Role of N-linked glycans, chaperone interactions and proteasomes in the intracellular targeting of apolipoprotein(a)

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Introduction
The atherogenic lipoprotein, lipoprotein(a) (Lp(a)), consists of low-density lipoprotein (LDL) in which apolipoprotein (apo) B100, the sole protein component of LDL, is attached by a disulphide linkage to apo(a) [1]. Apo(a) is a large, highly heterogeneous glycoprotein that resembles plasminogen in structure. It contains multiple domains with homology with plasminogen kringle (K) 4, followed by domains homologous with the plasminogen K5 and protease domains [2]. As many as 34 different apo(a) size isoforms exist, from less than 300 to more than 800 kDa, owing to variation in the number of K4 domains (from 12 to more than 50) encoded in the apo(a) gene [3,4]. Additional polymorphisms independent of size are also present at the apo(a) locus; there are estimated to be more than 100 apo(a) alleles [5].

Plasma levels of Lp(a) vary over a 1000-fold range between individuals [6]. More than 90% of the variation is determined by polymorphism at the apo(a) gene locus [7]. Differences in Lp(a) levels between individuals are determined by differences in hepatic Lp(a) production rates [8,9]. Newly secreted apo(a) binds to the hepatocyte cell surface, where it assembles with LDL to form Lp(a) [10]. Several factors are likely to contribute to the characteristic secretion rate associated with each apo(a) allelic variant, including the rate of apo(a) gene transcription and/or apo(a) mRNA stability [11–13], and the rate of apo(a) mRNA translation [14]. Post-translational mechanisms also have an important role [15–18]. Here we summarize our recent studies to examine the role of endoplasmic reticulum (ER) chaperone proteins, N-linked glycosylation and proteasomes in determining the apo(a) secretion rate.

Post-translational mechanisms are major determinants of plasma Lp(a) levels
Studies done in collaboration with Dr. Robert Lanford with the use of primary cultures of baboon hepatocytes demonstrated that post-translational mechanisms have a major role in determining Lp(a) production rate [15–18]. Apo(a) is synthesized as a lower-molecular-mass precursor containing high-mannose, endoglycosidase H-sensitive N-linked glycans that has a prolonged residence time in the ER before maturation and secretion. Maturation involves Golgi-specific modification of N-linked glycans and the addition of O-linked carbohydrate side chains [15]. An analysis of hepatocytes from baboons expressing different apo(a) isoforms demonstrated that apo(a) allelic variants exhibit marked differences in the kinetics and efficiency with which they are transported out of the ER. Larger isoforms are retained longer and a greater portion of these isoforms is targeted for pre-secretory degradation [16,18]. This probably accounts for the uniformly low plasma Lp(a) levels associated with large isoforms [19]. Smaller apo(a) isoforms tend to be secreted more efficiently [and in general are associated with higher plasma Lp(a) levels] unless other polymorphisms are present that decrease apo(a) intracellular transport. For example, a large in-frame deletion was recently identified in the protease domain of a baboon apo(a) isoform [20] that completely prevents the transport of this protein out of the ER [17]. The efficiency of apo(a) transport out of the ER is therefore a major determinant of plasma Lp(a) levels.

We have used hepatocytes isolated from mice transgenic for a 17-K4 form of human apo(a) and for the entire human apoB gene (Lp(a) transgenic mice [21]), to examine the factors that determine the efficiency of apo(a) export from the ER. The characteristics of human apo(a) synthesis, folding, intracellular processing, secretion and assembly with apoB in transgenic mouse hepatocytes seem very similar to those described for baboon apo(a) [22]. However, in contrast with

Abbreviations used: 6AHA, 6-aminohexanoic acid; apo, apolipoprotein; CST, castanospermine; ER, endoplasmic reticulum; HA, influenza virus haemagglutinin; LDL, low-density lipoprotein; Lp(a), lipoprotein(a).

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baboon apo(a) proteins of similar size, the human apo(a) isoform expressed in the mouse hepatocytes was subjected to more extensive presecretory degradation [22,23].

**Presecretory degradation of apo(a) is mediated by the proteasome**

To determine the protease responsible for the presecretory degradation of apo(a), the effect of several different protease inhibitors on apo(a) intracellular stability was analysed in pulse–chase experiments. Hepatocytes were labelled for 15 min with 35S-labelled amino acids and then chased for 30 min [to measure apo(a) synthesis] or 6 h in the presence of the various protease inhibitors. In the experiment shown in Figure 1 (A), under control conditions, 13% of apo(a) had undergone maturation and secretion by 6 h of chase, and 59% of the protein had been degraded. The addition of lysosomal protease inhibitors had no effect on apo(a) secretion or degradation (results not shown). However, addition of the proteasome inhibitors acetyl-leucyl-leucyl-norleucinal or lactacystin essentially abolished apo(a) degradation (3% and 13% degradation at 6 h respectively). No increase in apo(a) secretion was observed in the presence of the proteasome inhibitors, but there was an increase in recovery of the intracellular apo(a) precursor at 6 h of chase (Figure 1A). The apo(a) precursor in these cells remained sensitive to digestion with endoglycosidase H (results not shown), suggesting that it accumulated in a pre-medial Golgi compartment [24]. These results suggest that in mouse hepatocytes a large portion of the apo(a) precursor is degraded from a pre-medial Golgi compartment by the proteasome.

The proteasome is a cytoplasmic enzyme complex that degrades proteins conjugated to multiple copies of the 76-residue peptide ubiquitin [25]. As a secretory protein, apo(a) is normally retained within the lumen of the secretory pathway. Recently, a degradation pathway for misfolded secretory proteins has been elucidated that involves the translocation of the proteins back across the ER membrane into the cytosol, followed by deglycosylation, conjugation to multi-ubiquitin chains and degradation by the proteasome [26–28]. The apo(a) precursor that accumulated in lactacystin-treated cells did not show a change in molecular mass (Figure 1A), as might be expected if apo(a) were deglycosylated [15] or ubiquitinated. In addition, the accumulated apo(a) was resistant to digestion with trypsin in microsome preparations (results not shown), suggesting that it was retained in the lumen of the secretory pathway. These results suggested either that the role of the proteasome in apo(a) degradation was indirect, or that proteasome activity was required for apo(a) translocation back across the ER membrane.

To investigate this issue, we used a more sensitive assay to look for the presence of ubiquitinated apo(a) degradation intermediates. Primary hepatocytes from Lp(a) transgenic mice were
transfected with a construct encoding an influenza-virus-haemagglutinin-tagged form of ubiquitin (HA-ubiquitin [29]). Control cells were transfected with His$_6$-tagged ubiquitin (His$_6$-ubiquitin [29]). The transfected cells were then treated with lactacystin, apo(a) was immunoprecipitated from the cell lysates and the immunoprecipitates were analysed by immunoblotting with an anti-HA antibody (Figure 1B).

In unfractionated cell lysates, a smear of anti-HA reactivity was observed in HA-ubiquitin-transfected cells that increased in intensity after treatment with lactacystin (Figure 1B). Because lactacystin inhibits proteasome-mediated degradation without preventing ubiquitination [30], this smear presumably represents different ubiquitinated cellular proteins. In apo(a) immunoprecipitates, a high-molecular-mass smear of anti-HA reactivity was observed that also increased in intensity in lactacystin-treated cells. Most of this reactivity was of higher molecular mass than native apo(a), and presumably represented apo(a) conjugated to multiple ubiquitin chains (Figure 1B). No anti-HA reactivity was observed in apo(a) immunoprecipitates from His$_6$-ubiquitin-transfected cells, or in apoB or albumin immunoprecipitates (Figure 1B).

These results suggest that at least a portion of apo(a) is targeted to the proteasome by translocation across the ER membrane and subsequent ubiquitination. Further studies will be required to determine whether all apo(a) presecretory degradation is mediated by this pathway.

**N-linked glycans and apo(a)-chaperone interactions**

N-linked glycans have an important role in protein secretion by maintaining protein solubility in the ER [31] and by mediating interactions with the lectin-like ER chaperones, calnexin and calreticulin [32,33]. A diagram of N-linked glycan addition and processing in the ER [24] is presented in Scheme 1. N-linked glycans are added co-translationally to secretory proteins as NacGlc$_3$Man$_n$Glc$_3$ precursors (Scheme 1). The outer and two inner glucose residues are then trimmed by ER glucosidases I and II respectively. If still unfolded, the protein is recognized by the ER resident enzyme UDP-glucose:glycoprotein glucosyltransferase [34], which reattaches a single glucose residue (Scheme 1). The monoglucosylated side chains are then bound by the ER chaperones calnexin and calreticulin, which help the protein to fold [32,33]. The reattached glucose can then be removed by ER glucosidase II. If the protein is still unfolded it will again be recognized by UDP-glucose:glycoprotein glucosyltransferase and will enter a reglucosylation/deglucosylation cycle involving cyclic interactions with calnexin and calreticulin until the protein has been folded and can proceed down the secretory pathway [32,33]. While still in the ER, the N-linked

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**Scheme 1**

Role of N-linked glycans and chaperone interactions in apo(a) intracellular targeting

Diagram of N-linked glycan addition and processing in the ER [24]. Calnexin and calreticulin interact with monoglucosylated side chains. The sites of action of glycoprotein processing inhibitors are indicated. Abbreviations: CST, castanospermine; dNMN, deoxymannojirimycin; GSD, glucosidase; GT, UDP-glucose:glycoprotein glucosyltransferase; MSD, mannosidase; OST, oligosaccharyltransferase; TM, tunicamycin. Numbers 1–5 refer to the carbohydrate structures studied in Table I.
glycans can also be trimmed by ER mannosidase (Scheme 1).

Using a variety of glycoprotein processing inhibitors, we analysed the role of N-linked glycans in apo(a) secretion, chaperone interactions and intracellular degradation in primary mouse hepatocyte cultures. The results of these studies are summarized in Table 1.

Addition of N-linked glycans was essential for the secretion of apo(a) and its interaction with calnexin and calreticulin. Unglycosylated apo(a) (Scheme 1, 1) aggregated in the ER and was not secreted or degraded. The interaction of apo(a) with calnexin and calreticulin was also prevented by trapping apo(a) in its triglucosylated form with ER glucosidase inhibitors (Scheme 1, 2). Under these conditions, apo(a) secretion was prevented and an increased portion of apo(a) was targeted for degradation. This suggests an important role for calnexin and/or calreticulin in apo(a) folding and export from the ER. However, in contrast with some other proteins [35], the results suggest that calnexin interaction is not required to target apo(a) to the degradation pathway. When apo(a) binding to calnexin and calreticulin was enhanced by trapping apo(a) in its monoglucosylated form (Scheme 1, 3), both the secretion and the degradation of apo(a) were prevented. Thus calnexin and/or calreticulin might be able to sequester apo(a) in the ER lumen and prevent its export for either secretion or degradation.

Recent studies have demonstrated a requirement for ER mannosidase activity in targeting of secretory proteins to the proteasome [36,37]. Inhibition of ER mannosidase activity resulted in a small increase in apo(a) secretion and a decrease in apo(a) degradation, perhaps suggesting a role for a lectin activity in targeting to the degradation pathway. The precise structure of the N-linked carbohydrate side chains on apo(a) therefore has a central role in apo(a) processing in the ER and its targeting to the secretory and degradation pathways.

**Apo(a) intracellular targeting can be regulated**

We have recently identified conditions under which the portion of apo(a) targeted to the secretory and degradation pathways can be modulated. In agreement with studies by Nassir et al. [38], we found that incubation of hepatocytes with high concentrations of fatty acids increased apo(a) secretion approx. 2-fold (results not shown). The mechanism of this effect is uncertain. Nassir et al. [38] suggested that apo(a) secretion might be coupled to lipoprotein assembly, possibly through the modulation of apo(a) translocation across the ER membrane. However, we have been unable to demonstrate any cytoplasmically exposed apo(a) in our mouse hepatocyte cultures, and apo(a) is secreted from non-hepatic cell lines that do not synthesize lipoproteins [39]. It is possible that co-expression with apoB decreases the secretion of apo(a) by means of non-productive interactions between apo(a) and misfolded apoB in the ER lumen. By this model, conditions that enhance apoB secretion (such as supplementation with

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fatty acids [40]) could indirectly increase apo(a) secretion by decreasing apo(a) retention in the ER. We are currently investigating the effect of apoB co-expression on apo(a) secretion.

We have also demonstrated that incubation of hepatocytes with high concentrations of the lysine analogue 6-aminohexanoic acid (6AHA), increases apo(a) secretion from Lp(a) transgenic hepatocytes more than 10-fold [23]. 6AHA decreases apo(a) degradation and increases the efficiency of apo(a) export from the ER. Interestingly, 6AHA can overcome the block in apo(a) secretion induced when apo(a) is trapped in its triglucosylated form, suggesting that 6AHA relieves the dependence of apo(a) folding on calnexin-calreticulin interaction [23]. 6AHA binds to the apo(a) kringle domains and might help to stabilize these domains during folding. Alternatively, because 6AHA prevents kringle-mediated apo(a)–protein interactions [41,42], it might prevent non-productive interactions of apo(a) with other proteins in the ER lumen.

Conclusions

Scheme 2 summarizes our current view of apo(a) processing in the ER. Apo(a) is co-translationally translocated into the ER lumen as an extended polypeptide chain (Scheme 2, 1). While still being translated, apo(a) begins to fold and passes through a series of folding intermediates [17,22] (Scheme 2, 2). Multiple chaperones interact with apo(a) during this process, including immunoglobulin heavy-chain binding protein, protein disulphide isomerase, calnexin [18] and calreticulin (this study). Once correctly folded (Scheme 2, 3), apo(a) can be transported out of the ER and secreted. Misfolding of only a single domain in apo(a) [17,20] (Scheme 2, 4) targets the protein for degradation. For degradation to take place, apo(a) is translocated back across the ER membrane [18], presumably after being unfolded by ER chaperone proteins (Scheme 2, 5). The identity of the chaperone(s) involved in this process is unknown.

Targeting of apo(a) to the secretory and degradation pathways is presumably determined by its ability to assume a correctly folded conformation [43]. A large size or the presence of polymorphisms in individual protein domains might increase the extent of apo(a) misfolding and intracellular degradation. Conversely, factors that enhance apo(a) folding efficiency, such as incubation with 6AHA, might increase the rate of apo(a) secretion and Lp(a) production. Attempts to reveal differences in folding efficiency between apo(a) variants that are secreted at different rates [17], or in cells treated with or without 6AHA [23], have been unsuccessful. However, the folding assay used will not detect non-covalent differences

Scheme 2

Processing of apo(a) in the ER

The pathways of apo(a) synthesis, folding, secretion and degradation are depicted. Numbers 1–5 refer to the different folded states of apo(a) that occur during these processes. For the sake of clarity the carbohydrate structures on apo(a) are not shown. See the text for details. Abbreviations: BiP, immunoglobulin heavy-chain binding protein; PDI, protein disulphide isomerase.
in apo(a) conformation or relatively subtle disulfide-bond-dependent changes that involve only one or a few of the many domains in apo(a) [17,20]. A major focus of future studies will be to develop more sensitive assays for the analysis of the conformation of apo(a) in the ER, to elucidate the precise mechanisms that regulate the export of apo(a) from this compartment.

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