICN Immunobiologicals (Costa Mesa, CA). Lactacystin was purchased from the E.J. Corey laboratory, Harvard Medical School, or from Calbiochem. HA- and His6-tagged ubiquitin expression constructs (30) were kindly provided by D. Bohman. All other chemicals were of analytical grade.

Hepatocyte isolation and culture

Baboon hepatocytes were isolated as described previously (31). Lobectomy was performed according to institutional guidelines under general anesthesia. Analgesics were provided 48 h post-operatively. Cells were cultured in serum-free medium (SFM; formula III) as described previously (31). All experiments were performed using confluent 60-mm or 35-mm dishes of cells, which had been in culture for 5–7 days.

Hepatocytes from two different animals were analyzed. Apo[a] isoforms in the baboon are classified into 12 size groups; A, the largest, through L, the smallest (27). One animal expressed an A isoform and had a plasma Lp[a] level of 17 mg/dL. This animal was heterozygous for a null apo[a] allele that does not give rise to any detectable apo[a] mRNA or protein. The second animal had no Lp[a] detectable in its plasma (null phenotype). Northern analysis of hepatic RNA from this null animal revealed a single apo[a] mRNA species predicted to give rise to an apo[a] isoform approximately the size of an I isoform.

Pulse-chase analysis of apo[a] synthesis, secretion, and intracellular degradation

Hepatocytes were pre-incubated in methionine- and cysteine-free SFM for 1 h and then labeled for 10 min in the same medium supplemented with 125 μCi/ml each of [^{35}S]cysteine and Expre^{35}S^{35}S label. The cells were then washed with phosphate-buffered saline (PBS) and chased for between 30 min and 8 h in complete SFM. Media and cell lysates were harvested and apo[a] was analyzed by immunoprecipitation and 3–10% gradient SDS-polyacrylamide gel electrophoresis (SDS-PAGE) after reduction with 2-mercaptoethanol, exactly as described previously (7). To quantitate apo[a] in each sample, autoradiographs were scanned using an LKB densitometer. Values were normalized to total trichloroacetic acid-precipitable counts (cells + media) or to total cell protein (BCA micro-protein assay; Pierce Chemical Co.).

To determine the influence of various compounds on apo[a] intracellular degradation, hepatocytes were pre-incubated and labeled as described above, then chased for 30 min or 6 h in serum-free medium. Drugs were added to the pre-incubation, labeling and chase media, as described for individual experiments. Apo[a], albumin, and apoB were then immunoprecipitated and quantitated as described above. The amount of apo[a] present at the 30-min chase time point was taken as the amount of apo[a] synthesized, and that remaining at 6 h was used to calculate the percent degradation. Immunoblot analysis of samples before and after immunoprecipitation demonstrated that the apo[a] immunoprecipitation reaction was essentially quantitative (>95% of apo[a] precipitated; data not shown).

Transfection of hepatocyte cultures

Hepatocytes were seeded into 10-cm dishes at a density of 1.6×10^6 cells per dish. Twenty-four hours after plating, 8.3 μg of DNA in a total volume of 167 μl of HBS (20 mm HEPES, 150 mm $NaCl_2$, pH 7.4) was mixed with 50 μl of DOTAP transfection reagent and 117 μl of HBS. After a 15-min incubation at room temperature, 5 ml of SFM was added and the mixture was then placed on the cells. Cells were incubated at 37°C for 6 h, then washed and placed in fresh SFM. Experiments were performed 48 h after transfection. Ubiquitin-transfected cells were harvested in buffer (7) containing 0.1% SDS and 5 mm N-ethylmaleimide (NEM) to inhibit isopeptidase activity.

Trypsin digests of permeabilized cells

Hepatocytes were permeabilized with digitonin essentially as described by Meigs and Simoni (32). Briefly, cells were washed once with CSK buffer (0.3 m sucrose, 0.1 m KCl, 2.5 mm $MgCl_2$, 1 mm Na-free EDTA, 10 mm PIPES, pH 6.8) and then incubated for

15 min at room temperature in CSK buffer containing 25 μg/ml digitonin. This protocol was found to release the maximum number of TCA-precipitable counts (31% of counts incorporated into total cellular protein during a 30-min label) without concomitant loss of ER lumenal proteins (data not shown). The cells were then washed once with ice-cold CSK buffer, and incubated on ice for 15 min in CSK buffer with or without 100 μg/ml tryspin and 1% Triton X-100. Each sample was then adjusted to the following concentrations of protease inhibitors: soy bean trypsin inhibitor, 200 μg/ml; leupeptin 2.5 mg/ml; pepstatin, 25 μg/ml; PMSF, 4 mm; aprotinin 200 μg/ml. All samples were adjusted to 1% Triton X-100, and then harvested by adding an equal volume of 1% Triton X-100/200 mm NaCl/50 mm Tris, pH 9.0 (EB). Portions of each sample were immunoprecipitated for apo[a], as described above, or immunoblotted (7) with anti-KDEL or anti-calnexin antiserum.

Digestion with EndoH and jack bean α–mannosidase

Apo[a] was immunoprecipitated from cell lysates as described above. For endoH digestion, 120 μl of 1% SDS containing 1% mercaptoethanol was added to the final protein A pellet and the samples were heated to 100°C for 5 min. Aliquots of the supernatant were then incubated overnight at 37°C in 30 mm sodium acetate, pH 5.7, containing 3 mIU of endoH. For digestion with jack bean α-mannosidase, 25 μl of 50 mm sodium citrate, pH 4.4 and 10 μg of enzyme were added to the final protein A pellet and the samples were incubated for 2 h at 37°C. All samples were analyzed by 3–10% SDS-PAGE.

Co-immunoprecipitation of apo[a] with chaperone proteins

To analyze the interaction of apo[a] with various chaperone proteins, cells were labeled and chased as described above. To harvest, the cells were placed on ice and washed twice with ice-cold PBS containing 20 mm N-ethylmaleimide (NEM). NEM was included in the incubations to prevent rearrangement of disulfide bonds in apo[a] (29) so as to help preserve apo[a]–chaperone interactions. The cells were then lysed in ice-cold 50 mm HEPES, pH 7.6, containing 0.3% 3-[(3-cholamidopropyl)-1-propanesulfonate (CHAPS), 200 mm NaCl, and 20 mm NEM. Aliquots of cell lysates were incubated for 3 h at 4°C with protein A-agarose plus pre-immune rabbit serum or anti-chaperone antibodies. Precipitates were then washed 3 times for 5 min at 4°C in 0.3% CHAPS buffer. To elute proteins precipitated by the anti-KDEL or anti-PDI antibodies, 50 μl of 1% SDS in 50 mm HEPES/200 mm NaCl was added, and the samples were heated to 100°C for 5 min. To each sample EB (1 ml) was added and the protein A pellet was removed by centrifugation. For calnexin immunoprecipitates, proteins were eluted with 50 μl of 10 mm EDTA in EB for 1 h at 30°C, 1 ml EB was added to each sample and the protein A pellet was removed, as above. Apo[a] was then immunoprecipitated from the co-precipitated protein solution and from the immunoprecipitate supernatants, as described above.

RESULTS

Apo[a] degradation is mediated by the proteasome pathway

Our earlier studies demonstrated that apo[a] is synthesized as a lower molecular weight precursor containing high mannose, endo-H sensitive, N-linked glycans characteristic of ER resident proteins. After a prolonged time period (30–120 min) apo[a] begins to exit the ER, acquires mature N- and O-linked glycans, and is secreted. However, a portion of apo[a] never appears in its mature form and is degraded (7, 23). The fraction of apo[a] directed to the secretory versus degradation pathways shows considerable variation among allelic variants, the most notable example being null apo[a] proteins, which are incapable of maturation and secretion (23).

To characterize the apo[a] degradation pathway, hepatocytes expressing a null apo[a] protein were initially used, as loss of this protein from cell lysates could be attributed entirely to degradation (23). To examine the kinetics of apo[a] degradation, hepatocytes were labeled for 10 min with ^{35}S-methinione and ^{35}S-cysteine, then chased for between 30 min and 8 h in the absence of labeled amino acids. Apo[a] was immunoprecipitated from the cell lysates and analyzed by SDS-PAGE. The amount of apo[a] at 30 min of chase was used to measure apo[a] synthesis, as pulse-chase experiments indicated maximum incorporation of counts into apo[a] at this time point (data not shown).

Newly synthesized null apo[a] remained stable in the cells for 2 h after synthesis (**Fig. 1A**). After 2 h, the amount of apo[a] recovered from the cells began to decline, and by 8 h of chase, only 31% of apo[a] remained inside the cells (Fig. 1A). As expected for a null apo[a] protein (23), all apo[a] recovered from the cells was in its endoH-sensitive precursor form. At no time point was any mature apo[a] detected in the cell lysates, and no apo[a] was recovered from the culture medium (Fig. 1 and data not shown).

To characterize the protease activity responsible for apo[a] degradation, the ability of various protease inhibitors to prevent apo[a] degradation was analyzed. Cells were labeled for 15 min and then chased in the presence of the various inhibitors. A 30-min time point was used to measure apo[a] synthesis under each condition. A 6-h time point was used to determine the influence on apo[a] degradation, as this represented roughly the half-life of the apo[a] protein (Fig. 1A).

In control, untreated hepatocytes, 36 ± 10% (mean ± SD, 10 experiments) of apo[a] remained at 6 h of chase (Fig. 1B). Aprotinin (a serine protease inhibitor), leupeptin (a serine/cysteine protease inhibitor), and ammonium chloride (a lysosomal protease inhibitor) had little effect on apo[a] degradation (28, 31, and 36% apo[a] remaining at 6 h, respectively). ALLM (calpain inhibitor type II) also did not significantly reduce apo[a] degradation (47% remaining at 6 h). In contrast, the related inhibitor, ALLN (calpain inhibitor type 1), markedly decreased the extent of degradation, with 84% of apo[a] remaining at 6 h (Fig. 1B) ($P < 0.001$ versus control at 6 h).

ALLN is an efficient inhibitor of the cytoplasmic proteasome, whereas ALLM is a poor proteasome inhibitor (33). To determine directly whether the proteasome mediates apo[a] degradation, the influence of the highly specific proteasome inhibitor, lactacystin (34), on apo[a] degradation was analyzed. Lactacystin inhibited apo[a] degradation in a concentration-dependent manner (data not shown). At 10 μm lactacystin, apo[a] degradation was virtually eliminated (97% of apo[a] remaining at 6 h, $P < 0.01$ versus control at 6 h; Fig. 1B). The effect of ALLN and lactacys-

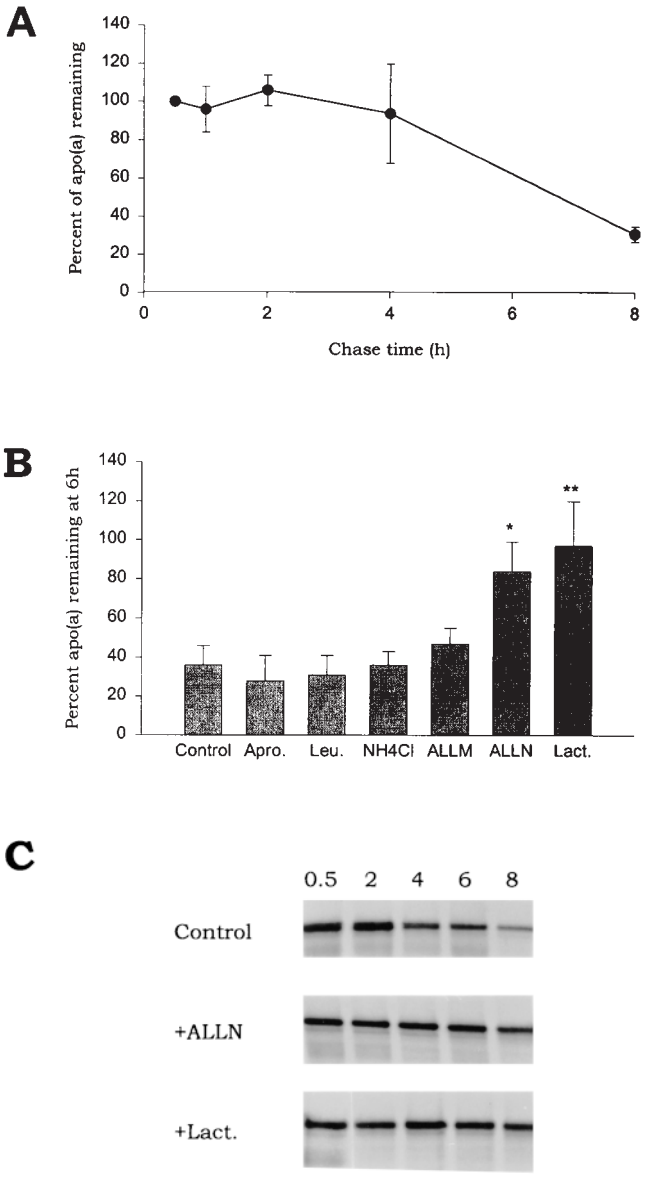

Fig. 1. Degradation of null apo[a] is mediated by the proteasome. A: Hepatocytes expressing a null apo[a] protein were labeled for 10 min with [^{35}S]methionine and [^{35}S]cysteine, and chased for between 30 min and 8 h in an excess of unlabeled amino acids. Apo[a] was then immunoprecipitated from the cells and culture media and analyzed by SDS-PAGE. Apo[a] was quantified by densitometric scanning, and normalized to total TCA-precipitable counts (cells + media). Values are expressed as the percentage of apo[a] recovered at each time point, with apo[a] recovered at 30 min of chase taken as 100%. Error bars represent the standard deviation for three experiments. B: Hepatocytes were labeled for 15 min and chased for 30 min or 6 h, as described above. Included in the chase period was no addition (Control), 10 μg/ml aprotinin (Apro.), 100 μg/ml leupeptin (Leu.), 10 mm ammonium chloride (NH_4Cl), 50 μg/ml ALLM (ALLM), 50 μg/ml ALLN (ALLN), or 10 μm lactacystin (Lact.). Apo[a] was quantified as in A. Results are presented as the mean ± SD % of apo[a] remaining at 6 h of chase under each condition, with apo[a] at 30 min for the same condition taken as 100%. C: Hepatocytes expressing the null apo[a] protein were labeled for 10 min and chased for 0.5, 2, 4, 6, or 8 h, in the presence of no addition (Control), 50 μg/ml ALLN, or 10 μm lactacystin, and apo[a] was analyzed by immunoprecipitation and SDS-PAGE. All procedures were performed as described under Experimental Procedures.

tin on null apo[a] intracellular stability was examined over an 8-h chase period (Fig. 1C). The amount of intracellular radiolabeled apo[a] in untreated hepatocytes began to decline after 2 h of chase. In contrast, apo[a] remained stable in ALLN- and lactacystin-treated cells throughout the 8-h time period (Fig. 1C). These experiments suggest that the intracellular degradation of apo[a] is mediated by the proteasome. Results comparable to those in Fig. 1 were obtained when two other null apo[a] proteins were analyzed (data not shown).

To determine whether the proteasome also degrades the portion of secreted apo[a] isoforms that do not undergo maturation and secretion, the influence of ALLN and lactacystin on the secretion and intracellular stability of a large, inefficiently secreted apo[a] isoform was analyzed (**Fig. 2A**). After a 15-min pulse and 30-min chase, only the precursor form of this apo[a] protein was visible inside the cells. By 6 h of chase, the mature form of apo[a] was also recovered from the cell lysates, and apo[a] was secreted into the culture medium (Fig. 2A). At the 6-h chase time point the amount of immature apo[a] recovered from ALLN- and lactacystin-treated cells was 167% and 178% of that in control cultures, respectively. Apo[a] secretion was increased to 194% and 224% of control levels, respectively, in ALLN- and lactacystin-treated cultures (Fig. 2A). Aprotinin, leupeptin, and ammonium chloride had no effect on the secretion or intracellular stability of this isoform (data not shown). That the precursor form of this apo[a] protein accumulated in the presence of lactacystin suggests that at least a portion of this isoform is degraded by a pathway similar to that for null apo[a]. The increase in apo[a] secretion with proteasome inhibitors, however, suggests that, unlike null apo[a], when degradation of a secreted apo[a] isoform is inhibited, the efficiency of its maturation and secretion can be increased. Alternatively, there may be a second proteasome-dependent degradation pathway that normally degrades a portion of the mature form of the protein. Inhibition of such a pathway would also lead to an increase in apo[a] secretion.

The influence of ALLN and lactacystin on apoB-100 and albumin secretion from primary hepatocytes was also examined (Figs. 2B and C). In the human hepatoma cell line, HepG2, a large portion of newly synthesized apoB-100 is subject to presecretory degradation by the proteasome (35). In contrast, we found that apoB-100 was efficiently secreted by our primary baboon hepatocytes, and that its secretion was not increased by the presence of either ALLN or lactacystin (Fig. 2C). The secretion of albumin was also quantitative in our experiments, and was not influenced by the presence of proteasome inhibitors (Fig. 2B).

Apo[a] accumulates in the lumen of the secretory pathway in the presence of lactacystin

The proteasome is a cytoplasmic enzyme complex, whereas secretory proteins, such as apo[a], are normally retained within the membrane-bound compartments of the secretory pathway. Translocation of proteins from the ER lumen to the cytoplasm has been demonstrated as a mechanism for exposure of degradation substrates to the

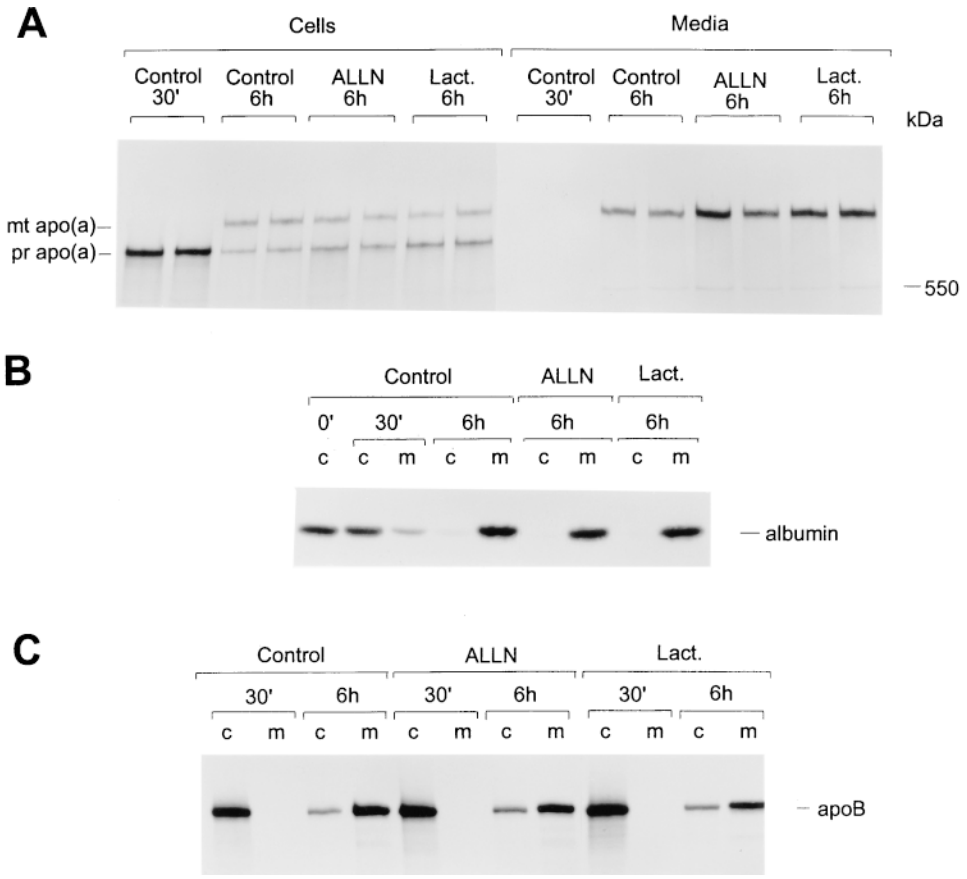

Fig. 2. ALLN and lactacystin increase apo[a] secretion. Hepatocytes expressing a large apo[a] isoform were labeled for 15 min and then harvested immediately (0′) or chased for 30 min or 6 h in the absence (Control) or presence of 50 μg/ml ALLN, or 10 μm lactacystin, as described in the legend to Fig. 1. Apo[a] (A), albumin (B), or apoB (C) were then immunprecipitated from the cells and culture media and analyzed by 3–10% SDS-PAGE as described in Experimental Procedures. The positions of the precursor (pr) and mature (mt) forms of apo[a] and of apoB (550 kDa) and albumin, are indicated.

proteasome (36–38). If apo[a] is degraded by this pathway, then it might be expected to accumulate in the cytoplasm in the presence of lactacystin. To investigate this issue, hepatocytes expressing the null apo[a] protein were labeled for 15 min and chased for 6 h in the presence or absence of lactacystin. The plasma membranes were then permeabilized with digitonin, the cells were treated with trypsin, and the amount of apo[a] remaining in each sample was analyzed by immunopreciptation (**Fig. 3**). To control for the integrity of the intracellular membranes, and for the efficiency of trypsin digestion, the ER chaperone proteins, BiP, GRP94, and calnexin were analyzed by immunoblotting (Fig. 3).

No apo[a] was recovered from the digitonin solution used to permeabilize the cells (data not shown). The amount of apo[a] recovered from undigested cells was 2.3-fold greater for lactacystin-treated versus control cultures (Fig. 3). When cells were exposed to trypsin in the presence of detergent to lyse the intracellular membranes, no apo[a] was recovered from the cell lysates (Fig. 3), confirming that apo[a] is a substrate for the enzyme. The ER lumenal protein, GRP94, was also absent from these samples, whereas BiP, another protein in the ER lumen, was found to be resistant to trypsin digestion under these conditions (Fig. 3). When cells were digested with trypsin in the absence of detergent, 76% and 77% of apo[a] was protected from trypsin in the control and lactacystin-treated cultures, respectively (Fig. 3). A similar portion of GRP94 was also protected from the enzyme under these conditions (Fig. 3). That digestion of cytoplasmically exposed proteins was complete under these conditions was confirmed by immunoblotting of the transmembrane ER resident protein, calnexin; trypsin caused a quantitative increase in the electrophoretic mobility of calnexin due to cleavage of its cytoplasmic C-terminal tail (Fig. 3). Thus, in both control and lactacystin-treated cells, the vast majority of apo[a] appeared to reside on the lumenal side of the ER membrane. The apo[a] protected by lactacystin from degradation thus appears to accumulate primarily in the lumen of the secretory pathway and not in the cytoplasm.

Ubiquitinated forms of apo[a] are present in lactacystin-treated cells

Our inability to demonstrate the presence of significant amounts of cytoplasmically exposed apo[a] in control

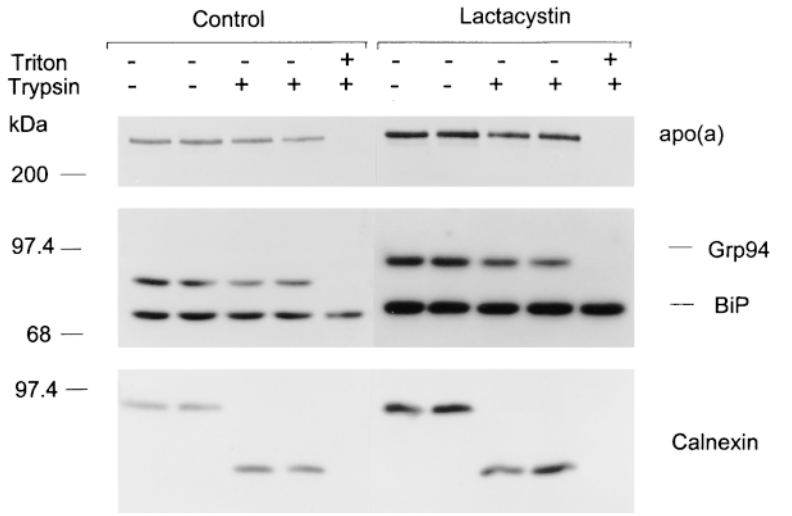

Fig. 3. Apo[a] accumulates in the lumen of the secretory pathway in the presence of lactacystin. Hepatocytes expressing a null apo[a] protein (Fig. 1) were labeled for 15 min and then chased for 6 h in the presence or absence of 10 μm lactacystin. The cells were then permeabilized with digitonin and incubated on ice in the presence or absence of 100 μg/ml trypsin and 1% Triton X-100 for 15 min as indicated. After addition of protease inhibitors, aliquots of the cell lysates were immunoprecipitated with anti-apo[a] antibody and analyzed by SDS-PAGE, or immunoblotted with anti-KDEL monoclonal antibody, or an antibody against the N-terminal region of calnexin. The anti-KDEL antibody recognizes both BiP and grp94 by immunoblotting. The positions of apo[a], Grp94, and BiP, and molecular weight markers are indicated. All procedures were performed as described in Experimental Procedures.

cells could be due to rapid degradation of this form of apo[a] by the proteasome. Inhibition of apo[a] degradation by lactacystin may not allow apo[a] to accumulate in the cystosol if the proteasome itself is involved in translocating apo[a] back across the ER membrane. To further examine whether apo[a] becomes exposed to the cytosol, we performed experiments to determine whether apo[a] targeted to the degradation pathway becomes ubiquitinated. Ubiquitin is a 76 amino acid cytosolic protein conjugated to proteins destined for degradation by the 26S proteasome (39). Primary hepatocytes expressing the null apo[a] protein were transfected with a plasmid encoding influenza virus hemaglutinnin (HA)-tagged ubiquitin. The cells were then incubated with lactacystin for 6 h, apo[a] was immunoprecipitated from the cell lysates, and the immunoprecipitates were immunoblotted with an anti-HA antibody. HA-ubiquitin-transfected cells that were not treated with lactacystin and lactacystin-treated hepatocytes that had been transfected with a His6-tagged ubiquitin construct were used as controls. ApoB and albumin were also immunoprecipitated from each cell lysate (**Fig. 4**).

A smear of HA-reactive bands was observed in unfractionated lysates of HA-ubiquitin-transfected cells. The intensity of these bands was greatly enhanced after treatment with lactacystin (Fig. 4). Because lactacystin inhibits degradation of ubiquitinated proteins without preventing their formation, this suggests that the smear of anti-HA reactivity represents multiple ubiquitinated cellular proteins. This smear of anti-HA-reactive material was absent from cell lysates of His6-tagged ubiquitin-transfected cells that had been treated with lactacystin (Fig. 4). The identity of the two prominent low molecular weight anti-HA reactive bands in the cell lysates is unknown, but presumably represents non-specific cross-reactivity with the antibody, as they were present in His6–ubiquitin-transfected cells.

A high molecular weight smear of bands was also recognized by the anti-HA antibody in apo[a] immunoprecipitates of HA–ubiquitin-transfected cells. The intensity of this smear increased markedly in lactacystin-treated cultures (Fig. 4). The molecular weight of the majority of this activity was larger than that of apo[a], consistent with these bands representing apo[a] conjugated to multiple ubiquitin molecules. No anti-HA reactivity was observed in apo[a] immunoprecipitates from lactacystin-treated His6–ubiquitin-transfected cells. As expected from the data in Fig. 2, no anti-HA reactive bands were present in apoB and albumin immunoprecipitates from any of the transfected cultures (Fig. 4).

These results suggest that at least a portion of apo[a] is targeted for degradation by the proteasome through con-

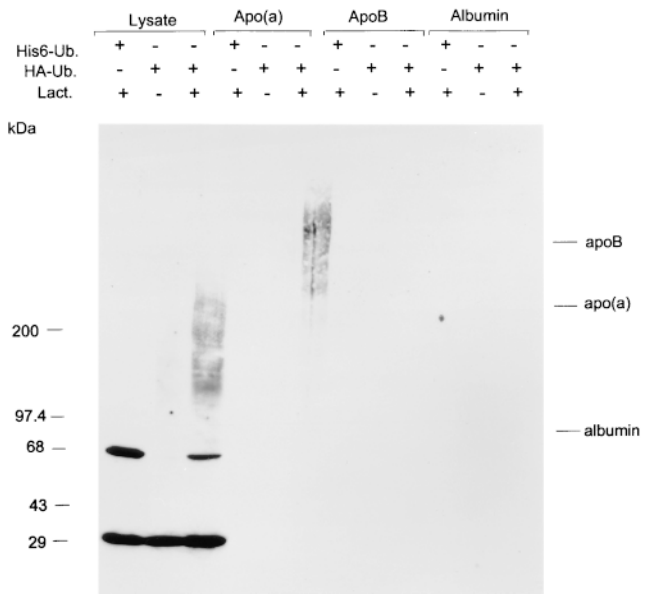

Fig. 4. Ubiquitinated apo[a] accumulates in the presence of lactacystin. Hepatocytes expressing the null apo[a] protein were transfected with a plasmid encoding either HA- or His6-tagged ubiquitin. Two days after transfection, the cells were incubated for 6 h with or without lactacystin. Aliquots (1/20) of total cell lysate, and apo[a], apoB and albumin sequentially immunoprecipitated from the remainder of each cell lysate, were then analyzed by immunoblotting with an anti-HA monoclonal antibody. The positions on the blot of molecular weight standards and of apo[a], apoB, and albumin, are indicated. On the original autoradiograph, a smear of anti-HA reactivity could be seen in total cell lysates and apo[a] immunoprecipitates from HA-transfected cells incubated without lactacystin.

jugation to multi-ubiquitin molecules. Such targeting presumably requires exposure of apo[a] to ubiquitin conjugating enzymes in the cytosol. Future studies will be required to determine whether all apo[a] is translocated to the cytosol for degradation.

Apo[a] remains endoH sensitive in the presence of lactacystin

Misfolded proteins are frequently targeted for degradation while residing in the ER. To address at which point in the secretory pathway apo[a] becomes a substrate for the proteasome, two approaches were taken (**Fig. 5**). In the first approach, we ascertained whether null apo[a], which accumulated intracellularly in the presence of lactacystin, remained sensitive to digestion with endoH (Fig. 5A). EndoH cleaves high mannose N-linked carbohydrate side chains, characteristic of ER-associated proteins (40). The electrophoretic mobility of apo[a] from both control and lactacystin-treated cultures was increased after digestion with endoH (Fig. 5A), confirming the presence of high mannose N-linked carbohydrate on each protein. As lactacystin does not prevent the intracellular trafficking events required for secretion (Fig. 2) this suggests that apo[a] degradation takes place in a compartment proximal to the medial–Golgi apparatus, where resistance of glycoproteins to endoH is acquired.

In the second approach, the influence of brefeldin A (BFA) on apo[a] degradation was examined (Fig. 5B). BFA blocks ER to Golgi transport and has been used to demonstrate ER-associated degradation of secretory proteins (41). When cells expressing the null apo[a] protein were labeled and chased in the presence of BFA, apo[a] degradation was inhibited (Fig. 5B; a mean of 86% apo[a] remaining at 6 h vs. 43% in control cells, $P < 0.05$, 5 experiments). However, BFA allows retrograde transport of proteins from the Golgi to the ER, and thus causes mixing of the ER and Golgi contents (42). This resulted in an increase in apo[a] molecular weight in BFA-treated cells due to modification of its carbohydrate content (Fig. 5B, and data not shown). To determine whether the inhibition of apo[a] degradation was due to a direct effect of BFA on intracellular transport or to an indirect effect mediated through the modification of apo[a] carbohydrate side chains, cells were labeled for 15 min and then chased for 2 h in the presence of BFA to allow modification of apo[a] carbohydrate to occur. The BFA was then removed, the cells were chased for an additional 4 h in the absence of BFA, and apo[a] was analyzed by immunoprecipitaton (Fig. 5B). Despite an increase in apo[a] molecular weight due to the early presence of BFA, apo[a] was once again a substrate for intracellular degradation in these cells (23% of the protein remaining at 6 h compared to 108% in cells where BFA was present throughout the 6-h chase; Fig. 5B). Thus, transport of apo[a] out of the ER appears to be required for degradation to occur. These results may indicate a role for the ER-Golgi intermediate compartment in apo[a] degradation, which has been implicated in the degradation of other secretory proteins (43).

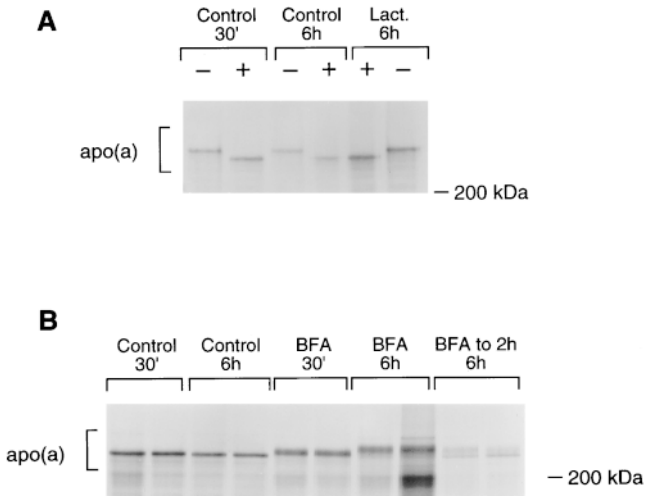

Fig. 5. Apo[a] degradation occurs in a compartment distal to the ER, but proximal to the medial-Golgi apparatus. A: Hepatocytes expressing the null apo[a] protein were labeled for 15 min and chased for 30 min or 6 h in the absence (Control) or presence of 10 μm lactacystin (Lact.). Apo[a] was then immunoprecipitated from the cell lysates, incubated without (−) or with (+) endoH, and analyzed by SDS-PAGE, as described in Experimental Procedures. B: Hepatocytes expressing the null apo[a] protein were labeled for 15 min, then chased for 30 or 6 h in the absence (Control) or presence of 10 μg/ml BFA. In the far right two lanes, BFA was added up to 2 h of chase, then removed and the chase was continued up to 6 h. Apo[a] was immunoprecipitated from the cell lysates and analyzed by SDS-PAGE. The positions of apo[a] and myosin (200 kDa) are indicated.

Role of N-linked carbohydrate processing in apo[a] intracellular degradation

N-linked glycans are added co-translationally to nascent secretory proteins, as $GlcNac_2Man_9Glc_3$ precursors (44) (where GlcNac is N-acetylglucosamine, Man is mannose, and Glc is glucose). While still in the ER, the outer and two inner glucose residues are removed by ER glucosidases I and II, respectively (44). If the protein is not properly folded after removal of the three glucose residues, it is recognized by the ER resident enzyme UDP-glucose:glycoprotein glucosyltransferase which re-attaches a single glucose residue (45). The protein then enters a deglucosylation/reglucosylation cycle until it is fully folded and no longer recognized by the glucosyltransferase enzyme. The glucosylation state of N-linked glycans can play a role in targeting secretory proteins for intracellular degradation (46, 47).

To examine the role of N-linked glycans in apo[a] degradation, castanospermine (CST), an inhibitor of ER glucosidases I and II (48), was used. When cells were preincubated, labeled and chased in the presence of CST, apo[a] degradation was unaffected (**Fig. 6A**; 44% and 46% of apo[a] remaining at 6 h of chase in control and CST-treated cultures, respectively, $P > 0.4$). Thus, trimming of the three glucose residues from N-linked glycans on apo[a] is not required for degradation to occur. Lactacystin inhibited apo[a] degradation under these conditions (data not shown), suggesting that the same degradation pathway was operable in control and CST treated cells.

Trimming of glucose residues from N-linked glycans occurs very rapidly after translation. Thus, when added post-translationally, CST can also be used to trap proteins in their monoglucosylated state (49). When hepatocytes were labeled in the absence of CST and then chased for 6 h in the presence of CST, apo[a] degradation was prevented (Fig. 6). Thus, the presence of monoglucosylated N-linked glycans can protect apo[a] from degradation.

To confirm that CST had indeed resulted in modification of apo[a] side chains, the apo[a] immunoprecipitated from CST-treated cells was digested with jack bean α mannosidase (Fig. 6B). This enzyme only removes deglucosylated N-linked glycans from proteins, and thus can be used to enhance the difference in electrophoretic mobility of glucosylated and non-glucosylated forms of apo[a] (49). Interestingly, apo[a] in control cells (no CST added at any time) was susceptible to digestion with the enzyme at both 30 min and 6 h of chase (Fig. 6B), indicating that under normal circumstances, the majority of apo[a] in the ER contains de-glucosylated side chains. However, when CST was present, either during the label and chase or during the chase period only, apo[a] became resistant to α mannosidase, confirming the efficacy of the CST treatment in each case (Fig. 6B).

These results suggest that, by trapping apo[a] in its monoglucosylated (but not triglucosylated) form, apo[a] degradation can be prevented.

The influence of CST on albumin secretion was also examined for comparison in these experiments. The presence of CST before and after labeling caused a partial inhibition of albumin secretion. The post-translational addition of CST, however, had no influence on recovery of albumin from the culture medium.

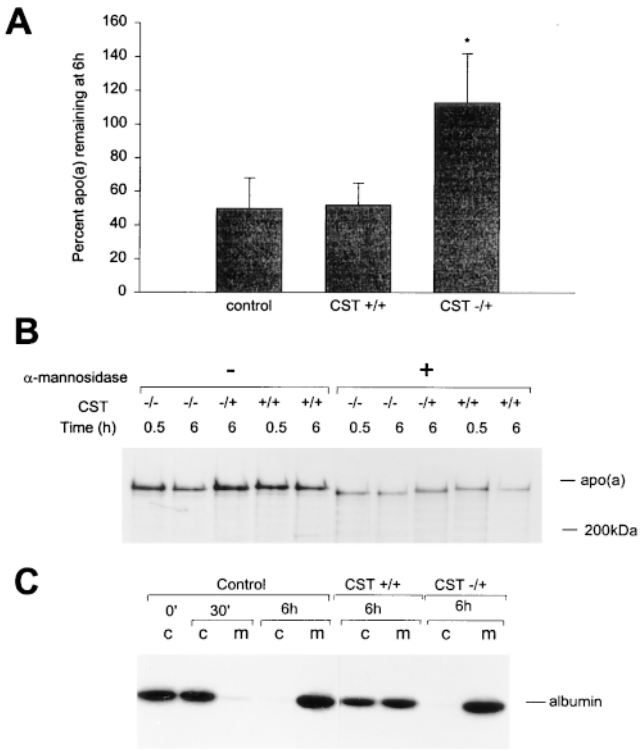

Fig. 6. Role of N-linked carbohydrate side chains in apo[a] degradation. A: Hepatocytes expressing null apo[a] were labeled for 15 min and chased for 0.5 or 6 h in the presence of no addition (control), or 1 mm CST; +/+ indicates that CST was present before, during, and after the labeling period (co-translational addition); −/+ indicates CST was present during the chase period only (post-translational addition). Results were quantified as described in the legend to Fig. 1B. Error bars show standard deviation. B: Cells were treated exactly as described for A: Apo[a] was then immunoprecipitated from the cell lysates and then analyzed by 3–10% SDS-PAGE, with or without prior treatment with jack bean α-mannosidase, as described in Experimental Procedures. The positions of apo[a] and myosin (200 kDa) are indicated. C: Albumin was immunoprecipitated from the cells (c) and media (m) of cultures treated as described above and analyzed by SDS-PAGE. An additional cell sample (0′), harvested immediately after the pulse, was also analyzed.

Role of ER chaperone proteins in apo[a] intracellular degradation

The ER chaperone, calnexin, binds the majority of its substrate proteins through specific interaction with monoglucosylated N-linked carbohydrate side chains, as part of the deglucosylation / reglucosylation cycle described above. Once a protein has reached its folded conformation, it is no longer a substrate for UDP-glucose:glycoprotein glucosyltransferase, calnexin no longer binds, and the protein can continue down the secretory pathway (50). As well as playing a role in protein folding and ER retention, calnexin has been proposed to either protect (46, 47) or target (51, 52) proteins for intracellular degradation. Our results with CST suggested that calnexin may play a role in regulating the intracellular degradation of apo[a]. To investigate this issue, the interaction of apo[a] with calnexin under conditions of altered carbohydrate processing was examined by co-immunoprecipitation in pulse-chase experiments (**Fig. 7** and **Fig. 8A**).

In control cells, after a 15-min pulse and either a 30-min or 6-h chase, 13% and 9% of apo[a] co-immunoprecipitated with calnexin, respectively (Fig. 7 and Fig. 8A). Lactacystin had no influence on the proportion of apo[a] associated with calnexin, although it clearly prevented apo[a] degradation in this experiment (Fig. 7). Surprisingly, BFA enhanced the association of apo[a] with calnexin to 161% and 138% of the 30-min control value, at 30 min and 6 h, respectively. As predicted, when either tunicamycin (TM), which prevents addition of N-linked glycans, or CST was added to the cells before and after labeling, the association of radiolabeled apo[a] with calnexin was prevented. Conversely, when CST was included only in the chase period, association of apo[a] with calnexin was markedly increased, to 254% and 223% of the 30-min control, at 30 min and 6 h of chase, respectively. In these latter CST-treated samples, 33% and 29% of apo[a] co-precipitated with calnexin at 30 min and 6 h (Fig. 8A). Due to technical reasons, this is likely to be an underestimate of the extent of apo[a]–calnexin interaction. Apo[a] did not co-precipitate with a preimmune serum control under any condition (Fig. 7), demonstrating the specificity of the co-precipitation reaction. This experiment confirms that calnexin interacts with apo[a] primarily through recognition of mono-

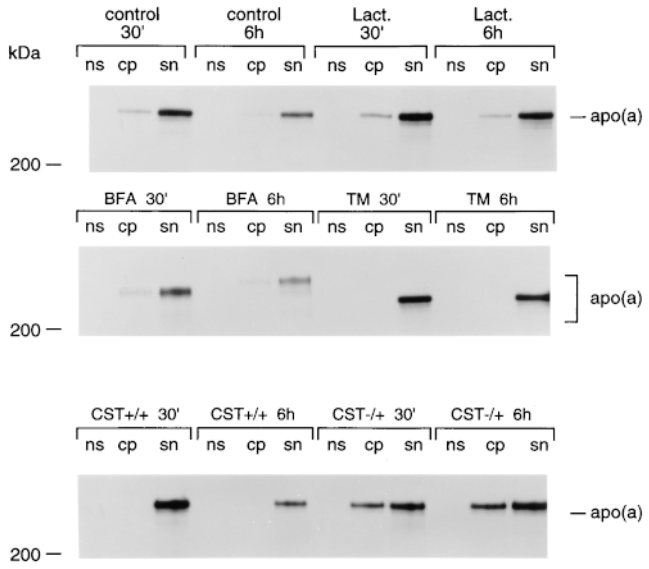

Fig. 7. Role of N-linked carbohydrate in the association of apo[a] with the ER chaperone, calnexin. Hepatocytes expressing the null apo[a] protein were labeled for 15 min and chased for 30 min or 6 h in the absence (control) or presence of 10 μm lactacystin (lact.), 10 μg/ml BFA, 10 μg/ml TM, or 1 mm CST; +/+ indicates that CST was present in all incubations; −/+ that CST was present only during the chase. Aliquots of the cell lysates were then immunoprecipitated with normal rabbit serum or with anti-calnexin antibody, and then apo[a] in the normal serum co-precipitate (ns) calnexin co-precipitate (cp) and that remaining in the ns supernatant (sn) was analyzed by immunoprecipitation and SDS-PAGE, as described in Experimental Procedures. The positions of apo[a] and myosin (200 kDa) are indicated.

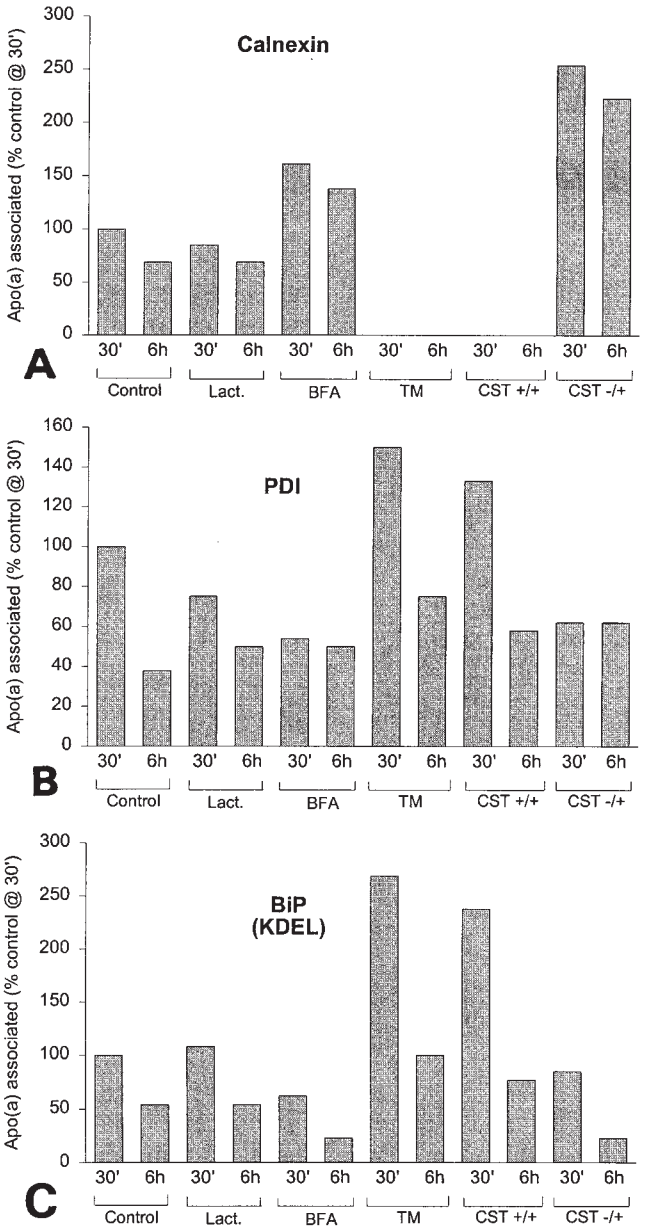

Fig. 8. Role of N-linked carbohydrate in the association of apo[a] with calnexin, PDI, and BiP. Cells expressing the null apo[a] protein were labeled for 15 min and chased for 30 min or 6 h in the presence of various compounds, exactly as described in the legend to Fig. 7. Cell lysates were then immunoprecipitated with anti-calnexin (A), anti-PDI (B), or anti-KDEL (C) antibodies. Apo[a] that co-precipitated with these antibodies and apo[a] that remained in the supernatant were then analyzed by immunoprecipitation and SDS-PAGE. The anti-KDEL antibody used in this experiment immunoprecipitates both BiP and Grp94. Apo[a] in each sample was quantified by densitometric scanning of autoradiographs. Results are expressed as the % of apo[a] in the total sample that co-precipitated with the chaperones under each condition, with the association at 30 min in control cells taken as representing 100%. Panel A represents quantitation of the autoradiographs shown in Fig. 7.

glucosylated side chains on apo[a], and suggests that increasing interaction with calnexin can protect apo[a] from degradation.

The association of apo[a] with the ER chaperone BiP (grp78, immunoglobulin heavy chain binding protein) and with PDI, a chaperone protein which also catalyzes the formation and isomerization of disulfide bonds in the ER (53), was also examined by co-immunoprecipitation (Fig. 8B and C).

Apo[a] co-precipitated with these chaperone proteins at both 30 min and 6 h of chase (Figs. 8B and C). Lactacystin again had very little influence on the extent of apo[a] association with these chaperones (Figs. 8B and C). In contrast to calnexin, however, BFA reduced the extent of apo[a] association with both PDI and BiP, whereas TM and CST, when present both before and after labeling, caused a marked increase in association, particularly at the 30-min chase time. Again in contrast to calnexin, post-translational addition of CST decreased the association of apo[a] with BiP and PDI (Fig. 8).

DISCUSSION

The portion of newly synthesized apo[a] protein targeted to the secretory versus the intracellular degradation pathways of the liver is influenced by allelic variation at the apo[a] locus and plays a role in determining inter-individual differences in Lp[a] production rate. In this study, we investigated the proteolytic mechanism responsible for apo[a] presecretory degradation and the factors

that regulate entry of apo[a] into this pathway. The results demonstrate that apo[a] degradation is mediated by the cytoplasmic proteasome, and can be prevented by conditions that enhance the interaction of apo[a] with the ER chaperone, calnexin. These studies support the emerging role of the proteasome as a major component of the ER quality control machinery and expand this role to one which contributes to the determination of plasma Lp[a] levels.

Intracellular degradation of a secretion-defective (null) apo[a] protein was almost entirely prevented by the highly specific proteasome inhibitor, lactacystin. Lactacystin also inhibited the degradation of a secreted apo[a] isoform and, as for null apo[a], caused accumulation of the precursor form of this protein inside the cell. This suggests that the portion of the secreted apo[a] isoform targeted for degradation is degraded by a similar pathway to null apo[a]. In contrast to the null apo[a] protein, however, lactacystin increased the proportion of the secreted isoform recovered in the culture medium. This suggests that when its degradation is inhibited, the efficiency with which apo[a] undergoes maturation can be increased. An alternative explanation is that a second proteasome-dependent degradation pathway exists for apo[a] which normally degrades a portion of the mature protein. Further studies will be required to distinguish between these possibilities. In either case, the proteasome clearly plays an important role in determining the rate of apo[a] secretion from hepatocytes.

In many cases, proteasome substrates are targeted for degradation through conjugation to multiple copies of the 76 amino acid protein, ubiquitin (39). Sequestration in the ER of the lumenal domains of transmembrane proteins and of soluble secretory proteins, such as apo[a], presents a barrier for access to the ubiquitin–proteasome pathway. Retrotranslocation from the ER lumen to the cytosol has been demonstrated as a mechanism for presentation of these protein substrates to the proteasome (36–38). The translocon, the protein-conducting channel also responsible for the co-translational translocation of proteins into the ER, has been demonstrated to play a role in the retrotranslocation process (36, 38). High molecular weight apo[a]–ubiquitin complexes were detected in cell lysates of hepatocytes transfected with an HA-tagged ubiquitin construct and after treatment with lactacystin. Thus, apo[a] appears to be targeted to the proteasome through conjugation to ubiquitin. However, the proportion of apo[a] ubiquitinated appeared to be small as the vast majority of apo[a] analyzed by Western blot (data not shown) or immunoprecipitation did not show an increase in molecular weight in the presence of lactacystin. In addition, trypsin digestion of permeabilized cells demonstrated that apo[a] accumulated primarily in the lumen of the secretory pathway in the presence of lactacystin, whereas the ubiquitin conjugation enzymes reside on the cytosolic side of the ER membrane. These data are similar to those reported for mutated carboxypeptidase Y (CPY) in yeast, which is degraded by the ubiquitin–proteasome pathway (54). The data may suggest that once apo[a] is translocated to the cytosol it is ubiquitinated and degraded very rapidly, making detection of ubiquitinated apo[a] difficult. If the proteasome itself is involved in the retrotranslocation process, blocking its action with lactacystin may inhibit translocation and prevent accumulation of cytosolic intermediates. Studies by Mayer, Braun, and Jentoch (55) also suggested a role for the proteasome in mediating retrotranslocation of mutated forms of SEC62 protein in yeast for degradation. Alternatively, it is possible that only a portion of apo[a] is degraded by this pathway and that a second proteasome-dependent degradation pathway for apo[a] exists in the lumen of the ER. Studies are currently underway to resolve this issue.

Our observation that intracellular apo[a] is largely sequestered in the lumen of the ER is in contrast to studies recently published by Nassir, Bonen, and Davidson (56), who concluded that a large portion of newly synthesized apo[a] was exposed to the cytosol due to incomplete co-translational translocation. This conclusion was based on proteinase K digestion of microsome preparations. However, in that study, no ER lumenal proteins were examined to control for the integrity of the membranes. In addition, apo[a] accessible to proteinase K was of the same molecular weight as that protected from the enzyme. However, untranslocated apo[a] would not be glycosylated, and thus should be easily distinguished from translocated, glycosylated apo[a] due to a large difference in molecular weight (29). Their "cystosolic" apo[a] is also unlikely to represent apo[a] retrotranslocated back across the membrane for degradation, as the apo[a] isoform studied was very efficiently secreted (56).

Apo[a] that accumulated intracellularly in the presence of lactacystin remained sensitive to endoH digestion. This provides strong evidence that apo[a] degradation takes place prior to movement of apo[a] into the medial-Golgi apparatus, where resistance to endoH is acquired (44). BFA, however, which prevents ER to Golgi transport, and has been used extensively to demonstrate a role for the ER in protein degradation (41, 57), protected apo[a] from degradation in a reversible manner, suggesting that movement out of the ER is required for apo[a] degradation to occur. The 2-h lag after synthesis before apo[a] degradation occurred is consistent with such a requirement. Raposo et al. (43) recently demonstrated accumulation of misfolded MHC class 1 molecules in an expanded intermediate compartment (located between the ER and Golgi apparatus), which was associated with components of the ubiquitin degradation system. Thus, more than one pathway of proteasome-mediated degradation may exist in the secretory pathway, one that occurs rapidly after protein synthesis, and a second that involves degradation of proteins in a compartment distal to the ER. Apo[a] may be a substrate for the latter degradation pathway. A formal possibility, however, is that BFA prevents apo[a] degradation through alteration of the architecture of the ER. We are currently pursuing studies to define the precise location in the secretory pathway where apo[a] is targeted to degradation.

Association with calnexin targets some ER-associated degradation substrates to the proteasome (51, 52), whereas

it protects others from degradation (46, 47). Calnexin binds to substrates primarily through interaction with monoglucosylated N-linked side chains, as part of a deglucosylation/reglucosylation cycle (50). In the current study, we demonstrated that newly synthesized apo[a] associates with calnexin in the ER. This interaction was prevented by inhibition of apo[a] N-linked glycosylation or by inhibition of the co-translational removal of N-linked glucose residues. Conversely, accumulation of apo[a] in its monoglucosylated form enhanced its interaction with calnexin.

Apo[a] degradation was unaffected when its interaction with calnexin was prevented and lactacystin protected apo[a] under these conditions, suggesting that calnexin is not required for delivery of apo[a] to the proteasome. In contrast, when apo[a]–calnexin interaction was enhanced, apo[a] degradation was prevented. Apo[a] secretion was also prevented under these conditions (data not shown). Thus, calnexin may protect apo[a] from degradation through sequestration from the proteolytic machinery, perhaps by retaining apo[a] in the ER. At least two other ER chaperone proteins, calreticulin (58) and Erp57 (59), interact primarily with monoglucosylated substrates, however, thus a role for these chaperones in protecting apo[a] cannot be ruled out.

The apo[a] glycoprotein has a highly repetitive and complex structure and is presumably targeted for presecretory degradation due to misfolding. The presumed folding defects are subtle, however, as null and secreted apo[a] proteins follow very similar folding pathways (29). For most allelic variants, apo[a] folding appears complete 60 min after synthesis, yet secretion and/or degradation may not begin until 2 h. We hypothesized that misfolded domains in apo[a] continue to unfold and refold, until a correct conformation is reached and secretion takes place, or until apo[a] is targeted to the degradation pathway. Consistent with this hypothesis are our findings that inhibition of degradation can increase apo[a] secretion (with the exception of null apo[a] proteins, which presumably possess a mutation precluding correct folding), and that apo[a] remains associated with chaperone proteins throughout its time in the ER. Chaperone interaction may also function to prevent secretion of misfolded apo[a]. The signal for entry of apo[a] into the degradation pathway, the intracellular site of degradation, and the precise role that the proteasome plays in this process, are issues which will be addressed in future investigations.

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