Influence of Allelic Variation on Apolipoprotein(a) Folding in the Endoplasmic Reticulum*

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Plasma levels of lipoprotein(a) (Lp(a)) vary over 1000-fold between individuals and are determined by the gene for its unique apolipoprotein, apo(a), which has greater than 100 alleles. Using primary baboon hepatocyte cultures, we previously demonstrated that differences in the ability of apo(a) allelic variants to escape the endoplasmic reticulum (ER) are a major determinant of Lp(a) production rate. To examine the reason for these differences, the folding of newly synthesized apo(a) was analyzed in pulse-chase experiments. Samples were harvested in the presence of N-ethylmaleimide to preserve disulfide-bonded folding intermediates, and apo(a) was analyzed by immunoprecipitation and SDS-polyacrylamide gel electrophoresis. apo(a) required a prolonged period (30–60 min) to reach its fully oxidized form. Multiple folding intermediates were resolved, including a disulfide-linked, apo(a)-containing complex. Unexpectedly, all allelic variants examined showed similar patterns and kinetics of folding. Even "null" apo(a) proteins, which are unable to exit the ER, appeared to fold normally. The ER glucosidase inhibitor, castanospermine, prevented apo(a) secretion, but did not inhibit folding. This suggests that an event which is dependent on trimming of N-linked glucoses, and which occurs after the folding events detectable in our assay, is required for apo(a) secretion. Differences in the ability to undergo this event may explain the variable efficiency with which apo(a) allelic variants exit the ER.

Apolipoprotein(a) (apo(a)) is a highly polymorphic, high molecular weight glycoprotein that circulates in plasma as a component of lipoprotein(a) (Lp(a)). Lp(a) is composed of low density lipoprotein in which apoB is attached to apo(a) by disulfide linkage (1, 2). As many as 34 different isoforms of apo(a) have been identified in human plasma, which vary in size from <300 to >800 kDa (3–6). Apo(a) has a highly complex, repetitive structure and is homologous with plasminogen (7). Apo(a) contains an inactive copy of the plasminogen protease domain and a single plasminogen kringle 5 (K5) domain, preceded by multiple domains with homology to plasminogen K4 (7). The size variation of apo(a) is due to differences in the number of K4 domains encoded in the apo(a) gene (8–11), which varies from approximately 12 to 51 (3). Each K4 repeat contains 3 internal disulfide bonds, 1 N-linked, and 6 potential O-linked glycosylation sites (7). Thus, a large apo(a) isomorph may contain in excess of 150 disulfide bonds, 50 N-linked and 300 O-linked carbohydrate side chains.

Lp(a) is only found in the plasma of primates (12) and the hedgehog (13, 14). In humans, plasma levels of Lp(a) vary from <1 to >100 mg/dl and are highly heritable (15). High plasma levels of Lp(a) are associated with an increased incidence of cardiovascular diseases (for review, see Ref. 16). Greater than 90% of the inter-individual variation in Lp(a) concentration is attributable to the apo(a) gene locus (17). There is an inverse correlation between apo(a) size and plasma Lp(a) level (5, 6, 10, 18); however, this relationship is not absolute (6, 10), and a particular isoform size may be associated with as much as a 200-fold difference in plasma Lp(a) concentrations in different individuals (19). Thus, sequence variations at the apo(a) locus independent of size also influence Lp(a) levels (20). “Null” apo(a) alleles, which do not give rise to any detectable plasma protein (5, 9), and which are distributed throughout the apo(a) allele size range (18, 21), are an extreme example of apo(a) sequence variations that influence Lp(a) levels. A number of polymorphisms have recently been identified at the apo(a) locus (20, 22–31), and it is estimated that there are greater than 100 apo(a) alleles (20). However, as yet, no identified polymorphism other than K4 number has been demonstrated to directly influence circulating Lp(a) levels.

Lp(a) is synthesized by the liver (32–34), and the rate of Lp(a) production determines plasma Lp(a) concentration (35–37). Due to the lack of a small animal model and the highly polymorphic nature of the apo(a) gene, the mechanisms governing Lp(a) production rate have remained poorly characterized. Some of the variation in Lp(a) production rate is determined by differences in hepatic apo(a) mRNA concentration (9, 38). “Transcript negative null” alleles that do not produce any detectable hepatic apo(a) mRNA transcript (9, 39) represent an extreme example of this type of regulation. Post-transcriptional mechanisms are also important (38). A sequence polymorphism in the 5′-untranslated region of human apo(a) was recently shown to influence the translation efficiency of apo(a) mRNA in vitro (31).

Baboons show very similar characteristics to humans in terms of plasma Lp(a) levels and apo(a) isoform sizes (40, 41). We have established primary cultures of baboon hepatocytes as a model system for the analysis of Lp(a) biogenesis (34). Since hepatocytes can be isolated from animals with selected Lp(a) phenotypes, this provides us with a unique opportunity to examine the influence of apo(a) allelic variation on Lp(a) production rate. Using this system, we have demonstrated that, in comparison to other secretory proteins, newly synthesized apo(a) has a prolonged residence time in the endoplasmic reticulum (ER) before it is processed to its mature form and

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1 The abbreviations used are: apo, apolipoprotein; CST, castanospermine; PBS, phosphate-buffered saline; DTT, dithiothreitol; ER, endoplasmic reticulum; Lp, lipoprotein; NEM, N-ethylmaleimide; PAGE, polyacrylamide gel electrophoresis; TM, tunicamycin.

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transported to the cell surface (21, 39, 42, 43). Differences in the efficiency with which apo(a) allelic variants undergo post-translational processing accounts, at least partially, for the inverse correlation between apo(a) size and plasma Lp(a) level (21). In addition, “transcript positive null” apo(a) phenotypes, in which there is no detectable plasma Lp(a), but which are associated with substantial levels of apo(a) mRNA in the liver (9, 39), are explained by the production of defective apo(a) proteins that are unable to exit the ER and are retained and degraded inside the cell (21). Thus, the efficiency with which apo(a) leaves the ER is a major determinant of plasma Lp(a) levels.

The ER lumen provides a specialized environment to promote the folding and assembly of newly synthesized transmembrane and secretory proteins. Misfolded and unassembled proteins are retained and degraded in the ER (44). Considering the complex structure of the apo(a) glycoprotein, we hypothesized that the long ER residence time of apo(a) may be due to the extended time required to reach its correct conformation. Differences in the proficiency with which apo(a) allelic variants fold may account for their varying abilities to escape the ER. To test this hypothesis, we analyzed the folding of a number of apo(a) allelic variants in the ER.

EXPERIMENTAL PROCEDURES

Materials—([35S]Cysteine and Expre35S35S) were from DuPont NEN. Protein A-agarose was from Repligen Corp. (Cambridge, MA). Sheep anti-human apoB was from Boehringer Mannheim, and goat anti-human Lp(a) was from Biodesign (Kennebunkport, ME). Methionine and cysteine-free Williams medium E was purchased from Life Technologies, Inc. Tunicamycin was from Sigma, and deoxymannojirimycin were from Genzyme Corp. (Cambridge, MA). All other chemicals were of analytical grade.

Hepatocyte Isolation and Culture—Hepatocytes were isolated as described previously (45). Lobectomy was performed according to institutional guidelines under general anesthesia; ketamine hydrochloride was used as an immobilizing agent, and anesthesia was maintained with sodium pentobarbital. Analgesics were provided 48 h post-operatively. Cells were cultured in a serum-free medium (SFM) formulation (formula III) as described previously (43), except for the omission of thyrotropin releasing factor. All experiments were performed using confluent 60-mm dishes of cells, which had been in culture for 5–7 days.

Apo(a) isoforms in the baboon are classified into 12 size groups; A, the largest, through L, the smallest (40). Hepatocytes from seven different animals were analyzed. Two animals expressed an I apo(a) isoform (plasma Lp(a) levels of 34 and 30 mg/dl) (Figs. 2 and 7). Three animals expressed an A isoform (19 mg/dl, Fig. 2C; 20 mg/dl, Fig. 2D; 17 mg/dl, Fig. 3), and one expressed a C isoform (34 mg/dl, Fig. 2B). Two animals (Fig. 4) had plasma Lp(a) levels <2 mg/dl and no apo(a) detectable in their plasma by immunoblotting. Northern analysis of hepatic RNA from these null phenotype animals revealed a single apo(a) mRNA species in each case, which gave rise to apo(a) proteins approximately the size of an I isoform.

Steady-state Labeling—For steady-state labeling experiments, hepatocytes were incubated for 20 h in SFM containing 0.1 × the normal concentration of methionine and cysteine plus 125 μCi/ml each of [35S]cysteine and Expre35S35S label. Culture media were clarified at 2000 × g for 10 min and adjusted to 1% Nonidet P-40. Cells were washed with PBS and lysed in 2 ml of extraction buffer (50 mM Tris, 150 mM NaCl, 1% Nonidet P-40, pH 9.0).

Effect of Glycosylation Inhibitors—To analyze the influence of glycosylation inhibitors on apo(a) maturation and secretion, hepatocytes were preincubated for 1 h in methionine and cysteine-free SFM in the presence or absence of tunicamycin (5 μg/ml), castanospermine, or deoxymannojirimycin (1 μM each). The cells were then labeled for 30 min in the same medium containing 125 μCi/ml each of [35S]cysteine and Expre35S35S label and then chased in complete SFM plus drugs for 5 h. Culture media and cells were harvested as described above.

Analysis of Apo(a) Folding—Apo(a) folding was initially analyzed essentially as described by Braakman et al. (46). Hepatocytes were preincubated for 1 h in methionine and cysteine-free SFM and then labeled for 10 min in the same medium containing 125 μCi/ml each of [35S]cysteine and Expre35S35S label. The cells were then either harvested immediately or were washed once with PBS and chased for various periods in complete SFM containing 0.5 mM cycloheximide. To harvest, the cells were placed on ice, washed twice with ice-cold phosphate-buffered saline (PBS) containing 20 mM N-ethylmaleimide (NEM), and then lysed in 1 ml of ice-cold extraction buffer containing 20 mM NEM. In later experiments, this protocol was modified to optimize detection of apo(a) folding intermediates. The experiments were performed as described above, except that the cells were labeled for 1 h. Five minutes before the end of labeling, 5 mM dithiothreitol (DTT) was added to the cultures. The hepatocytes were then washed twice with PBS, chased, and harvested, as above.

Immunoprecipitation and SDS-PAGE—Samples were immunoprecipitated exactly as described previously (42). Prior to electrophoresis, immunoprecipitates were heated to 100 °C for 5 min in gel sample buffer with (reduced samples) or without (nonreduced samples) 10% 2-mercaptoethanol. Proteins were resolved by 3–10% SDS-PAGE and fluorography (42).

Immunoblotting—Aliquots of cell lysates were resolved by 3–10% SDS-PAGE, either with or without prior reduction with 2-mercaptoethanol, and immunoblotted for apo(a), exactly as described previously (42).

RESULTS

Folding of Newly Synthesized Apo(a)—Apo(a) folding was initially analyzed using a pulse-chase protocol, essentially as described by Braakman et al. (46). Baboon hepatocytes were labeled for 10 min with [35S]methionine and cysteine and chased for 0, 10, 30, or 60 min in unlabeled medium. Cells were lysed in the presence of the alkylating agent, NEM, to preserve partially disulfide-bonded apo(a) folding intermediates, and apo(a) was immunoprecipitated and analyzed, with or without prior reduction with 2-mercaptoethanol, by 3–10% SDS-PAGE.

Fig. 1 shows results obtained with hepatocytes expressing a
low molecular weight apo(a) isoform (I isoform), the mature form of which has a molecular mass similar to that of apoB (550 kDa). When samples were analyzed after reduction, a single apo(a) protein, representing the newly synthesized apo(a) precursor, could be seen at the 0, 10, and 30 min time points. At 60 min, the fully glycosylated, mature form of apo(a) also appeared (Fig. 1). Mature apo(a) contains fully processed N-linked and O-linked glycans, and its appearance is a marker for movement of apo(a) out of the ER and through the Golgi apparatus (42). Densitometric scanning of the autoradiograph determined that 13% of apo(a) was in the mature form at 60 min, consistent with our previous studies in which we analyzed apo(a) proteins of similar size (21). An almost undetectable decrease in the electrophoretic mobility of the reduced apo(a) precursor was observed with increasing chase times (Fig. 1). Control experiments established that this was due to decreased binding of NEM at later time points as a result of increased intramolecular disulfide formation. NEM presumably increases the electrophoretic mobility of apo(a) by enhancing binding of SDS through electrostatic interaction.

When analyzed under nonreducing conditions, a single form of apo(a) was again seen immediately after the pulse (Fig. 1). Nonreduced apo(a) exhibited an increased mobility in comparison to the fully reduced protein, indicating some limited cotranslational disulfide bond formation (46, 47). The electrophoretic mobility of nonreduced apo(a) increased at later chase times, reflecting the formation of further disulfide bonds. At the 30-min time point, a form of apo(a) with a greatly decreased mobility also appeared (comp., Fig. 1). Control immunoprecipitations and immunoblotting experiments (see below) confirmed that this band contains apo(a). Since it disappears upon reduction, it most likely represents a disulfide-linked complex of apo(a) with itself or with another protein(s). While in this form, apo(a) apparently continued to fold, since the mobility of the complex also increased with time (Fig. 1, and see below). The mobility of monomeric apo(a) did not reach a maximum until 60 min of chase, concomitant with the appearance of the mature protein. Mature apo(a) had a mobility between that of the precursor’s monomeric and complexed forms (Fig. 1). In comparison to other characterized secretory proteins, apo(a) has a greatly prolonged ER residence time. These data demonstrate that the long ER residence time of apo(a) coincides with a prolonged period required for apo(a) to fold.

Apo(a) Folding after Post-translational Reduction—Due to the short labeling time required, the above folding assay was not sensitive enough to resolve folding intermediates of most apo(a) variants. Secretory proteins in the ER of living cells can be rapidly reduced by addition of the membrane-permeable reducing agent, DTT (48). This effect is reversible; on removal of DTT, the reduced proteins fold and are secreted normally (47–50). To increase the sensitivity of the apo(a) folding assay, an alternative protocol was adopted in which hepatocytes were labeled for 1 h with [35S]cysteine and [35S]methionine. During the last 5 min of labeling, 5 mM DTT was added to the cultures to reduce and unfold radiolabeled apo(a) that had accumulated in the ER. The cells were then washed and chased for times...
between 0 and 120 min, and apo(a) folding intermediates were resolved as in Fig. 1.

Fig. 2A shows results obtained with hepatocytes expressing the same (I) apo(a) isoform as in Fig. 1. Under reducing conditions, apo(a) folding was seen as a slight decrease in electrophoretic mobility (“NEM shift”), with the mature form of the protein appearing at 60 min of chase. (9% of apo(a) had matured at this time point. The absence of radiolabeled mature apo(a) after 55 min of labeling may appear contradictory to Fig. 1, where mature apo(a) was easily seen at 60 min. However, the experiment in Fig. 1 also included a 10-min pulse, making the 60-min chase time in that experiment effectively a 70-min time point.) Under nonreducing conditions, apo(a) folding was seen as a progressive increase in apo(a) electrophoretic mobility, reaching a maximum at 60 min of chase, coincident with the appearance of the mature protein. The high molecular weight apo(a)-containing complex was also observed, this time as early as 10 min of chase (Fig. 2A). Treatment with 5 mM DTT did not fully unfold apo(a), since the mobility of apo(a) at the start of the chase was greater than that of the fully reduced protein (Fig. 2A). Increasing the concentration of DTT to 20 mM did not cause any further decrease in apo(a) electrophoretic mobility (data not shown). This suggests the presence of a number of disulfide bonds in oxidized apo(a) that are not readily accessible to reduction by DTT.

The kinetics and pattern of apo(a) folding observed with the two different protocols were almost identical. The increased sensitivity achieved with the latter assay allowed the folding of a number of apo(a) allelic variants to be compared.

**Influence of Apo(a) Size on Folding Pattern—**Our previous studies demonstrated that large apo(a) isoforms tend to have longer ER residence times than small isoforms (21). The kinetics and patterns of folding of large and small apo(a) proteins were therefore compared (Fig. 2). Similar to the I isoform depicted in Fig. 2A, folding of a C (Fig. 2B) and two A (Fig. 2, C and D) apo(a) isoforms was observed as a barely discernible NEM shift in reduced samples. However, in contrast to the I isoform, and consistent with their larger size (21), less than 1% of each of these isoforms had matured by 1 h of chase. By the 2-h chase time point, only 5, 4, and 6% (Fig. 2, B–D, respectively) of the proteins were in the mature form. Under nonreducing conditions, a similar pattern of apo(a) folding intermediates as that observed for the I isoform was seen for the C and A isoforms. In each case, the electrophoretic mobility of apo(a) increased with time, reaching a maximum by 60 min of chase (Fig. 2, B–D). A high molecular weight apo(a)-containing complex was also observed, this time as early as 10 min of chase (Fig. 2A). Increasing the concentration of DTT to 20 mM did not cause any further decrease in apo(a) electrophoretic mobility (data not shown). This suggests the presence of a number of disulfide bonds in oxidized apo(a) that are not readily accessible to reduction by DTT.

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Apo(a) Folding

**Fig. 5.** Analysis of null apo(a) folding in the absence of DTT and by immunoblotting. Hepatocytes expressing null protein II (Fig. 4) were analyzed. A, apo(a) folding in the absence of DTT was analyzed exactly as described in the legend to Fig. 1. B, unlabeled hepatocytes were treated for 5 min with 5 mM DTT, washed twice with PBS, and chased for 0, 60, or 120 min before harvest in the presence of NEM. Aliquots of each cell lysate were then resolved by 5–10% SDS-PAGE and immunoblotted for apo(a), as described under "Experimental Procedures." In each experiment, samples were analyzed with (reduced) or without (non-reduced) prior reduction with 2-mercaptoethanol. The position of the apo(a) precursor (pr apo(a)), the apo(a)-containing complex (comp.) and apoB100 (550 kDa; arrowhead) are indicated. ns, premune serum immunoprecipitation control.

**Fig. 6.** Role of N-linked glycans in apo(a) secretion. A, schematic representation of N-linked carbohydrate addition and trimming in the ER and the site of action of the inhibitors tunicamycin (TM) and castanospermine (CST). B, hepatocytes expressing a B apo(a) isofrom were labeled for 30 min and chased for 5 h in the presence of 5 µg/ml TM or 1 mM CST or deoxynojirimycin (dMN), and apo(a) was immunoprecipitated from the cell lysates (c) and culture media (m), as described under "Experimental Procedures." The positions of the precursor (pr apo(a)) and mature (apo(a)) forms of apo(a) and of apoB100 (550 kDa) are indicated.

ER and are completely retained and degraded inside the cell (21). To determine whether this phenotype is caused by aberrant folding, the folding of two null apo(a) proteins was analyzed (Fig. 4). The proteins were of similar size, which, based upon electrophoretic mobility, was equivalent to that of an apo(a) isofrom precursor. Steady-state labeling of hepatocytes expressing these proteins confirmed the null phenotype (Fig. 4A). In each case a single apo(a) protein representing the unprocessed null apo(a) precursor (21) was observed in the cell lysate, and no apo(a) was detectable in the culture medium (Fig. 4A). ApoB was efficiently secreted from each cell type, demonstrating normal functioning of the secretory pathway (Fig. 4A).

Unexpectedly, the pattern and kinetics of folding of the null proteins were essentially indistinguishable from those of secreted apo(a) proteins. Under reducing conditions (Fig. 4B) folding was observed as an NEM shift, although no mature apo(a) was seen, even after 2 h of chase (Fig. 4B). Under nonreducing conditions (Fig. 4C), apo(a) folding was observed as an increase in electrophoretic mobility with time, reaching a maximum by 60 min of chase. High molecular weight apo(a)-containing complexes were also apparent in each case. Thus, despite their inability to exit the ER, these null apo(a) proteins follow an apparently normal folding pathway. A gross inability to fold thus does not explain the null apo(a) phenotype.

Null apo(a) protein II in Fig. 4 was synthesized at a high level in comparison to most apo(a) allelic variants. Analysis of the folding of this protein in unperturbed cells (no DTT treatment), as described for the secreted apo(a) isoform in Fig. 1, also revealed an apparently normal folding pathway (Fig. 5A). In this case, the high molecular weight apo(a) containing complex was apparent as early as the 0 chase time point, suggesting that the complex forms during, or immediately after, translation. This experiment confirms that the use of DTT did not artificially induce a normal folding pathway for the null proteins. The folding of null apo(a) protein II was also analyzed by
immunoblotting (Fig. 5B). A single form of apo(a) was seen after DTT treatment in the nonreduced samples, whereas at the later time points, proteins corresponding to fully folded apo(a) and the high molecular weight complex were recognized by the anti-apo(a) antibody. This confirms the identity of the intermediates observed in radiolabeled folding experiments as apo(a).

An interesting observation for each of the null proteins was the presence of a minor band below the null apo(a) protein in the cell lysate (Fig. 4). This band was not observed with normally secreted apo(a) proteins (Figs. 1 and 2, and data not shown) and may be a marker for the null phenotype. In addition, a second high molecular weight apo(a)-containing band was apparent in nonreduced samples from null animal I. Further experimentation will be required to determine the significance of these observations.

**Influence of Glycosylation Inhibitors on Apo(a) Maturation and Folding**—Apo(a) is highly glycosylated. N-Linked glycosylation occurs co-translationally in the ER lumen (51) and plays a significant role in the folding and secretion of some glycoproteins. To begin to investigate the factors that influence the exit of apo(a) from the ER, the role of N-linked glycans in apo(a) maturation and secretion was examined. Two inhibitors were used, tunicamycin (TM), which prevents N-linked glycosylation (52), and castanospermine (CST), which inhibits the trimming of glucose residues from N-linked glycans in the ER (53) (Fig. 6A). Hepatocytes expressing a B apo(a) isoform were labeled for 30 min and then chased for 5 h in the presence of TM or CST, and apo(a) in the cell lysates and in the culture media was analyzed by immunoprecipitation. The effect of deoxymannojirimycin, which inhibits the trimming of mannose residues from N-linked glycans after transport to the Golgi apparatus (53), was examined as a control.

TM caused a reduction in the molecular weight of the apo(a) precursor, due to inhibition of N-linked glycosylation, and completely prevented apo(a) maturation and secretion (Fig. 6B). CST also prevented maturation of apo(a) (Fig. 6B). Thus, both addition and trimming of N-linked carbohydrate are required for apo(a) to exit the ER. In contrast, deoxymannojirimycin did not prevent apo(a) secretion (Fig. 6B).

The anti-apo(a) antibody was unable to efficiently immunoprecipitate reduced, unglycosylated apo(a), so the effect of TM on apo(a) folding could not be determined. The folding pattern of apo(a) in the presence of CST, however, was almost indistinguishable from that in control cells (Fig. 7); in each case, folding was observed as a NEM shift in reduced samples and as a gradual increase in electrophoretic mobility, reaching a maximum at 60 min, in nonreduced samples. The high molecular weight apo(a)-containing complex was also clearly visible. The small increase in apo(a) molecular weight in CST-treated cells, most notable in the nonreduced samples, was due to the presence of the untrimmed glucose residues. The only other difference between CST and control samples was the absence of mature apo(a) in CST-treated cells (Fig. 7). Thus, CST induced an apo(a) phenotype similar to that of a null apo(a) protein. Combined with the results in Figs. 1–4, this suggests that an event that is dependent on the trimming of glucose residues from N-linked carbohydrate on apo(a) and that occurs after the bulk of apo(a) folding is required for apo(a) secretion.

**DISCUSSION**

We have previously demonstrated that newly synthesized apo(a) has a prolonged residence time in the ER and that allelic differences in the ability of apo(a) to escape this compartment are a major determinant of plasma Lp(a) levels (21, 39, 42). Since only correctly folded proteins are permitted to exit the ER (44), we examined the folding of newly synthesized apo(a) and the influence of allelic variation on the kinetics and efficiency of this process. The results revealed a complex and kinetically extended apo(a) folding pathway. Unexpectedly, no significant differences were found in the kinetics and patterns of folding of a variety of apo(a) allelic variants. Even null apo(a) proteins, which fail to escape the ER, appeared to fold normally. These studies suggest that an event downstream of the folding events detectable in our assay must occur for apo(a) to be secreted and that allelic differences in the ability of apo(a) to undergo this event contribute to the extreme inter-individual variation in Lp(a) production rate.

The variously disulfide-bonded folding intermediates of apo(a) were detected in pulse-chase experiments by exploiting differences in their electrophoretic mobility on SDS-PAGE. For many proteins, folding intermediates are hard to resolve, due to small differences in electrophoretic mobility or to rapid folding kinetics (46, 49, 54, 55). However, its large size and numerous disulfides made apo(a) ideally suited to this type of analysis. Our experiments revealed that the majority of disulfides in apo(a) were formed post-translationally and that folding was not complete until 30–60 min after synthesis. A unique characteristic was the formation of a high molecular weight, disulfide-linked, apo(a)-containing complex. The complex seems unlikely to represent aggregated apo(a), since apo(a) apparently continued to fold while in this form. The complex was confined to the ER, since only a single form of mature apo(a) was observed. Potential roles of the complex could be in targeting apo(a) for secretion, ER retention, or degradation. Alternatively, the complex could be involved in protecting Cys-4057 in apo(a), which is required for disulfide formation with apoB (56, 57), from inappropriate inter- or intramolecular interactions.
during the folding process. Comparison of reduced and non-reduced apo(a) immunoprecipitates did not identify other radiolabeled proteins that could be a component of the complex (data not shown). However, ER resident proteins tend to have long half-lives and may not incorporate sufficient radioactivity for detection during the short labeling periods used. Future studies will address the composition of the complex and its role in apo(a) secretion.

Similar patterns and kinetics of apo(a) folding were observed whether analyzed in unperturbed cells or after post-translational reduction of apo(a) with DTT. The latter assay allowed longer labeling times to be used and permitted comparison of the folding patterns of multiple apo(a) allelic variants. Similar protocols have been used to study the folding of a number of secretory and transmembrane proteins (47–50). In each case the characteristic folding pattern is unchanged by DTT treatment.

Unexpectedly, our studies failed to reveal any significant differences in the kinetics and patterns of folding of apo(a) proteins with very different ER residence times and efficiencies of secretion. We had expected that large apo(a) isoforms would take longer to fold than small isoforms and that folding and maturation times would coincide. However, this was not the case. A particularly striking example was the A isoform analyzed in Fig. 3. Although folding was apparently complete by 60 min, this protein did not begin to leave the ER until at least 3 h of chase. We had also anticipated that null apo(a) proteins would exhibit aberrant folding patterns. However, all null proteins examined to date appear to fold normally, yet are retained and degraded in the ER.

Several hypotheses can be put forward to explain these observations. The bulk of the apo(a) protein consists of a tandem array of identical K4 domains (7), variation in the number of which is responsible for the size polymorphism of apo(a) (3). The K4 repeats each contain three internal disulfide bonds (7) and are present as a series of globular domains (58). Since the majority of disulfides in apo(a) are formed post-translationally, the similar kinetics of folding of large and small apo(a) isoforms could be explained if the K4 units fold independently of each other and at similar rates. Braakman et al. (54) have provided evidence that proteins undergo cycles of folding, unfolding, and re-folding in the ER. It is possible that not all of the K4 domains in apo(a) initially fold correctly and that some must unfold and refold before their final conformation is reached. The greater number of K4 domains in large isoforms may increase the frequency of this event, resulting in longer ER residence times. These comparatively small changes in conformation, occurring after the bulk of apo(a) has folded, may not be detectable on our gel system. Similarly, null apo(a) proteins may possess a mutation(s) that prevents one or more K4 domains from ever reaching their correct conformation, resulting in complete ER retention.

The mutant kringle may have no influence on the folding of neighboring domains and again produce no detectable change in apo(a) mobility on SDS-PAGE. Apo(a) variants with unusually long ER residence times, such as the A isoform in Fig. 3, may possess sequence variations that decrease the efficiency of folding but which eventually allow at least a portion of apo(a) to reach its correct conformation and be secreted.

An alternative explanation could be that, after all the disulfides in apo(a) have been formed, an additional event is required before secretion can occur. Differences in the ability of apo(a) allelic variants to undergo such an event could explain the different kinetics and efficiencies with which they leave the ER. Apo(a) may need to interact with another protein, such as a chaperone protein, or to be released from an ER “receptor.” Alternatively, further folding events, which do not involve re-arrangement of disulfide bonds, may be required.

To begin to dissect the precise requirements for apo(a) to exit the ER, we analyzed the role of N-linked carbohydrate in apo(a) secretion. Both addition and processing of N-linked glycans was found to be required for apo(a) to escape the ER, since both TM, which prevents addition of N-linked glycans (52), and CST, which inhibits the trimming of glucose residues from N-linked glycans in the ER (53), prevented apo(a) maturation and secretion. TM causes aggregation of some secretory proteins (59). We were unable to ascertain the influence of TM on apo(a) folding due to lack of recognition of reduced, unglycosylated apo(a) by the anti-apo(a) antibody. This in itself could be indicative of aggregate formation, but further studies will be required to address this issue. CST prevents interaction of secretory proteins with the ER chaperone, calnexin, which recognizes substrates by binding to monoglucosylated N-linked carbohydrate side chains (60). Although CST prevented apo(a) secretion, it had no apparent effect on the pattern or kinetics of apo(a) folding. Again, this could reflect our inability to detect more subtle conformational changes in apo(a) caused by CST and may suggest a role for calnexin in apo(a) secretion. Alternatively, CST may inhibit an event downstream of folding, which requires carbohydrate trimming, and which is essential for apo(a) secretion. Clearly, further studies will be required to address the many outstanding questions regarding the requirements for apo(a) exit from the ER and the precise mechanism by which apo(a) allelic variation influences the kinetics and efficiency of this process.

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