Role of Calnexin, Calreticulin, and Endoplasmic Reticulum Mannosidase I in Apolipoprotein(a) Intracellular Targeting[†]

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ABSTRACT: Apolipoprotein(a) [apo(a)] is a component of atherogenic lipoprotein(a) [Lp(a)]. Differences in the extent of endoplasmic reticulum (ER) associated degradation (ERAD) of apo(a) allelic variants contribute to the >1000-fold variation in plasma Lp(a) levels. Using human apo(a) transgenic mouse hepatocytes, we analyzed the role of the ER chaperones calnexin (CNX) and calreticulin (CRT), and ER mannosidase I in apo(a) intracellular targeting. Co-immunoprecipitation and pulse−chase analyses revealed similar kinetics of apo(a) interaction with CNX and CRT, peaking 15−30 min after apo(a) synthesis. Trapping of apo(a) N-linked glycans in their monoglucosylated form, by posttranslational inhibition of ER glucosidase activity with castanospermine (CST), enhanced apo(a)−CNX/CRT interaction and prevented both apo(a) secretion and ERAD. Delay of CST addition until 20 or 30 min after apo(a) synthesis [when no apo(a) had yet undergone degradation or Golgi-specific carbohydrate modification] allowed a portion of apo(a) to be secreted or degraded. These results are consistent with a transient apo(a)−CNX/CRT association and suggest that events downstream of CNX/CRT interaction determine apo(a) intracellular targeting. Inhibition of ER mannosidase I with deoxymannojirimycin or kifunensine had no effect on apo(a) secretion, but inhibited proteasome-mediated apo(a) ERAD even under conditions where apo(a)−CNX/CRT interaction was prevented. These results suggest a role for an additional, mannose-specific, ER lectin in targeting secretory proteins to the proteasome for destruction.

Lipoprotein(a) [Lp(a)][1] is an unusual lipoprotein (*1*) found in only humans, Old World primates (*2*), and the hedgehog (*3*). Lp(a) essentially consists of low-density lipoprotein (LDL) in which apolipoprotein (apo) B100, the sole protein component of LDL, is attached to an additional glycoprotein, apo(a), by a disulfide bond (*1*). Apo(a) is synthesized by the liver (*4*−*6*). Most (*7*−*10*), although not all (*11*, *12*), studies support that the association of apo(a) with apoB to form Lp(a) occurs after apo(a) has been secreted by the hepatocyte. Inheritance at the highly polymorphic apo(a) gene locus determines a greater than 1000-fold interindividual variation in plasma levels of Lp(a) (*13*). High Lp(a) levels (>30 mg/dL) are associated with an increased incidence of cardiovascular disease (*14*).

We are interested in defining the molecular determinants of apo(a) intracellular targeting, since allelic differences in the extent of apo(a) ER-associated degradation (ERAD) have a major impact on the plasma levels of Lp(a) (*15*, *16*). More than 30 size isoforms of apo(a) are found in human plasma (*17*−*19*) due to variation in the number (from ∼12 to 51) of plasminogen kringle 4 (K4)-like domains encoded in the apo(a) gene (*20*). Each K4 domain in apo(a) is posttranslationally modified in the ER, forming three disulfide bonds and acquiring an N-linked glycan. Apo(a) folding requires a comparatively extended time period (30−60 min) (*21*), and apo(a) interacts with multiple ER chaperone proteins (*16*). Large apo(a) proteins have longer ER residence times and tend to be subject to more ERAD than small apo(a) isoforms (*15*, *16*, *22*), accounting for the inverse correlation between apo(a) size and plasma Lp(a) levels (*17*, *18*). Other amino acid sequence variations can also influence the extent of apo(a) ER retention and degradation (*15*, *21*, *23*). These unique properties make apo(a) a valuable tool with which to analyze the mechanisms of protein processing and quality control in the ER.

ERAD of apo(a) is mediated by the cytoplasmic proteasome (*16*, *24*). A pathway has recently been elucidated whereby misfolded secretory and ER transmembrane proteins are targeted for proteasome-mediated destruction via retrotranslocation across the ER membrane (*25* and references cited therein). The precise mechanisms responsible for recognition and targeting of ERAD substrates for retrotranslocation have not been determined, however, but it seems likely that more than one pathway exists. For some proteins, such as mutant forms of α_1-antitrypsin (α_1AT), binding by

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[1] Abbreviations: Lp(a), lipoprotein(a); 6AHA, 6-aminohexanoic acid; α_1-AT, α_1-antitrypsin; apo, apolipoprotein; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CNX, calnexin; CRT, calreticulin; CST, castanospermine; dMNN, deoxymannojirrimycin; ER, endoplasmic reticulum; ERAD, ER-associated degradation; K, kringle; LDL, low-density lipoprotein; PAGE, polyacrylamide gel electrophoresis; SFM, serum-free medium.

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the ER chaperone calnexin (CNX), which primarily recognizes substrates containing monoglucosylated N-linked glycans (*26*), is essential for targeting to ERAD (*27, 28*). Conversely, in other instances, CNX interaction protects proteins from ERAD (*29*−*31*). Apo(a) falls into the latter category; conditions that prevent formation of monoglucosylated N-linked glycans on apo(a) prevent apo(a)−CNX interaction yet do not inhibit apo(a) ERAD. Conversely, trapping apo(a) in its monoglucosylated form enhances the interaction with CNX, prevents both apo(a) secretion and ERAD, and sequesters apo(a) in the ER lumen (*16, 24*). In addition, while the Sec61 translocon has been implicated in mediating the retrotranslocation of ERAD substrates (*25*), targeting of apo(a), and perhaps a subset of other proteins, to the proteasome may involve a transport step out of the ER; Brefeldin A, which prevents ER to Golgi transport (*32*), inhibits apo(a) degradation (*16*). Apo(a) that accumulates intracellularly in the presence of proteasome inhibitors, however, remains sensitive to endoglycosidase H digestion, suggesting that degradation occurs prior to the medial Golgi (*16*). The ER−Golgi intermediate compartment (ERGIC) has been implicated in the proteasome-mediated degradation of misfolded MHC class I molecules (*33*).

ER mannosidase processing of N-linked glycans also plays a role in targeting proteins to ERAD (*28, 34*−*37*). In the case of mutant forms of α_1AT, trimming by ER mannosidase I is thought to promote degradation by enhancing the interaction of α_1AT with CNX (*28*). However, it is not clear that this represents a general model since, in yeast, mannosidase processing enhances ERAD independently of the presence of CNX (*35*). In addition, the role of ER mannosidase in ERAD of proteins such as apo(a), whose degradation is independent of CNX interaction, is unclear.

We have recently characterized Lp(a) production in primary cultures of hepatocytes derived from mice transgenic for a 17 K4 human apo(a) isoform and for human apoB (*38*). This model system provides the opportunity to analyze human apo(a) secretion and Lp(a) formation in a primary hepatocyte background. The characteristics of apo(a) synthesis, secretion, and assembly with apoB to form Lp(a) in mouse hepatocyte cultures are similar to those described for endogenous apo(a) in baboon hepatocytes (*24, 38, 39*). In the current study, we utilized Lp(a) transgenic hepatocytes to further investigate the precise roles of CNX, the related ER chaperone, calreticulin (CRT), and N-linked glycan processing in apo(a) intracellular targeting.

EXPERIMENTAL PROCEDURES

Materials. Expre^{35}S^{35}S label was from DuPont NEN. Protein A agarose was from Repligen Corp. (Cambridge, MA). Rabbit anti-human Lp(a) was from Cortex Biochem (San Leandro, CA). Rabbit anti-calnexin and anti-calreticulin antibodies were from StressGen Biotechnologies Corp. (Victoria, BC, Canada). Castanospermine (CST) and deoxymannnojirimycin (dMNM) were from Roche Molecular Biochemicals. Kifunensine and swainsonine were kindly provided by Dr. Mark Lehrman. All other reagents were of analytical grade.

Hepatocyte Isolation and Culture. Mouse hepatocytes were isolated and cultured in a serum-free medium (SFM) formulation exactly as described previously (*38*). Donor mice (*40*) (kindly provided by Dr. Helen Hobbs) were hemizygous for a human apo(a) cDNA encoding 17K4 domains, and either homozygous or hemizygous for a yeast artificial chromosome encoding full-length human apoB. Throughout the manuscript, these mice are referred to as Lp(a) transgenic mice. All experiments were performed with cells that had been in culture for 48−72h.

Radiolabeling, Immunoprecipitation, and SDS−*PAGE*. To examine the association of apo(a) with CNX and CRT, cells were preincubated for 1 h in methionine- and cysteine-free SFM, labeled for 15 min with the same medium supplemented with 125 μCi/mL Expre^{35}S^{35}S label, and then chased for between 0 and 120 min in complete SFM. The cells were then placed on ice and washed twice with ice-cold PBS containing 20 mM *N*-ethylmaleimide (NEM) to preserve apo(a) in its variously folded forms (*21*). The cells were then lysed in ice-cold extraction buffer (EB: 0.3% CHAPS, 200 mM NaCl, 50 mM Hepes, pH 7.4) and incubated with protein A Sepharose and antibodies to the chaperone proteins for 3 h at 4 °C. The protein A pellets were washed 3 times for 5 min with EB at 4 °C with shaking. Coprecipitated proteins were eluted in EB containing 10 mM EDTA and 1% Triton X-100 for 30 min at 37 °C, and then diluted 20-fold in buffer containing 1% Triton X-100, 0.3% CHAPS, 100 mM NaCl, 50 mM Tris, pH 9.0. Apo(a) was immunoprecipitated from the coprecipitates and the supernatants saved from each sample, and analyzed on 4−10% SDS−polyacrylamide gels, as described previously (*7*), except that a rabbit anti-Lp(a) antibody was used.

Analysis of Apo(a) Degradation. Apo(a) degradation under various culture conditions was analyzed as described previously (*16, 39*). Briefly, cells were labeled for 15 min as above then chased for between 30 min and 6 h in unlabeled media. Apo(a) in the cells and media at each time point was analyzed by immunoprecipitation and SDS−PAGE. Various compounds were added as detailed for individual experiments. The amount of apo(a) present at 30 min of chase was taken as an estimate of apo(a) synthesis [peak incorporation of counts into the apo(a) precursor is observed at this time point; refs *16, 38*]. The total amount of apo(a) (cells + media) remaining at 6 h of chase was then used to estimate the extent of apo(a) degradation. The long chase time point is required due to the long half-life of the apo(a) precursor (*16*).

Quantitation of Apo(a). Autoradiographs were scanned with an Arcus II or Duoscan T1200 desktop scanner (Agfa), and bands were quantified using Scanalytics ONE-Dscan software. Analysis of serially diluted samples demonstrated that this method was quantitative over at least a 16-fold range (data not shown).

RESULTS

All experiments were performed using primary hepatocytes isolated from mice transgenic for human apoB and a 17K4 form of human apo(a) [Lp(a) transgenic mice; see Experimental Procedures].

Kinetics of Apo(a) Interaction with Calnexin and Calreticulin. Previous studies demonstrated that apo(a) binds to both CNX and CRT in the ER (*16, 24*). To further examine the role of CNX and CRT in apo(a) intracellular targeting, we determined the kinetics of apo(a) interaction with the chaperones by co-immunoprecipitation in pulse−chase experiments (Figure 1).

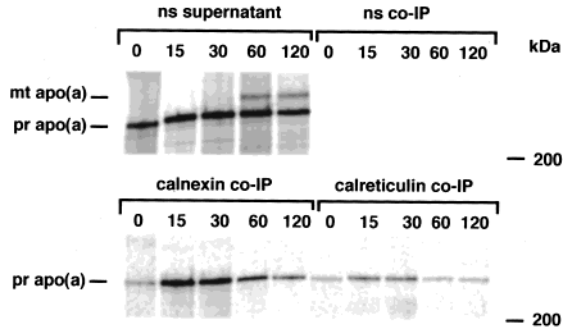

FIGURE 1: Kinetics of interaction of apo(a) with calnexin and calreticulin. Lp(a) transgenic hepatocytes were labeled for 15 min with [^{35}S]Met and [^{35}S]Cys, and chased for between 0 and 120 min in unlabeled medium. Cell lysates were immunoprecipitated with preimmune rabbit serum (ns) or with antibodies against calnexin or calreticulin. Coprecipitated proteins were eluted, and apo(a) in the coprecipitates (co-IP) and that remaining in the supernatants of the ns samples (ns supernatant) was analyzed by immunoprecipitation and 4−10% reducing SDS−PAGE. All techniques were performed as described under Experimental Procedures. The positions of the precursor (pr) and mature (mt) forms of apo(a) and of the 200 kDa molecular mass standard are indicated. Approximately 2-fold longer autoradiographic exposures are presented for the co-IP samples than for the supernatant samples. Results from one of three independent experiments are shown.

Cells were labeled for 15 min and chased for periods of up to 2 h (Figure 1). No apo(a) was coprecipitated from the cell lysates when preimmune serum was substituted for CNX and CRT antibodies at any time point analyzed (Figure 1; ns co-IP). In the supernatants of these samples, however, the apo(a) precursor was clearly present at the early chase times (0, 15, and 30 min), while at 60 and 120 min of chase the higher molecular weight, mature form of the protein was also apparent (Figure 1; ns supernatant). The increase in molecular weight of apo(a) on maturation is due to the addition of O-linked glycans and Golgi-specific modification of N-linked glycans after exit of the precursor from the ER (*7*).

The apo(a) precursor, but not mature apo(a), coprecipitated with both CNX and CRT at all chase times analyzed. In each case, the interaction peaked between 15 and 30 min of chase and by 60 min declined to a lower level (Figure 1; 2.4-fold reduction in the extent of association with each chaperone between 15 and 60 min of chase). Apo(a) requires between 30 and 60 min to fold after translocation into the ER lumen (*21*). The kinetics of apo(a) interaction with CNX and CRT are thus consistent with a role for these chaperones in apo(a) folding.

Apo(a) Is Targeted to ERAD after Release from CNX/CRT. Results in Figure 1 also suggested that the interaction of the apo(a) precursor with CNX and CRT was transient, since the extent of association markedly declined by 60 min of chase, yet the majority of apo(a) remained in its precursor form at this time point with only a small portion having undergone Golgi-specific modification (Figure 1) or degradation (Figure 1 and Figure 2E). This implies that targeting of apo(a) to either ERAD or maturation and secretion may occur after release of apo(a) from CNX/CRT.

To examine this issue, we determined the effect of the ER glucosidase inhibitor castanospermine (CST) on apo(a) secretion and intracellular degradation when added at various chase times in pulse−chase experiments. The rationale for this approach is illustrated in Figure 2A, which depicts the processing of N-linked glycans in the ER. CNX and CRT interact only with apo(a) possessing monoglucosylated N-linked glycans (see below and refs *16*, *24*). Monoglucosylated glycans can be produced through the co-translational

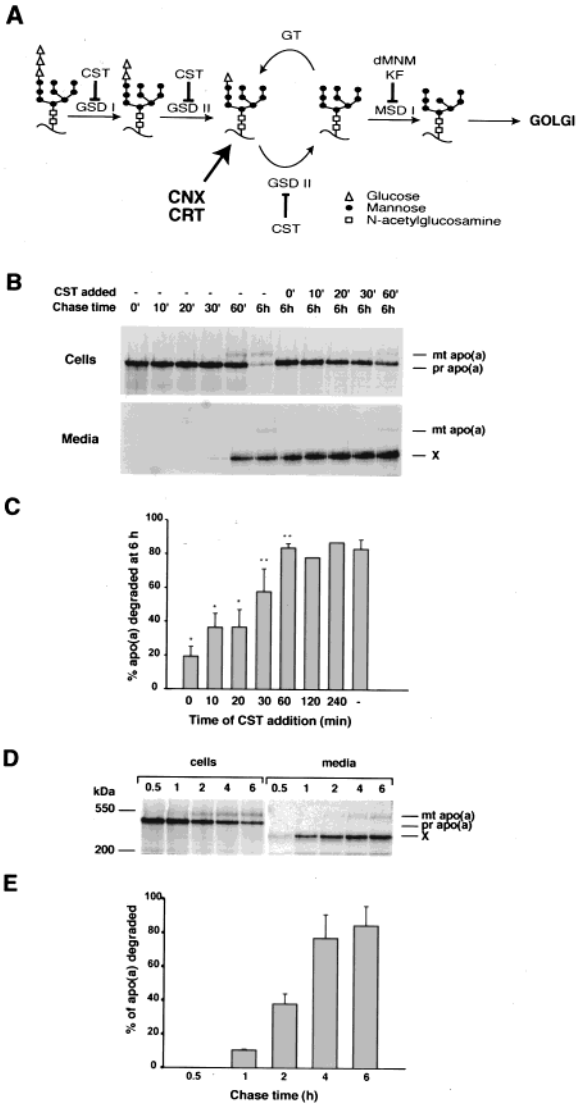

FIGURE 2: Effect of posttranslational addition of CST on apo(a) ERAD. (A) Schematic representation of N-linked carbohydrate processing in the ER. GSD, glucosidase; CST, castanospermine; GT, UDP-glucose:glycoprotein glucosyltransferase; MSD, mannosidase; dMNM, deoxymannojirimycin; KF, kifunensine. (B) Hepatocytes were labeled for 15 min and chased for the indicated times. CST (1 mM) was either not added (−) or added at various chase times, as indicated. Apo(a) was immunoprecipitated from the cells and media and analyzed by SDS−PAGE. The positions of the precursor (pr) and mature (mt) forms of apo(a) and of a protein nonspecifically precipitated from the culture media (X) (*7*) are indicated. (C) Hepatocytes were analyzed as in (B). The extent of apo(a) degradation at 6 h of chase under each condition was determined as described under Experimental Procedures. Results are presented as mean ± SE; n = at least 4 in each case, except for the 120 and 240 min samples where $n = 1$. *, $p < 0.01$ vs control (−) cells. **, $p < 0.05$ versus CST added at time 0. (D) Control cells (no inhibitors added) were labeled for 15 min and chased for between 0.5 and 6 h. Apo(a) was immunoprecipitated and analyzed by SDS−PAGE, as described under Experimental Procedures. (E) Cells were analyzed as in (D). The extent of apo(a) degradation at each time point was determined as described under Experimental Procedures. Results are presented as mean ± SE for 2 experiments.

removal of the outer two glucose residues from the nascent glycan chain by ER glucosidases I and II, or as part of a reglucosylation/deglucosylation cycle involving cyclic addition of a single glucose by UDP-glucose:glycoprotein glucosyltransferase to the fully deglucosylated side chain and the subsequent removal of this residue by ER glucosidase II (Figure 2A). When CST is added posttranslationally (i.e., to the chase only in a pulse−chase experiment), it can trap radiolabeled apo(a) in its monoglucosylated form by preventing the ER glucosidase II-mediated deglucosylation of monoglucosylated glycans (*16*). Under these conditions, interaction of apo(a) with CNX and CRT is enhanced, and both apo(a) secretion and degradation are inhibited (see below and refs *16*, *24*). We reasoned that if the interaction of apo(a) with CNX and CRT, and hence its association with the deglucosylation/reglucosylation cycle, was transient, then the ability of CST to inhibit apo(a) secretion and degradation should depend on the time of its addition after apo(a) synthesis.

To examine the effect of CST on apo(a) degradation, cells were labeled for 15 min and then chased for 6 h with CST added at 0, 10, 20, 30, 60, 120, or 240 min of chase. Parallel dishes of cells were harvested at each time point to determine the extent of apo(a) maturation and degradation at the point of CST addition (Figure 2B). The amount of apo(a) present in control cells at 30 min of chase was used as a measure of apo(a) synthesis. The total amount of apo(a) remaining (cells + media) at 6 h was used to determine the extent of degradation (see Experimental Procedures).

Under control conditions (no CST added), 83 ± 15% (mean ± SD, $n = 7$) of apo(a) synthesized during the 15 min pulse was degraded by 6 h of chase (Figure 2B,C). Consistent with previous observations (*16*, *39*), when CST was added immediately after the pulse (0 chase time), only 19 ± 15% of apo(a) was degraded by 6 h (Figure 2B,C). When addition of CST was delayed until 10, 20, or 30 min of chase, an intermediate amount of apo(a) degradation was observed (37 ± 18%, 37 ± 21%, and 58 ± 30%, respectively; Figure 2B,C). For the 30 min sample, significantly more apo(a) was degraded than in cells treated with CST at 0 min of chase. Apo(a) that accumulated in the presence of CST was in its intracellular, precursor form (Figure 2B). When CST addition was delayed until 60 min of chase or later, no significant effect on apo(a) degradation compared to control cells was observed (Figure 2B,C).

Consistent with previous studies (*16*), in control cells (no CST added at any time) only ~10% of apo(a) had undergone degradation by 60 min of chase (Figure 2D,E), and by 2 h less than 40% of apo(a) was degraded (Figure 2D,E). Thus, although we cannot be certain of the time required for CST to inhibit ER glucosidase activity after addition to the cells (the related inhibitor *N*-butyldeoxynojirimycin requires less than 5 min to inhibit ER glucosidase activity in CHO cells; ref *41*), degradation of apo(a) prior to CST action cannot explain the differential effect of CST on apo(a) ERAD when added at different chase times. These results therefore support a transient association of apo(a) with CNX and CRT and suggest that apo(a) is targeted to ERAD after release from CNX/CRT.

Apo(a) Released from CNX/CRT Can Also Be Targeted to Secretion. The effect of delayed CST addition on apo(a) secretion was difficult to assess in the above experiments due to the small amount of apo(a) that underwent maturation even in untreated cells (Figure 2B). To explore the influence of CST on apo(a) secretion, we examined its effect in the presence of the lysine analogue 6-aminohexanoic acid (6AHA). 6AHA acts as a chemical chaperone for apo(a), greatly increasing its efficiency of secretion and decreasing apo(a) ERAD (*39*). However, 6AHA is unable to overcome the ER retention of apo(a) induced by posttranslational addition of CST (*39*). 6AHA was therefore used as a tool to accentuate differences in apo(a) secretion under the different culture conditions.

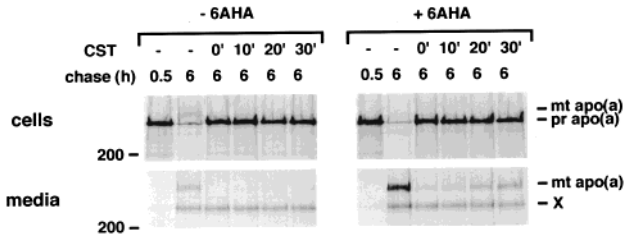

FIGURE 3: Effect of posttranslational addition of CST on apo(a) secretion. Hepatocytes were labeled for 15 min and chased for 0.5 or 6 h. CST (1 mM) was either not added (−), or added at 0, 10, 20, or 30 min of chase, as indicated. 6AHA (200 mM) was present in the chase media where indicated. Apo(a) in the cells and media was analyzed by immunoprecipitation and SDS−PAGE. The positions of the precursor (pr) and mature (mt) forms of apo(a), the 200 kDa molecular mass standard, and of a protein nonspecifically immunoprecipitated from the culture media (X) (*7*) are indicated.

Consistent with previous results (*39*), 6AHA increased apo(a) secretion from control cells 13.6-fold by 6 h of chase, but was unable to overcome the block in secretion induced by addition of CST to the cells at the 0 chase time (Figure 3). However, when CST addition was delayed until 20 or 30 min of chase, a portion of apo(a) was secreted by 6 h in 6AHA-treated cells (Figure 3) [when CST was added at 30 min, 2.4-fold more apo(a) was secreted in the presence of 6AHA than from control cells with neither CST nor 6AHA added]. Addition of CST to the chase also caused partial inhibition of apo(a) degradation in this experiment, as evidenced by the increased amount of the apo(a) precursor remaining in the cells at 6 h of chase in comparison to control cultures (Figure 3). The differences in secretion could not be explained by differences in the rate of movement of apo(a) out of the ER between control and 6AHA-treated cultures, since even in the presence of the lysine analogue no apo(a) had yet undergone maturation by 30 min of chase (Figure 3). These results are again consistent with a transient association of apo(a) with the deglucosylation/reglucosylation cycle.

The results in Figures 1−3 suggest that the interaction of the apo(a) precursor with CNX and CRT is transient, and that once released from these chaperones apo(a) can be targeted to either secretion or degradation. Thus, a quality control mechanism downstream of CNX/CRT interaction is involved in intracellular retention of apo(a) and in regulating apo(a) targeting in the secretory pathway.

ER Mannosidase Activity Enhances Apo(a) ERAD. To examine the potential role of ER mannosidase processing in apo(a) intracellular targeting, we determined the effect of the ER mannosidase inhibitor deoxymannojirimycin (dMNM) on apo(a) interaction with CNX and CRT (Figure 4A) and

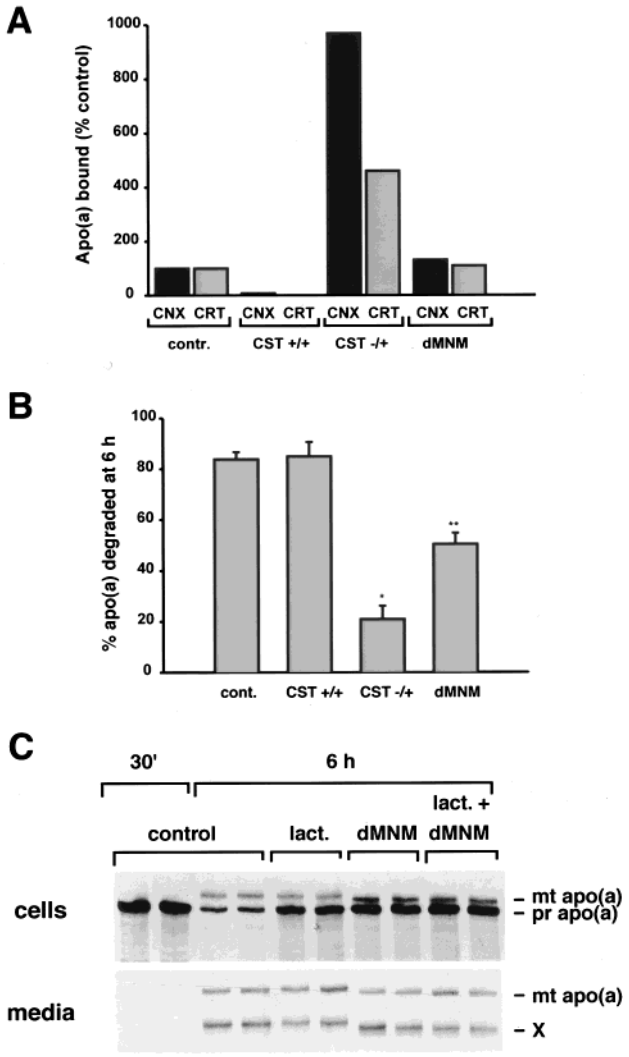

FIGURE 4: Role of ER mannosidase activity in apo(a) ERAD. (A) Hepatocytes were labeled for 15 min and chased for 30 min in the absence of added inhibitors (contr.), with 1 mM CST added to the preincubation (1 h), labeling, and chase media (CST +/+), with 1 mM CST added to the chase only (CST −/+), or with 2 mM dMNM added to all incubations. Association of apo(a) with CNX and CRT in each sample was analyzed as in Figure 1. Results are presented as the relative extent of association of apo(a) with CNX and CRT, with the amount in control cells taken as 100%. (B) Hepatocytes were labeled for 15 min and chased for 6 h with inhibitors added as in (A). The extent of apo(a) degradation under each condition was determined as described under Experimental Procedures. *, $p = 4 \times 10^{-10}$ versus control cells; **, $p = 2 \times 10^{-6}$ versus control cells. (C) Hepatocytes were labeled for 15 min and then chased for 30 min or 6 h under control conditions, in the presence of 10 μM lactacystin (lact.), 2 mM dMNM, or both lactacystin and dMNM. Apo(a) in the cells and media was analyzed by immunoprecipitation and SDS−PAGE. The positions of the precursor (pr) and mature (mt) forms of apo(a) and a protein nonspecifically immunoprecipitated from the culture media (X) are indicated. Inhibition of mannosidase activity resulted in a small decrease in the molecular weight of mature apo(a).

on apo(a) intracellular degradation (Figure 4B). In each case, CST-treated cells were examined as controls.

As previously observed (*16*), when CST was added both before and after labeling in pulse−chase experiments to trap radiolabeled apo(a) in its triglucosylated form (Figure 2A), interaction of apo(a) with both CNX and CRT was prevented (Figure 4A). Apo(a) degradation was unaffected under these conditions (Figure 4B). Conversely, when CST was added

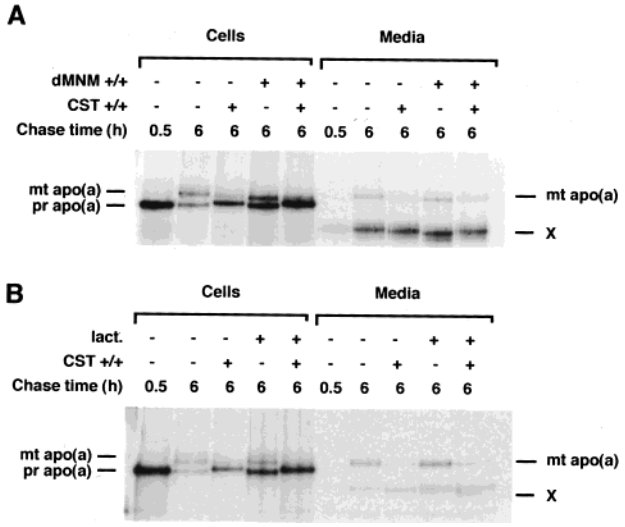

FIGURE 5: Role of ER mannosidase in apo(a) ERAD is independent of apo(a)−CNX/CRT interaction. (A) Hepatocytes were labeled for 15 min and chased for 0.5 or 6 h, as described under Experimental Procedures. Where indicated, CST (1 mM) and/or dMNM (2 mM) were added before (for 1 h), during, and after labeling. (B) Hepatocytes were labeled for 15 min and chased for 0.5 or 6 h. Where indicated, CST (1 mM) was added before during and after labeling, or 10 μM lactacystin (lact.) was added to the chase only. Apo(a) was immunoprecipitated from the cells and media and analyzed by SDS−PAGE. The positions of the precursor (pr) and mature (mt) forms of apo(a) and of a protein nonspecifically precipitated from medium samples (X) are indicated.

only to the chase to trap apo(a) in its monoglucosylated form (Figure 2A), a large increase in apo(a)−CNX/CRT interaction was observed (Figure 4A). Under these conditions, apo(a) degradation was largely prevented (Figure 4B). Inhibition of ER mannosidase activity with dMNM had no discernible effect on apo(a) interaction with CNX or CRT (Figure 4A). However, a significant decrease in apo(a) degradation was observed [50 ± 12% of apo(a) degraded at 6 h versus 84 ± 10% in control cells; $p < 0.00001$] (Figure 4B). This was reflected in an increase in the amount of apo(a) precursor remaining in the cells at 6 h, and no increase in apo(a) secretion was observed (Figure 4C and data not shown). Thus, as documented for a number of other ERAD substrates (*28*, *34*−*37*), ER mannosidase activity appears to enhance apo(a) degradation.

Experiments in which both dMNM and the proteasome inhibitor lactacystin were added to cells confirmed that dMNM inhibited proteasome-mediated degradation of apo(a), as the effects of dMNM and lactacystin on apo(a) degradation were not additive [Figure 4C; % apo(a) degraded in control cells, cells treated with lactacystin alone, dMNM alone, or both lactacystin and dMNM = 93, 61, 37, and 35, respectively].

ER Mannosidase Enhances Apo(a) ERAD Independently of CNX Interaction. Results in Figure 4A,B suggested that dMNM inhibited apo(a) degradation independently of any effect on apo(a)−CNX association. To confirm this, we analyzed the effect of dMNM on apo(a) degradation in cells that had been treated with CST to accumulate apo(a) in its triglucosylated form and prevent interaction with CNX and CRT (Figure 5A). Under control conditions (no inhibitors added), 85% of apo(a) was degraded after a 6 h chase (Figure 5A). When CST was added to prevent removal of the three glucose residues from apo(a) N-linked glycans, 71% of

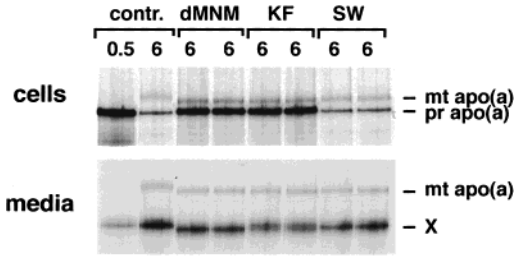

FIGURE 6: ER mannosidase I promotes apo(a) ERAD. Hepatocytes were labeled for 15 min and chased for 0.5 or 6 h as indicated, under control conditions (contr.) or with 2 mM dMNM, 1 μg/mL kifunensine (KF), or 2 μg/mL swainsonine (SW) added to the chase media only. Apo(a) in the cells and media was analyzed by immunoprecipitation and SDS−PAGE, as described under Experimental Procedures. The positions of the precursor (pr) and mature (mt) forms of apo(a) and of a protein nonspecifically precipitated from medium samples (X) are indicated.

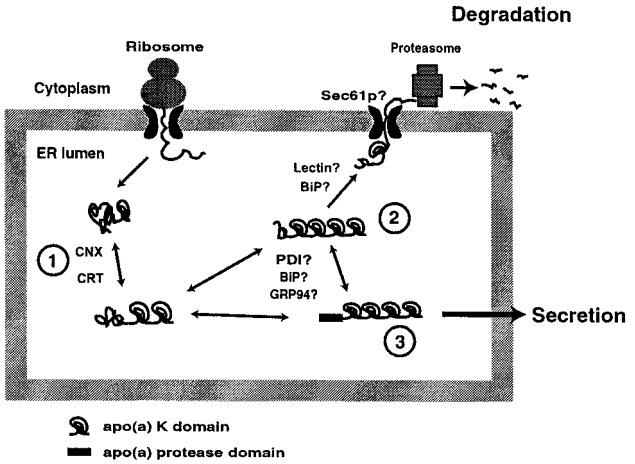

FIGURE 7: Schematic representation of apo(a) processing in the ER. Apo(a) is co-translationally translocated into the ER lumen, after which it passes through a series of folding intermediates (step 1). CNX and CRT play an important role in the folding process. However, the interaction of apo(a) with CNX and CRT is transient, and the decision to target apo(a) to ERAD (step 2) or secretion (step 3) occurs after release from CNX and CRT. Other chaperones, such as PDI, GRP94, and BiP, presumably play a role in completion of apo(a) folding and targeting to steps 2 and 3. A role for a Man_8-specific ER lectin in targeting apo(a) for retrotranslocation across the ER membrane is also indicated. The mechanism of apo(a) retrotranslocation and whether this involves the Sec61 protein channel or transport to a post-ER compartment (not indicated) are uncertain. Previous studies have demonstrated that a deletion in the protease domain of apo(a) is sufficient to cause complete ER retention and ERAD of apo(a) (*21*, *23*).

apo(a) was degraded by 6 h, and apo(a) maturation and secretion was almost entirely prevented (Figure 5A). dMNM inhibited apo(a) degradation in both control and CST-treated cells [15% and 5%, respectively, of apo(a) degraded by 6 h; Figure 5A]. In CST-treated cultures, the molecular weight of the apo(a) precursor was increased slightly, confirming retention of the glucose residues (Figure 5A). Thus, dMNM inhibited apo(a) degradation under conditions in which apo(a)−CNX interaction was prevented. Furthermore, we confirmed that degradation of triglucosylated apo(a) was mediated by the proteasome, as lactaycstin also inhibited apo(a) degradation in CST-treated cells (Figure 5B).

ER Mannosidase I Activity Enhances Apo(a) Degradation. The above results suggest that the role of ER mannosidase in apo(a) ERAD is distinct from that in α_1AT degradation. However, at least two separate mannosidase activities have been documented in the ER, both of which are inhibited by dMNM (*42*, *43*). ER mannosidase I is responsible for enhancing α_1AT degradation (*28*). To determine which ER mannosidase was involved in apo(a) degradation, we utilized the inhibitors kifunensine (KF) and swainsonine (SW) (Figure 6). KF selectively inhibits ER mannosidase I (*42*), whereas SW inhibits Golgi mannosidase II (*43*).

Both dMNM and kifunensine inhibited apo(a) degradation (for the experiment shown in Figure 6, 23 and 26% degradation by 6 h in the presence of dMNM and 27 and 22% in the presence of KF, compared to 75% in control cells), resulting in accumulation of the apo(a) precursor in cell lysates (Figure 6). In contrast, swainsonine had no effect on apo(a) degradation (81% degraded by 6 h; Figure 6). All three mannosidase inhibitors clearly affected apo(a) carbohydrate processing, however, since each produced an observable decrease in the molecular weight of mature apo(a) (Figure 6).

DISCUSSION

In the current study, we analyzed the roles of CNX, CRT, and ER mannosidase I in apo(a) intracellular targeting. Our results suggest the following model, as summarized in Figure 7. CNX and/or CRT appear to play an important role in apo(a) folding (step 1 in Figure 7) as conditions that prevent interaction with the chaperones largely prevent apo(a) secretion. However, the interaction of apo(a) with CNX/CRT is transient, and the ultimate decision to target apo(a) to degradation (step 2 Figure 7) or secretion (step 3 Figure 7) occurs after release from the chaperones. Other chaperones, such as GRP94, PDI, or BiP, each of which interacts with apo(a) (*16*), may be required to complete the apo(a) folding pathway and allow secretion to occur, or to ultimately target misfolded apo(a) to the degradation pathway. A role for an additional lectin in apo(a) intracellular targeting is suggested by the dependence of apo(a) degradation on ER mannosidase I processing. This effect is observed independently of apo(a)−CNX interaction, and may represent an activity required to target apo(a) for retrotranslocation across the ER membrane. The mechanism of apo(a) retrotranslocation and whether this involves the Sec61 protein channel or transport to a post-ER compartment (*16*) (not indicated) are uncertain.

Athough CNX and CRT can bind to misfolded proteins independently of the presence of carbohydrate (*44*, *45*), we found that apo(a) interaction with both CNX and CRT was dependent upon the presence of monoglucosylated N-linked glycans on apo(a) (refs *16*, *24* and Figure 4). The peak of apo(a) interaction with the chaperones occurred 15−30 min after apo(a) synthesis (Figure 1). These kinetics and the fact that apo(a) secretion is inhibited when its interaction with CNX and CRT is prevented (refs *16*, *24* and Figure 4) suggested an important role for these chaperones in apo(a) folding. Using a gel-based folding assay (*21*), however, we were unable to demonstrate any effect on apo(a) folding when apo(a) interaction with CNX and CRT was either enhanced or prevented by the co- or post-translational inhibition of glucosidase II (ref *21*, and data not shown). This may reflect an inability of the folding assay to detect disulfide bond-independent changes in conformation, or differences restricted to one or a few domains in apo(a) (*21*, *23*, *39*). More sensitive assays will be required to determine

the precise role of CNX and CRT in the apo(a) folding pathway. Due to the similar characteristics of apo(a) association with CNX and CRT, we were unable to distinguish the individual roles of these chaperones in apo(a) intracellular targeting.

The kinetics of apo(a) interaction with CNX and CRT (Figure 1) and the time-dependent effect of posttranslational addition of CST on apo(a) ER retention (Figures 2 and 3) suggested that the interaction of the apo(a) precursor with both CNX and CRT was transient. Furthermore, the results suggested that once released from CNX/CRT apo(a) could be targeted to either degradation (Figure 2) or secretion (Figure 3). Since apo(a) can be retained in a pre-medial Golgi compartment and targeted to proteasome-mediated degradation in the absence of CNX/CRT interaction (ref *21* and Figure 5), these results suggest that CNX and CRT are not required for targeting apo(a) to ERAD, or for intracellular retention of apo(a), and that these chaperones do not make the ultimate decision to allow apo(a) secretion to occur. It is possible that the dissociation of apo(a) from CNX and CRT reflects transport of apo(a) to a post-ER pre-medial Golgi compartment. Misfolded vesicular stomatitis virus G protein has been shown to cycle between the ER and ERGIC in association with BiP, but not CNX, which remains in the ER (*46*). In addition, BFA inhibits apo(a) degradation, yet apo(a) that accumulates in the presence of lactacystin remains endoH-sensitive (*21*). This may suggest a role for the ERGIC in apo(a) degradation, as has been demonstrated for misfolded MHC class I molecules (*33*). Alternatively, dissociation of apo(a) from CNX/CRT may reflect a conformational change in the apo(a) molecule such that it is no longer recognized by the ER glucosyl transferase enzyme (GT) or perhaps by the chaperones themselves (*44*, *45*). Further studies will be required to address this issue. Apo(a) interacts with a number of other ER chaperones (e.g., protein disulfide isomerase, GRP94, and BiP; ref *16*) that may play a role in apo(a) intracellular retention and targeting to degradation. BiP in particular has been implicated in targeting proteins for ERAD, retrotranslocation of ERAD substrates across the ER membrane, and retaining misfolded proteins in the ER (*47*−*49*). Preliminary studies suggest that the kinetics of apo(a) interaction with BiP and PDI are distinct from those of its interaction with CNX/CRT, with a constant proportion of the precursor coprecipitated with antibodies against these chaperones over time (data not shown). Studies are currently underway to address the role of these chaperones in apo(a) intracellular targeting.

A number of recent studies have demonstrated a role for ER mannosidase I in targeting proteins to ERAD (*28*, *34*−*37*). It has been suggested that this enzyme may act as a molecular clock, regulating the disposal of misfolded glycoproteins (*28*). Liu et al. (*28*) recently proposed that ER mannosidase I enhances ERAD of mutant α_1AT in mammalian cells by enhancing the interaction of α_1AT with CNX, which is required for targeting of α_1AT to ERAD. This is because glucosidase II shows reduced activity against the Man_8-containing glycans produced by ER mannosidase I, thus enhancing retention of monoglucosylated glycans on α_1AT and increasing CNX interaction. Similarly, we found that inhibition of ER mannosidase I inhibited apo(a) ERAD (Figures 4−6). In contrast to α_1AT, however, apo(a) ERAD is not dependent on CNX interaction. In addition, we found that ER mannosidase I enhanced proteasome-mediated degradation of apo(a) even under conditions where apo(a)−CNX interaction was prevented by treatment with CST (Figure 5). While this paper was in preparation, it was reported that ERAD of CD1b proteins in human cell lines and of mutant α_2-plasmin inhibitor in CHO cells is inhibited by dMNM in the presence of CST (*50*, *54*). Consistent with observations by Chung et al. (*54*), these findings suggest that ER mannosidase I is able to cleave $Glc_3Man_9GlcNAc_2$ to $Glc_3Man_8GlcNAc_2$. Although, to our knowledge, this activity has not been directly demonstrated for the mammalian enzyme, the orthologous yeast ER α1,2-mannosidase does possess this activity (*51*, *52*).

Similar to our findings, the role of yeast ER α1,2-mannosidase in ERAD is independent of the presence of the yeast CNX homologue (*35*). In addition, the presence of a single glucose residue on N-linked glycans was found to retard ERAD of mutant carboxypeptidase Y in yeast, a protein whose degradation is also enhanced by trimming of glycans to the Man_8 form (*37*). Other very recent studies also suggest that the role of ER mannosidase activity in ERAD is independent of CNX interaction (*53*, *54*). Together with our results and those of Liu et al. (*28*), these studies suggest pleiotropic roles for ER mannosidase in ERAD and imply a role for an additional ER lectin in targeting proteins to the degradation pathway. Since both secreted proteins and those targeted for ERAD are processed by ER mannosidase I, such a lectin must distinguish between folded and misfolded/unassembled proteins. As for the GT enzyme (*55*), this could represent an additional property of the lectin itself, or could be furnished by associated chaperone proteins.

The formal possibility exists that dMNM and KF inhibit apo(a) ERAD by competition for lectin binding rather than through their inhibition of ER mannosidase I. In addition, it is possible that the carbohydrate trimming events that target apo(a) for degradation occur during cycling of apo(a) between the cis-Golgi and the ER, since the Golgi mannosidase I enzymes are also inhibited by dMNM and KF. Studies in yeast that demonstrate a direct role for $Man_8GlcNac_2$ isomer B in ERAD (*37*) argue against these possibilities. However, direct analysis of the N-linked glycans on apo(a) that accumulates when its degradation is prevented by proteasome inhibitors will be required to address these contingencies.

In conclusion, our studies further define the roles of CNX and CRT in apo(a) intracellular targeting, and provide additional insights into the roles of these chaperones in ER quality control processes. Our studies also support a pleiotropic role for ER mannosidase I in targeting proteins to ERAD, and suggest that all the molecular components of the ERAD system have yet to be identified. Future studies will be designed to further delineate the precise molecular events regulating the intracellular targeting of atherogenic apo(a).

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