Disulfide Bonds Are Required for Folding and Secretion of Apolipoprotein B Regardless of Its Lipidation State*

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Apolipoprotein (apo) B-100, an essential protein for the assembly and secretion of very low density lipoproteins depends on lipid binding (lipidation) for its secretion. Seven of its 8 disulfides are clustered within the N-terminal 21%. The role of these disulfides in the secretion of lipidated or unlipidated truncated forms of apoB was studied in C127 cells expressing apoB-17, apoB-29, or apoB-41. These cells do not express microsomal triglyceride transfer protein yet secrete apoB-41 on triacylglycerol-rich lipoproteins while apoB-29 and apoB-17 are secreted with little or no lipid, respectively. Dithiothreitol utilized in pulse-chase studies prevented the cotranslational formation of disulfides and when added posttranslationally reduced native disulfides. As a result, the secretion of reduced apoB forms was blocked and they were retained in the cells. Reduced apoB polypeptides were rescued following removal of dithiothreitol, as they underwent post-translational disulfide bonding, attained their mature form, and were subsequently secreted. Together the data suggest that in C127 cells the formation of native disulfides is critical for the folding and secretion of apoB independent of its length, its requirement for lipidation or microsomal triglyceride transfer protein expression. Therefore, these cells provide an appropriate model to study the folding of apoB in great detail.

The assembly and secretion of very low density lipoproteins (VLDL)¹ and chylomicrons in humans is directed by full-length apolipoprotein (apo) B (apoB-100) and its truncated form apoB-48, respectively (1–3). The capacity to recruit large amounts of neutral lipids, triacylglycerols (TAG), and cholesterol esters is unique to apoB and is thought to be conferred by regions along its sequence that are enriched in amphipathic β-strands with a strong lipid-binding motif (4, 5). For example, when arranged as 11-mers β-strands located between the N-terminal 21% (apoB-21) and 41% (apoB-41) could form anti-parallel amphipathic β-sheets in which the hydrophobic face could bind TAG (6). Indeed, progressive C-terminal truncations in vivo (e.g. naturally occurring mutations) (7, 8) or in vitro (9–13) lead to diminished capacity of apoB to recruit TAG. Thus, while the N-terminal 37% (apoB-37) is capable of assembling VLDL (7), forms corresponding to apoB-32.5 and shorter are secreted primarily on high density lipoprotein-like particles (7, 13).² Shorter forms encompassing the major region of the α-1 domain predicted to be globular (4), such as apoB-17, are secreted primarily lipid-free (10–12, 15). It has therefore been proposed that the region between apoB-29 and apoB-41 which is enriched in β-sheets is critical for the recruitment of TAG to form VLDL (13).² Concurrently, apoB-37 and longer forms are absolutely dependent on lipidation to fold into their native form and be secreted. Their folding is further dependent on microsomal triglyceride transfer protein (MTP) that mediates lipidation and is expressed primarily in the liver and the intestine (16). When neutral lipids are limited (3, 17) or when MTP activity is diminished either due to mutations (18, 19) or due to the presence of specific inhibitors (20, 21) apoB is incapable of folding into its mature form and is subsequently targeted for degradation (22) via the ubiquitin-dependent proteasome pathway (21, 23–26).

In addition to lipidation, the folding of apoB also involves glycosylation and disulfide bond formation, all of which occur in the endoplasmic reticulum (ER). The ER lumen provides an optimal milieu for proper folding of proteins that enter the secretory pathway since (a) it provides oxidizing environment (27) necessary to support the formation of native disulfide bridges and (b) it contains folding catalysts and molecular chaperones which mediate the folding of nascent proteins. The oxidizing environment in the ER is maintained primarily by reduced and oxidized glutathione at 1:1–3:1 ratio ([GSH]/[GSSG]), respectively (27). A recently identified protein, ERO1, seems to play a critical role in protein oxidation in the ER of eukaryotic cells (28–30) by maintaining oxidizing conditions. The formation of native disulfides is catalyzed by protein-disulfide isomerase, a member of the thioredoxin superfamily (31), which functions both by introducing disulfides and by rearranging nonnative disulfides that form co-translationally (31). A number of other members of thioredoxin family have been identified in the ER (e.g. ERp72 and ERp57/ER60) (33, 34), however, their role in catalyzing disulfide bonding in vivo has not been established.

The redox state in the ER can be manipulated in vivo by low levels of the reducing agent, dithiothreitol (DTT), which rapidly equilibrates across membranes to alter the redox state of the ER, thereby abrogating the formation of disulfide bonds, leading to the retention of reduced proteins in the ER (35–38). This manipulation has the following advantages: (a) the secretory

¹ The abbreviations used are: VLDL, very low density lipoproteins; apo, apolipoprotein; TAG, triacylglycerol; DTT, dithiothreitol; MTP, microsomal triglyceride transfer protein; ER, endoplasmic reticulum; DMEM, Dulbecco’s modified Eagle’s medium; PAGE, polyacrylamide gel electrophoresis; PL, phospholipids.

² M. Carraway, H. Herscovitz, V. I. Zannis, and D. M. Small, submitted for publication.
pathway remains unperturbed, (b) the inhibitory effects are fully reversible upon removal of DTT, and (c) fully or correctly folded proteins are not affected (35, 36, 39).

ApoB-100 (40, 41) and apoB-28 (42) were demonstrated to be sensitive to DTT reduction which led to their failure to fold into their mature form and as a result, their secretion was blocked. These findings suggested that disulfide bonds are critical for the folding of apoB. However, while apoB-28 could fold and be secreted following removal of DTT (42), apoB-100 was incapable of refolding and instead, was rapidly degraded (40, 41), making it impossible to isolate folding intermediates in the maturation of apoB-100 for further characterization. On the other hand, important information has been derived from studies using apoB-28 as a model for early folding events of apoB (42). However, at least 37% of apoB is required for the formation of TAG-rich lipoproteins (7). Therefore, we extended these studies to include apoB-41, apoB-29, and apoB-17 which have different requirements for lipidation representing different stages in the maturation of apoB. ApoB-17 has 6 disulfides, while both apoB-29 and apoB-41 have 7 of the 8 disulfides in apoB-100 (43). These apoB forms were expressed by stably transfected mammary-derived C127 cells. These cells are unique since they do not express MTP, yet secrete apoB-41 exclusively on high density lipoprotein-like particles containing a TAG-rich core (44). In contrast, apoB-17 is secreted primarily lipid-poor (15) while apoB-29 is secreted either lipid-free or associated with mostly polar lipids.2 We show that these apoB forms are sensitive to DTT regardless of their lipidation state, and that the inhibitory effects of DTT on their secretion are fully reversible. Therefore, these cells provide a unique model system in which folding intermediates during different stages of maturation can be isolated to study the details of the folding pathway of apoB, including lipidation and characterization of cellular factors that mediate folding, such as molecular chaperones and folding catalysts.

**EXPERIMENTAL PROCEDURES**

**Materials**—Dulbecco’s modified Eagle’s medium (DMEM), fetal calf serum, and Dulbecco’s phosphate-buffered saline were from Life Technologies Inc. Dithiothreitol (DTT), cycloheximide, leupeptin, aprotinin, and iodoacetamide were from Sigma. N-Acetyl-L-leucinyl-L-leucinyl-L-norleucinal (ALLN) was from Roche Molecular Biochemicals. 4-(2-Aminoethyl)-benzylsulfonyle fluoride was from Calbiochem. Gelatin- and Protein G-Sepharose for that 4 °C. ApoB was captured on Protein G-Sepharose, washed in lysis buffer followed by phosphate-buffered saline, and solubilized in sample buffer (45) containing 8 M urea. Proteins were resolved by 7% SDS-PAGE (45), followed by autoradiography using a PhosphorImager screen (Molecular Dynamics). ApoB was quantified using “Imagequant” software and normalized to cell protein (46).

**Statistics**—Statistical analysis was performed using a software package RS1 (BBN Research Systems).

**RESULTS**

**Disulfide Bonds in ApoB-17 Are Formed Co-translationally**—The formation of disulfide bonds often results in the generation of more compact intermediates that can be distinguished from reduced forms by their faster migration on non-reducing SDSPAGE. However, since apoB is a large protein and most of its disulfides are formed between adjacent cysteines, the shift in the migration of the disulphide-bonded form compared with its reduced form is virtually undetectable. Since 7 out of 8 disulfides occur in the first 21% of apoB the difference in migration between reduced and oxidized forms is easily observed in the shorter forms of apoB. Therefore, we chose apoB-17, which contains 6 disulfides, to determine the rate of the formation of disulfide bridges. Cells were pretreated with puromycin to synchronize the translating ribosomes, and then pulse-labeled for the indicated times, treated with iodoacetamide and lysed. ApoB-17 was immunoprecipitated from cell lysates and analyzed on nonreducing gels. Fig. 1 shows that full-length apoB-17 was detected within 4 min after the beginning of the pulse (lane 3). Furthermore, nascent apoB-17 even at its earliest appearance migrated faster than its reduced counterpart (compare lane 3 to lane 1), and its mobility did not change with time (lanes 3–10), indicating that most if not all the native disulfides were formed either co- or immediately post-translationally.

**DTT Blocks the Secretion of ApoB-17 in a Reversible Fashion without Perturbing the Secretory Pathway**—To determine whether the formation of native disulfides is critical for folding and secretion of apoB we used DTT, to prevent the formation of disulfides by changing the redox state in the ER (35). First, it was necessary to ensure that exposure to DTT does not perturb

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**FIG. 1.** Disulfide bonds in apoB-17 are formed co-translationally. Cells were incubated in medium lacking methionine and cysteine for 15 min. 30 μg Puromycin was added and incubation continued for additional 45 min in the same medium. Cells were then washed and pulse-labeled for the indicated times with [35S]methionine/cysteine. ApoB-17 was immunoprecipitated with polyclonal antibodies to apoB, resolved on 7% nonreducing SDS-PAGE, and imaged by PhosphorImager. The sample applied to lane 1 was reduced with 20 mM DTT to serve as a reference for fully reduced apoB-17. Note a steady increase in labeling of apoB-17.
the secretory pathway in C127 cells. To that end we studied the effects of 2 or 4 mM DTT on the secretion of apoA-I which lacks disulfide bonds. C127 cells transfected to express and secrete apoA-I (47) were pulse-labeled for 4 min with or without 2 or 4 mM DTT, and then chased for 2 h in the presence or absence of the corresponding concentration of DTT. Such experiments showed that the amount of labeled apoA-I secreted into the medium following incubation with DTT was indistinguishable from the corresponding controls (data not shown). Thus, the secretory pathway in C127 cells is not perturbed by either 2 or 4 mM DTT.

Next, we determined the effects of 4 mM DTT on the secretion of apoB-17 (15). Cells were pulse-labeled for 4 min in the presence of 4 mM DTT, washed, and then chased for 2 h with or without DTT. A, secreted and cellular apoB-17 was immunoprecipitated as described in the legend to Fig. 1, resolved by 7% SDS-PAGE under reducing conditions, imaged by PhosphorImager, and quantified using “Imagequant” software. The amount of apoB-17 (arbitrary units) in cells and media recovered after 2 h of chase was normalized to the amount of cellular protein and expressed as percent of the pool synthesized during the pulse. The data represent the mean ± S.E. of four independent experiments. 2 of which were carried out in duplicates. Black and gray bars represent cellular and secreted apoB-17, respectively. Asterisk indicates p = 0.0001. B, the migration of apoB-17 recovered from cells incubated under the conditions shown in panel A was analyzed on nonreducing 7% SDS-PAGE.

FIG. 2. DTT prevents disulfide bond formation in apoB-17 and blocks its secretion in a reversible manner. Cells were pulse-labeled for 4 min in the presence or absence of 4 mM DTT, washed, and then chased for 2 h with or without DTT. A, secreted and cellular apoB-17 was immunoprecipitated as described in the legend to Fig. 1, resolved by 7% SDS-PAGE under reducing conditions, imaged by PhosphorImager, and quantified using “Imagequant” software. The amount of apoB-17 (arbitrary units) in cells and media recovered after 2 h of chase was normalized to the amount of cellular protein and expressed as percent of the pool synthesized during the pulse. The data represent the mean ± S.E. of four independent experiments, 2 of which were carried out in duplicates. Black and gray bars represent cellular and secreted apoB-17, respectively. Asterisk indicates p = 0.0001. B, the migration of apoB-17 recovered from cells incubated under the conditions shown in panel A was analyzed on nonreducing 7% SDS-PAGE.
not shown). These data suggest that post-translational formation of disulfide bonds after removing DTT occurred rather quickly but not as rapid as co-translational disulfide bonding.

**DTT Blocks the Secretion of ApoB-29 in a Reversible Manner**—Next, apoB-29, which contains 7 of the 8 disulfides in apoB-100 (43) was analyzed. Although a small fraction is secreted lipid-poor, the majority of the secreted pool of apoB-29 is associated with particles containing about 26% lipids (by weight), most of which are polar lipids such as phospholipids (PL) and diacylglycerols. As depicted in Fig. 3 about 35% of the labeled pool was secreted by 2 h, and about 80% of the pool synthesized during the pulse was recovered at the end of the chase. Like apoB-17, DTT blocked the secretion of apoB-29 both co- and post-translationally (p = 0.0001). Furthermore, it was rescued by removing DTT from the chase, so that it could undergo post-translational disulfide bonding and subsequent maturation into its secretion-competent form. Reduced apoB-29 like apoB-17 was relatively stable, as about 60% was recovered after a 2-h chase in media containing DTT.

**DTT Blocks the Secretion of ApoB-41 in a Reversible Manner**—ApoB-41 like apoB-29 contains 7 disulfides (43). Unlike apoB-17 or apoB-29, it absolutely requires lipids, both TAG and PL, for its folding and is secreted exclusively on large high density lipoprotein-like lipoproteins containing a TAG-rich core (44). As depicted in Fig. 4, only about 17% of apoB-41 pool synthesized during the pulse was secreted by 2-h of chase, presumably due to insufficient lipids (primarily TAG) available to form TAG-rich lipoproteins. Furthermore, unlike apoB-17 and apoB-29, only 70% of the pool of apoB-41 synthesized during the pulse was recovered after a 2-h chase, indicating that about 30% was degraded. Despite the differences between apoB-41 and the shorter forms of apoB in their dependence on lipidation, the effects of DTT on the secretion of apoB-41 were very similar to those observed for both apoB-17 and apoB-29. Thus, the secretion of apoB-41 was blocked by DTT when added either co- or post-translationally (p = 0.0001), and its inhibitory effects on the secretion of apoB-41 were fully reversible if DTT was removed from the chase medium (Fig. 4). The amount of apoB-41 recovered by the end of the chase in the presence of DTT was similar to control levels, indicating that reduced apoB-41 did not exhibit increased propensity to degradation compared with controls. The slight increase in the recovery of apoB-41 following addition of DTT into the chase supports the presumed DTT-sensitive protease in the secretory pathway (48).

**Half of ApoB-29 Pool Folds into a DTT-resistant Form within 5 Min of Chase Compared with 45 Min Required for ApoB-41 to Achieve DTT Resistance**—DTT at relatively low concentrations reduces disulfide bonds during early stages of folding since they are accessible to the surrounding milieu. However, as folding progresses, disulfide bonds become inaccessible to reduction and the protein is expected to complete its folding and be secreted. The experiments depicted in Figs. 2–4 demonstrated that the addition of DTT immediately post-translationally was still effective in preventing the folding of apoB into a secretion-competent form, suggesting that the folding of all apoB forms into a DTT-resistant form is relatively slow. To estimate the half-time of the folding of apoB into a DTT-resistant form, cells were pulse-labeled without DTT to allow the formation of native disulfides, and then chased for 2 h. DTT was added into the chase immediately (chase 0), or after specified times (e.g. 5, 15, 45, and 90 min). As depicted in Fig. 5 neither apoB-29 nor apoB-41 were DTT-resistant immediately after their synthesis, as only about 26 and 12% of the respective control levels were secreted. Even at 15 min into the chase the majority of the labeled pool of apoB-41, about 70%, was still sensitive to DTT reduction. However, after 45 min half of the pool destined for secretion achieved DTT resistance, and by 90 min it was completely secreted. In contrast, 50% of the secretable pool of apoB-29 became resistant to DTT within 5 min of chase, and by 45 min it was fully resistant.

**Prolonged Exposure to DTT Progressively Diminishes the Ability of ApoB-41 to Fold Post-translationally into a Secretion-competent Form**—The experiments shown in Figs. 2–4 indicated that the inhibitory effects of DTT on the secretion of all 3 apoB forms were fully reversible, if DTT was removed from the chase media. Thus, following a relatively short exposure to DTT, the reduced, secretion-incompetent apoB polypeptides could refold post-translationally. It would be expected, however, that upon longer exposure to DTT reduced, misfolded apoB will be ultimately targeted for degradation by the quality control in the ER (49). We therefore, examined how long can reduced apoB-41 remain in the cells in a form that is still competent for refolding into its native form upon removal of DTT. We chose apoB-41 since its folding is absolutely depend-
The amount of apoB secreted following a 2-h chase without DTT was expressed as percent of control values recovered from both cells and media incubated without DTT. These findings suggest that apoB-41 was irreversibly misfolded presumably because it failed to refold into a secretion-competent form following a prolonged exposure to DTT. Interestingly, however, a major fraction of apoB-41, about 90% of control levels was still present in the cells after 90 min exposure to DTT (Fig. 6), corresponding to about 70% of control levels, accumulated in the media. However, after 60 and 90 min of chase with DTT, only about 17 and 9%, respectively, of control values was secreted, indicating that apoB failed to refold into a secretion-competent form following a prolonged exposure to DTT. Interestingly, however, a major fraction of apoB-41, about 90% of control levels was still present in the cells after 90 min exposure to DTT (Fig. 6), corresponding to about 70% of control values recovered from both cells and media incubated without DTT. These findings suggest that apoB-41 was irreversibly misfolded presumably because it was targeted for degradation. However, the actual degradation process seemed to be rather slow.

**DISCUSSION**

Disulfide bonds often stabilize the structure of proteins. Therefore, perturbation of their formation by reducing agents such as DTT reportedly leads to a failure of such proteins to fold into their native forms (35, 36). In the present report truncated forms of human apoB with different requirements for lipiddation expressed in a heterologous system have been tested for their sensitivity to DTT to assess the role of proper disulfide bonding in the folding and secretion of apoB. The studies presented clearly demonstrate that despite substantial differences in their lipiddation, apoB-17, apoB-29, and apoB-41 all showed sensitivity to DTT. Thus, exposure to DTT either co- or immediately post-translationally led to a failure of all forms to achieve secretion competence (Figs. 2–4) which resulted in their intracellular accumulation presumably in a reduced form (Fig. 2B). Thus, consistent with other reports (40, 42, 52, 53), the formation of native disulfide bridges is an early event in the folding of apoB, which is necessary for subsequent folding steps to take place, regardless of length or requirement for lipiddation to be secreted. Nevertheless, some differences between these apoB forms are evident. While addition of DTT post-translationally resulted in a significant reduction in the secretion of all forms (Figs. 2–4), the magnitude of the reduction differed, such that the secretion of apoB-17, apoB-29, and apoB-41 corresponded, respectively, to 6, 25, and 10% of control values. Thus, a significantly ($p < 0.05$) larger fraction of the nascent pool of apoB-29 was secreted compared with both apoB-17 and apoB-41, indicating that apoB-29 folded faster than apoB-17 and apoB-41 into a DTT-resistant form. In fact, within 5 min of chase 50% of the nascent pool of apoB-29 destined for secretion folded into a DTT-resistant form, while it took about nine times as long for 50% of the secretable pool of apoB-41 to become insensitive to DTT (Fig. 5). Thus, disulfide bonds in apoB-29 became rapidly inaccessible to the reducing milieu generated by DTT, and it achieved DTT resistance long before its folding was completed as judged by its much slower rate of secretion (data not shown). However, it remains undetermined whether all or only those disulfides critical for the secretion of apoB (52, 53) became rapidly solvent inaccessible to confer DTT resistance. Consistent with the findings reported here, apoB-28 (which is identical to apoB-29 in our study) expressed in either hepatoma cells which express MTP (e.g. HepG2 or McA-RH7777 cells) or in Chinese hamster ovary cells which lack MTP, similarly folded very rapidly into a DTT-resistant form (42). Remarkably, apoB-29 in C127 cells became rapidly resistant to 4 mM DTT that is double the concentration used in

**FIG. 5.** ApoB-29 folds into a DTT-resistant form faster than apoB-41. Cells expressing apoB-29 or apoB-41 were pulse-labeled for 5 and 15 min, respectively, and then chased for 2 h without DTT (controls). DTT was added into the chase of a second set of cells at the indicated times. Secreted apoB was immunoprecipitated, resolved by 7% SDS-PAGE, and quantified as described in the legend to Fig. 2A. The amount of apoB secreted following a 2-h chase without DTT was expressed as percent of the pool synthesized during the pulse. This value was set at 100%. ApoB secreted following addition of DTT into the chase was expressed as percent of the pool synthesized during the pulse and then expressed as percent of apoB secreted by control cells. The data represent the mean ± S.D. of three independent experiments carried out in duplicates, except for time points 5 and 60 min (apoB-29) which represent the mean of two independent experiments.

**FIG. 6.** Prolonged exposure to DTT progressively diminishes the ability of apoB-41 to fold post-translationally into a secretion-competent form. Cells were pulse-labeled for 15 min in the presence of DTT and then chased with DTT. At the indicated times cells were washed in medium lacking DTT and incubated for 2 h in the same medium to allow for folding and secretion. A parallel set of cells was pulsed and chased without DTT, and then washed and incubated for additional 2 h without DTT. Cellular and secreted apoB-41 was resolved by 7% SDS-PAGE and quantified as described in the legend to Fig. 2A. The amount of apoB recovered at the end of the incubation without DTT was expressed as percent of the pool synthesized during the pulse and was set at 100% (controls). The amount of apoB recovered following different exposure periods to DTT was expressed as percent of the pool synthesized during pulse in the presence of DTT and then expressed as percent of apoB recovered under control conditions. The data represent the mean ± S.D. of three independent experiments carried out in duplicate, except for time points 15 and 90 min which represent the mean of two independent experiments.

**FIG. 5.** ApoB-29 folds into a DTT-resistant form faster than apoB-41. Cells expressing apoB-29 or apoB-41 were pulse-labeled for 5 and 15 min, respectively, and then chased for 2 h without DTT (controls). DTT was added into the chase of a second set of cells at the indicated times. Secreted apoB was immunoprecipitated, resolved by 7% SDS-PAGE, and quantified as described in the legend to Fig. 2A. The amount of apoB secreted following a 2-h chase without DTT was expressed as percent of the pool synthesized during the pulse. This value was set at 100%. ApoB secreted following addition of DTT into the chase was expressed as percent of the pool synthesized during the pulse and then expressed as percent of apoB secreted by control cells. The data represent the mean ± S.D. of three independent experiments carried out in duplicates, except for time points 5 and 60 min (apoB-29) which represent the mean of two independent experiments.
Disulfide Bonds in ApoB Folding

**Fig. 7.** Folding intermediates during the maturation of apoB. Under oxidizing conditions (e.g. in the absence of DTT), native disulfide bonds are formed either co- or immediately post-translationally to yield intermediate 1 which is DTT-sensitive. With time, intermediate 1 folds into a more advanced form, intermediate 2, which is DTT-resistant but is presumably still secretion-incompetent. Intermediate 2 further folds into a secretion-competent form, and is ultimately secreted. When apoB is synthesized in the presence of DTT disulfide bonds are not formed and reduced apoB (intermediate 3) accumulates in the cells as it is incapable of achieving a mature form (and is therefore secretion-incompetent). Intermediate 3 is also formed when DTT is added shortly after synthesis, since the native disulfides that formed co-translationally are solvent accessible and therefore prone to DTT-induced reduction. However, if DTT is removed after a short exposure, native disulfide bonds form post-translationally to yield oxidized apoB (intermediate 1) that can then proceed on to successfully fold into a secretion-competent form. However, a prolonged exposure to DTT (1 h or greater) diminishes the capacity of reduced apoB to fold post-translationally (e.g. it is irreversibly misfolded) and therefore, accumulates in the cells as reduced, misfolded protein (intermediate 4) that is ultimately targeted for degradation.

HepG2 and Chinese hamster ovary cells (42). The findings reported by Ingram and Shelness (42) together with those presented in this report strongly suggest that the rapid folding of apoB-29 into a DTT-resistant form is independent of MTP or other cell-specific factors. However, folding of apoB-29 into a DTT-resistant intermediate does not determine its final secreted form, nor does it ensure that it can attain a secretion-competent form (42). Secretion competence appears to be dependent on the cell type. Thus, when expressed in hepatoma cells (e.g. HepG2 or McA-RH777 7cells) apoB-28 proceeds to bind sufficient lipids to be secreted on lipoproteins with a peak density of 1.17 g/ml (42). However, when expressed in C127 cells apoB-29 is secreted either lipid-free or associated with lipoproteins containing primarily polar lipids, such as diacylglycerol and PL with a peak density of 1.24 g/ml (42). Yet in Chinese hamster ovary cells apoB-28 is incapable of achieving secretion competence and requires co-expression of MTP to be secreted (42). Thus, folding events that occur after apoB-29 folds into a DTT-resistant form are critical for its maturation into its secretion-competent form and appear to be dependent on cell-specific factors.

In contrast to apoB-29, disulfides in apoB-41 remained solvent accessible for a prolonged period of time. Furthermore, the rate it achieved resistance to DTT almost paralleled its secretion rate (data not shown), suggesting that at least those disulfides critical for the secretion of apoB (52, 53) became solvent inaccessible just before it attained its fully folded form. Although unlikely, it is also possible that a sub-pool of apoB-41 can achieve secretion competence even when some of its disulfides remain solvent accessible, and therefore, DTT-sensitive.

The differences in the rate by which apoB-41 and apoB-29 achieve DTT resistance suggest that they proceed through different intermediates to attain their mature form. Indeed, while apoB-29 is secreted primarily with PL and diacylglycerols, apoB-41 requires a TAG-rich core to be secreted (44). However, lipidation alone may not be responsible for these differences. First, apoB-17 which is secreted primarily unlipidated (15) like apoB-41 also appears to be more sensitive to DTT compared with apoB-29, as only 6% of apoB-17 was secreted when DTT was added into the chase media, compared with 25% of apoB-29 (compare Figs. 2a and 3). Second, apoB-100 in HepG2 cells despite its dependence on lipidation folds very rapidly into a DTT-resistant form (40). Thus, other factors appear to play a role in the folding process. First, C127 cells do not express MTP which is essential for the lipidation of apoB in vivo (16) and in all other cells studied to date (54–57). Based on the structure of lipovitellin (58) and its homology to apoB and MTP (59, 60) it was proposed that an incomplete “lipid pocket” could form within the N-terminal region apoB (60), since it is missing the β-sheet that lines the bottom of the putative lipid pocket in lipovitellin. MTP may provide the missing β-sheet upon its binding to apoB, thereby promoting the formation of a complete lipid pocket (60). The presence of a putative complete lipid pocket in apoB may play an important role in the initial steps of recruiting PL and TAG (60) so that the completion of the proposed first step in the process of VLDL assembly to form the primordial particles (50, 51) is rather fast. In the absence of MTP in C127 cells, the putative incomplete lipid pocket in apoB-41 may lead to a slower, and perhaps less efficient lipidation process during which disulfides may remain solvent accessible longer. It is also possible that TAG recruitment in C127 cells proceeds via an alternative mechanism, which may be mediated by an MTP analogue. If so, its presumed binding to apoB-41 to mediate lipidation may change the conformation of apoB-41 to allow for at least the disulfides critical for its secretion to remain solvent accessible until lipidation is complete.
Second, the differences between apoB-100 and apoB-41 could stem from a higher requirement of apoB-100 to MTP to form lipid-rich lipoproteins compared with forms shorter than apoB-53 which can be secreted following the formation of high density lipoprotein-like lipoproteins (61). Third, the maturation of apoB like other secretory proteins seems to be mediated by molecular chaperones (55, 62–65). We have demonstrated that ER-resident molecular chaperones, such as GRP94, ERP72, and calreticulin interact both with apoB-100 in HepG2 cells and with apoB-17, -29, and -41 in C127 cells. We further showed that the interactions between these chaperones and apoB-100 have different characteristics compared with truncated apoB forms (64). Such differences may partly account for differences between full-length and truncated apoB forms in the rate and the path of folding.

Two other important features characterize the model system described in this study. First, reduced apoB-41, when synthesized in the presence of either 2 or 4 mM DTT, can fold and be secreted upon DTT removal (3) (Fig. 4). Thus, the formation of native disulfide bonds as well as lipidation can occur post-translationally allowing apoB-41 to achieve secretion competence. This has a clear advantage of isolating distinct intermediates of apoB that lead to the formation of small core-containing lipoproteins.

Second, reduced, misfolded apoB-41 is not prone to rapid degradation even after exposure to 4 mM DTT for as long as 1 h albeit it is incapable to fold into a secretion-competent form (Fig. 6). In contrast, reduced apoB-100 synthesized in the presence of 2 mM DTT remains irreversibly misfolded even when chased in media lacking DTT, as it is unable to achieve secretion competence (40). Furthermore, it was demonstrated that following exposure of HepG2 cells to DTT a larger fraction of the recovered pool of apoB remained associated with the translocon and was ubiquitinated (63). This form is presumably rapidly degraded, consistent with its lower recovery following 1 h exposure to DTT (40, 41). In general, misfolded or incorrectly folded proteins are targeted for degradation, by molecular chaperones which provide quality control in the ER (49). Thus, molecular chaperones not only assist in the efficient translocation of apoB-100. Consequently, C-terminal domains may be exposed to the cytosol, thereby becoming accessible to ubiquitinating enzymes (63). This may lead to increased susceptibility to proteasome-mediated degradation (23). Reduced apoB-41 on the other hand may be efficiently translocated into the ER despite impaired disulfide bonding, being less accessible to the cytosolic degradative machinery. Whether misfolded apoB-41 is ultimately targeted for proteasome-dependent degradation remains to be determined. Interestingly, following exposure to DTT the interaction of ERP72 and GRP94 both with apoB-41 and apoB-100 is elevated presumably to prevent aggregation and attempt to mediate folding. These interactions, however, do not lead to post-translational folding of apoB-100 or apoB-41 after a prolonged exposure to DTT (Figs. 6 and 7).

In Summary—Taken together our study suggests that (a) proper disulfide bonding is a critical step in the folding of apoB forms expressed in C127 cells regardless of their lipidation state. Thus, the capacity of apoB-41 to recruit TAG cannot overcome DTT-induced misfolding. (b) Unlike apoB-29, apoB-41 remains DTT-sensitive for the major part of its folding. (c) Disulfide bonding and subsequent folding steps, including lipidation can efficiently occur post-translationally in C127 cells. Thus, through the use of DTT at least four distinct intermediates in the folding path of apoB were identified (Fig. 7). Together with the fact that misfolded apoB forms in these cells unlike apoB-100 appear to be sequestered away from the degradative machinery for a prolonged period of time, these cells provide a unique model system for further detailed analysis of the folding of apoB including subcellular localization, lipidation, and characterization of cellular factors (e.g. folding catalysts and molecular chaperones) involved in the folding of apoB.

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