Folding of the Amino-terminal Domain of Apolipoprotein B Initiates Microsomal Triglyceride Transfer Protein-dependent Lipid Transfer to Nascent Very Low Density Lipoprotein

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The initial assembly of apolipoprotein B100 (apoB) into lipoprotein particles occurs cotranslationally. To examine steps required to initiate this process, the intracellular folding and assembly of the amino-terminal 28% of apoB (apoB28) was examined using several criteria including nonreducing gel electrophoresis, sensitivity to dithiothreitol (DTT)-mediated reduction, and buoyant density gradient centrifugation. In hepatoma cells, after a 1 min pulse with radiolabeled amino acids, labeled apoB28 migrated during gel electrophoresis in the folded position and was resistant to reduction in vivo with 2 mM DTT. A similar rate and extent of folding was observed in Chinese hamster ovary cells, a microsomal triglyceride transfer protein (MTP)-negative cell line that can neither lipitate nor efficiently secrete apoB28. Amino-terminal folding of apoB28 was essential for its subsequent intracellular lipidation as apoB28 synthesized in hepatoma cells under reducing conditions remained lipid poor (d > 1.25 g/ml) and was retained intracellularly. Upon DTT removal, reduced apoB28 underwent a process of rapid (t1/2 ~ 2 min) post-translational folding followed by a slower process of MTP-dependent lipidation. As with the cotranslational assembly pathway, post-translational lipidation of apoB28 displayed a strict dependence upon amino-terminal folding. We conclude that: 1) folding of the amino-terminal disulfide bonded domain of apoB is achieved prior to the completion of translation and is independent of MTP and events associated with buoyant lipoprotein formation and 2) domain-specific folding of apoBs amino-terminal region is required to initiate MTP-dependent lipid transfer to nascent apoB in the hepatic endoplasmic reticulum.

In addition to the protein and lipid biosynthetic capacities present in most cell types, the biogenesis of very low density lipoprotein requires at least two dedicated gene products: apoB, the major protein component of very low density lipoprotein (1–6), and the microsomal triglyceride transfer protein (MTP), a soluble lipid transfer protein localized to the ER of hepatocytes and intestinal epithelial cells (7). As the initial assembly of apoB with lipids occurs cotranslationally (8), it was predicted that the amino-terminal domain may play a critical role in initiating the process of lipoprotein particle assembly (7, 9). Indeed, the amino-terminal ~18% of apoB (apoB18) is atypical relative to the rest of the protein: (i) it is globular and highly disulfide bonded and is positioned within the amino-terminal 11% of the protein (10), (ii) it demonstrates a relatively lower affinity for plasma low density lipoprotein particles than internal and carboxyl-terminal domains (2, 11–13), and (iii) in transfected cells, apoB18 lacks the capacity to recruit a significant amount of lipid and its secretion can be achieved independently of MTP (14–17).

While the amino-terminal domain of apoB is incapable of forming core-containing lipoproteins on its own, several lines of evidence suggest that it nonetheless plays an essential role in lipoprotein secretion. When disulfide bond formation was disrupted in the amino-terminal domain of apoB by preincubating HepG2 cells with 2 mM DTT, the ability of apoB to form a secretable lipoprotein was irreversibly blocked (9). In addition, internal domains of apoB, which are otherwise incapable of undergoing secretion, could be rendered secretion competent by appending them to the carboxyl-terminal end of apoB17 (18). While proper folding of the amino-terminal domain of apoB appears essential to promote its secretion, due to apoB's large size (4,536 amino acid residues) and hydrophobicity, it has not yet been experimentally established which stage of the lipoprotein assembly and secretion process is dependent upon this amino-terminal folding event.

In the current report, this and other mechanistic aspects of the assembly and secretion of apoB-containing lipoproteins were explored by focusing on the biogenesis of the amino-terminal 28% of apoB (apoB28). This form of apoB has been shown previously to be of sufficient size to undergo secretion in the form of a buoyant, lipid core-containing lipoprotein particle (8, 14, 15, 19). Furthermore, the secretion of apoB28 is MTP-dependent, a hallmark of apoB biogenesis (7, 20–22). While capable of MTP-dependent lipoprotein assembly, the apoB28 polypeptide chain is sufficiently small (1270 amino acid residues) to enable resolution of reduced and folded forms by non-reducing SDS-PAGE. This allowed us, using techniques that are not applicable to full-length apoB48 or apoB100, to directly monitor the relationship between intracellular folding of apoB and its capacity to undergo MTP-dependent lipidation in the ER.

The studies described here indicate that folding of the amino-terminal domain of apoB28 occurs either during or shortly after translation and can be achieved independently of MTP. Furthermore, the data indicate that folding of this domain is essential for apoB28's capacity to undergo subsequent MTP-dependent assembly with lipids. The requirement for amino-
terminal folding was observed for both the normal cotranslational assembly pathway as well as during the post-translational folding and lipidation that occurs when apoB28 was artificially reduced in the ER and then allowed to refold. These studies provide compelling evidence that the initiation of lipoprotein formation in the hepatic ER proceeds via two distinct stages: the amino-terminal globular domain of apoB undergoes a cotranslational folding event that occurs autonomously and prior to appreciable lipid recruitment. Once completed, this folding event facilitates the capacity of apoB's downstream sequences to undergo MTP-dependent assembly with lipids. The requirements for the production of small, dense apoB28-containing lipoprotein particles described here, may also be necessary to initiate the formation of native lipoproteins containing apoB48 or -100.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction and DNA modification enzymes were from New England Biolabs. Tissue culture media and supplements were obtained from ICN (Irvine, CA) or MediaTech (Washington, D.C.). Cytochrome c, DTT, and IAA were from Sigma. Tran 35S-label (an ~5:1 mixture of [35S]Met and Cys) was from ICN. Anti-FLAG M2 monoclonal antibody was from Eastman Kodak Scientific Imaging Systems (New Haven, CT). Polyclonal antibodies to human apoB100 and albumin were obtained from Boehringer Mannheim. Centricon-10 and Centriprep-10 centrifugal concentrators were from Amicon (Beverly, MA).

**Construction of an apoB28-containing Expression Plasmid**—A form of apoB28 containing a 8-amino acid carboxyl-terminal epitope tag was cloned to plasmid pB53LII (obtained from Dr. Zemin Yao, University of Ottawa) was ligated to containing plasmid pSV2neo (29) at a mass ratio of 20:1 as described (28). Stably transfected CHO cells were selected in 700 g/ml G418. Individual clones were assayed (Figs. 6 and 7), cycloheximide was included in the chase to block new protein synthesis. After the pulse in the presence of DTT, chase media was supplemented with 35 g/ml L-proline. Labeling was performed in the presence DTT.

**Gradient Fractionation of Intracellular ApoB28**—Post-nuclear membranes were prepared by Dounce homogenization as described previously (26). Membranes were suspended in 300 g/ml of membrane buffer (26) and adjusted to 0.1 mM sodium carbonate, pH 11.5, by addition of 0.1 M stock. Samples were diluted to 2 ml with 100 mM sodium carbonate, pH 11.5, and incubated on ice for 60 min with occasional low speed vortexing. Membranes were pelleted by centrifugation at 100,000 rpm (412,000 g) for 15 min at 4 °C in a Beckman TL100 tabletop ultracentrifuge using the TLA 100.3 rotor. Supernatant fractions were transferred to clear polystyrene tubes and adjusted to 0.005% phenol red, 180 mM NaCl, pH 7.5, 300 mM bovine serum albumin and sufficient dilute HCl (~108 g/ml of a 1.5 dilution of concentrated HCl) to bring the pH to 7.5. The neutralized sample was adjusted to 1.25 g/ml with solid KBr and a final volume of 3 ml. Samples were transferred to polylamellar quiescent tube, and centrifuged in the TLA 100.3 rotor at 100,000 rpm for 12–15 h at 15 °C. The top 1 ml (< 1.25 g/ml) and bottom 2 ml (d > 1.25 g/ml) fractions were recovered with a tube slicer (Beckman Instruments). Fractions were concentrated to ~50 il using Centricon 10 centrifugal concentrators (Amicon) and diluted to 2 ml with phosphate-buffered saline. After another round of centrifugation/concentration, samples were diluted into 1 ml of lysis buffer (1% Triton X-100, 25 mM Tris-HCl, pH 7.5, 300 mM NaCl, 2.5 mM bovine serum albumin, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 1 mM p-mercaptoethanol) and subjected to immunoprecipitation with an anti-FLAG M2 monoclonal antibody as described (26). Pellets from carbonate extraction were washed 2 × with 1 ml of TBS and resuspended in 200 μl of 1% SDS, 25 mM Tris, pH 7.4, and boiled for 10 min. Samples were diluted with 800 μl of lysis buffer and subjected to immunoprecipitation as described (26).

**Density Gradient Centrifugation of Secretory ApoB28**—Eight ml of 1.25 g/ml KBr in phosphate-buffered saline was added to an SW41 polylamellar centrifuge tube (Beckman Instruments) and overlaid with 1 ml of conditioned media that had been adjusted to 1.20 g/ml with solid KBr. The 1-ml sample was then overlaid with 3 ml of 1.05 g/ml tubes. Tubes were centrifuged at 37,000 rpm (160,000 × g) for 48 h at 20 °C in an SW41 rotor. In Fig. 7, 9 ml of conditioned media were first centrifuged to 1 ml using a Centricon Centriprep-10 concentrator prior to adjusting to 1.20 g/ml. Tubes were fractionated from the top using an Autodensiflow Gradient Fractionator (Labconco Industries) into 1 ml samples. The density of each fraction was determined gravimetrically. For immunoprecipitation, gradient fractions were exchanged into phosphate-buffered saline using Centricon-10 centrifugal concentrators as described above. After diluting into lysis buffer, fractions were immunoprecipitated with anti-FLAG M2 monoclonal antibody.

**RESULTS**

**ApoB28F: A Model System for Examination of Early Stages of Hepatic Very Low Density Lipoprotein Assembly**—Because of the cotranslational kinetics of lipoprotein assembly, steps required to initiate small particles formed by the amino-terminal ~25% of apoB may be the same as those required for apoB48 and apoB100-containing lipoproteins. We therefore investigated whether an epitope-tagged form of apoB28 could serve as an appropriate model system for analyzing the relationship between apoB folding and subsequent steps required for the initiation of lipoprotein particle assembly.

A plasmid encoding the amino-terminal 28% of apoB with the 8-amino acid FLAG epitope (23, 24) and termination codon appended to its carboxyl terminus (apoB28F; “Experimental Procedures”) was transiently transfected into HepG2 cells (a
ApoB28F Undergoes Rapid Folding into a DTT Resistant Form—Utilizing both mobility changes during nonreducing SDS-PAGE as well as onset of DTT resistance as criteria, the folding kinetics of apoB28F were examined in transfected McA-RH7777 cells. The underlying basis for the ability of proteins to undergo a transition from DTT sensitivity to DTT resistance in the ER is not fully understood. The transition may reflect conformational changes in the protein and/or different extents or stages of chaperone association (34, 35). Nonetheless, for several model proteins previously examined, the rate of onset of DTT resistance in the ER closely parallels the time required to achieve a native conformation competent for anterograde transport in the secretory pathway (34, 36).

Stably transfected McA-RH7777 cells were pulse-labeled for 1 min with [35S]Met/Cys and chased for various times prior to adjusting the chase media to 2 mM DTT (30). After incubating with DTT for 5 min (a condition that allows elongation of nascent polypeptide chains (37)), cells were placed on ice and treated with an excess of IAA (30). ApoB28F immunoprecipitated from cell lysates was resolved by nonreducing SDS-PAGE. As observed in Fig. 2A, lanes 1–7, the predominant form of apoB28F migrated with the folded standard (lane R) and at no time after the pulse did addition of DTT cause appreciable reduction. Since proteins labeled during the 1-min pulse were, by definition, nascent polypeptides at the time that [35S]Met/Cys was added to the cells, and it is estimated that −3−4 min is required to synthesize apoB28F (~300–400 amino acids per minute (37)), these results indicate that the disulfide bonds in apoB28F progressed to the DTT resistant form either before or shortly after (within 1 min) translation was completed. Although the anti-FLAG antibody allowed us to monitor only full-length protein, use of a polyclonal antibody with the potential to recognize amino-terminal epitopes of apoB28F (38) failed to detect an appreciable spectrum of nascent polypeptide chains under our labeling conditions (not shown). Furthermore, it may be difficult to detect and interpret SDS-PAGE mobility shifts based on differences in disulfide bond formation within a spectrum of nascent polypeptide chains.

The rapid onset of DTT resistance observed for apoB28F is in marked contrast to all other disulfide bonded proteins examined to date which undergo a time-dependent post-translational transition from DTT sensitivity to DTT resistance in the ER (9, 34, 36, 39, 40). As an example, the onset of DTT resistance of albumin in HepG2 cells was examined. Although 11 of the 17 disulfide bonds in albumin form cotranslationally, with the remainder forming almost immediately after translation (41, 42), the disulfide bonds in albumin remained sensitive to
Met/Cys. Post-nuclear membranes were prepared and sub-some nonhepatic cell lines (43). McA-RH7777 and CHO cells, apoB28F by the MTP-independent pathway that may exist in explored whether or not CHO cells were capable of lipidating capacity to form secretable lipoproteins (17). Although CHO tors such as MTP. For this purpose, apoB28F was stably trans-

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tions—

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FIG. 3. Folding of the amino-terminal domain of apoB occurs independently of MTP and buoyant lipoprotein formation. In A, five 100-mm dishes each of CHO and McA-RH7777 cells (McA) stably transfected with apoB28F were labeled for 2 h with [35S]Met/Cys. A total post-nuclear membrane fraction was prepared and subjected to extraction with 0.1 M sodium carbonate, pH 11.5. After centrifugation, the pellet fraction (P) was subjected to immunoprecipitation with the anti-FLAG M2 monoclonal antibody. The supernatant fractions (S) were adjusted to 1.25 g/ml with KBr (final volume ~3 ml) and subjected to equilibrium density gradient centrifugation. The top 1 ml (T) and bottom 2 ml (B) of each gradient were immunoprecipitated with anti-FLAG M2 monoclonal antibody. Immunoprecipitates from carbonate pellet and gradient fractionated supernatant fractions were analyzed by reducing SDS-PAGE and fluorography. In B, stably transfected CHO cells were pulse-labeled and chased in the presence of DTT as described for Fig. 2.

FIG. 4. Reduced apoB28F is unable to form a buoyant li-

The first period of DTT sensitivity in the ER (34, 36, 40).

dT-mediated reduction well after completion of synthesis (Fig. 2A, lane 1). Approximately 3 min was required for half of the labeled albumin to achieve DTT resistance after a 1-min pulse (Fig. 2B, lane 4). Other disulfide bonded secretory pro-
tests examined in this way often display considerably longer periods of DTT sensitivity in the ER (34, 36, 40).

Rapid Folding of the Amino-terminal Domain of ApoB Oc-
curs Independently of MTP and Buoyant Lipoprotein Forma-

tion—We investigated whether the rapid folding of apoB28F, as measured by its almost immediate onset of DTT resistance, was an autonomous property of the apoB protein or whether folding was dependent upon the presence of liver specific factors such as MTP. For this purpose, apoB28F was stably trans-
fected into CHO cells, an MTP negative cell line that lacks the capacity to form secretable lipoproteins (17). Although CHO cells cannot efficiently secrete apoB28F (not shown), we also explored whether or not CHO cells were capable of lipidating apoB28F by the MTP-independent pathway that may exist in some nonhepatic cell lines (43). McA-RH7777 and CHO cells, stably transfected with apoB28F, were labeled for 2 h with [35S]Met/Cys. Post-nuclear membranes were prepared and sub-

To examine the intracellular consequences of blocking amino-terminal folding, the buoyant density of apoB28F extracted from cells that were labeled under control and reducing conditions was examined. For these experiments transiently transfected HepG2 cells were used because, unlike McA-

RH7777 cells, they express detectable amounts of serum albumin which is useful as an endogenous disulfide bonded control protein (not shown). Forty-eight hours after transfection, HepG2 cells were pulse-labeled for 10 min in the absence or presence of 2 mM DTT. Where DTT was used, it was added 1.5 min prior to addition of label. Under these conditions, ~80% of the labeled pool of apoB28F is reduced (“Experimental Procedures”). After metabolic labeling, post-nuclear membrane frac-
tions were prepared from control and DTT-treated cells and extracted with sodium carbonate, pH 11.5, to release their lumenal contents. Pellet and supernatant fractions were ana-

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DTT was added to transfected CHO cells immediately after the 1-min pulse, the folded form of apoB28F represented ~70% of total (Fig. 3B, lane 2). This result was somewhat different from that obtained in McA-RH7777 cells where ~90% of apoB28F resisted DTT immediately after the 1-min pulse (Fig. 2A, lane 1). Both of these results, however, were distinct from the behavior of albumin (Fig. 2B) and other proteins whose disulfide bonds remain completely sensitive to DTT-mediated reduction for a characteristic period of time after completion of translation. That the majority of apoB28F in CHO cells achieved DTT resistance immediately after the pulse indicated that its capacity to fold rapidly occurred independently of MTP, and inde-

pendently of events necessary for buoyant lipoprotein assem-

bly. This conclusion was further supported by the finding that, when apoB28F was reduced and then allowed to refold, the rate and extent of post-translational folding in CHO and McA-

RH7777 cells was also similar (see below).

Reduced ApoB28F Remains Lipid Poor in the Hepatic ER—To examine the intracellular consequences of blocking amino-terminal folding, the buoyant density of apoB28F extracted from cells that were labeled under control and reducing conditions was examined. For these experiments transiently transfected HepG2 cells were used because, unlike McA-

RH7777 cells, they express detectable amounts of serum albumin which is useful as an endogenous disulfide bonded control protein (not shown). Forty-eight hours after transfection, HepG2 cells were pulse-labeled for 10 min in the absence or presence of 2 mM DTT. Where DTT was used, it was added 1.5 min prior to addition of label. Under these conditions, ~80% of the labeled pool of apoB28F is reduced (“Experimental Procedures”). After metabolic labeling, post-nuclear membrane frac-
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To examine the contribution of MTP and events associated 

with buoyant lipoprotein formation on apoB28F folding, the experiment in Fig. 2 was repeated in CHO cells. When 2 mM
Fig. 5. Post-translational folding of albumin and apoB28F.
Transiently transfected HepG2 cells in 150-mm tissue culture dishes were preincubated for 1.5 min with 2 mM DTT and then labeled with \[^{35}S\]Met/Cys for 10 min, also in the presence of 2 mM DTT. Chase media lacking DTT was added and individual dishes were incubated for the indicated times. Cells were transferred to ice, treated with 100 mM IAA, and lysed. Aliquots of each detergent lysate were subjected to immunoprecipitation with anti-albumin antibody (A) or anti-FLAG M2 monoclonal antibody (B). Immune pellets were analyzed by 6% nonreducing SDS-PAGE and fluorography.

either fully reduced or folded, depending upon the labeling condition (not shown). These results indicate that folding of the amino-terminal domain is an essential prerequisite for apoB28F’s ability to recruit lipid in the hepatic ER.

Post-translational Folding of ApoB28F—While disulfide bond formation is in many cases cotranslational, proteins artificially reduced in the ER by DTT can fold post-translationally and undergo efficient secretion (30, 39, 47). It was previously shown that reduced apoB100 was incapable of achieving secretion competence post-translationally (9). However, because of its large size it was impossible to assess whether this was due to an inability to achieve post-translational disulfide bond formation or whether subsequent steps required for its assembly and secretion were blocked. To examine this question, transiently transfected HepG2 cells were pulse-labeled for 10 min in the presence of DTT to produce reduced apoB28F. After the pulse, the cells were chased for various periods of time in the absence of DTT. After each chase period, cells were placed on ice, treated with IAA, and prepared for immunoprecipitation. As observed in Fig. 5A, upon removal of DTT, reduced albumin underwent a process of post-translational folding as evidenced by its almost immediate (within 0.5 min) conversion to a diffuse series of electrophoretic forms representing intermediates in folding. Within 4 min, the diffuse grouping of bands was predominantly converted to a more rapidly migrating species corresponding to the folded form. This form has been shown previously to undergo efficient secretion (9, 30). When apoB28F was examined in the same extracts, it also underwent a time-dependent conversion from the reduced to the folded form which, based on nonreducing SDS-PAGE mobility, was complete within 4 min. In addition to its comigration with native folded apoB28F, post-translational folding of apoB28F was achieved within 4 min of DTT removal (not shown). This post-translational folding reaction was not facilitated by MTIF or events associated with apoB28F lipification as similar results were observed when the experiment was repeated in CHO cells (not shown). It appears, therefore, that the amino-terminal domain of apoB28F is capable of relatively rapid post-translational folding in the ER.

Post-translational Lipidation of ApoB28F Is Dependent Upon Folding of the Amino-terminal Domain—Since apoB28F can fold post-translationally, we examined whether post-translational folding was accompanied by a process of post-translational lipidation. Transiently transfected HepG2 cells were pulse-labeled in the presence of DTT for 10 min and then chased for 0, 5, or 15 min in the presence of DTT. At the end of the labeling period, post-nuclear membranes were prepared and treated with sodium carbonate, pH 11.5. Carbonate pellets and supernatant fractions were analyzed as described for Figs. 3A and 4B. In panel A, the percentage of carbonate-extractable apoB28F in the top (d < 1.25 g/ml) fractions was determined by densitometry and plotted (%d < 1.25 = T/(T + B) × 100). Values for the 0, 5, and 15 min chase in the absence of DTT represent the means ± S.D. (n = 3). Values for the 15-min chase in the presence of DTT represent mean ± S.D. (n = 2).

As observed in Fig. 6A, lanes 2 and 3, cells labeled for 10 min in the presence of DTT contained predominantly lipid-poor apoB28F in the carbonate supernatants (i.e. most was recovered in the bottom fraction of the gradient). Although it was previously demonstrated that post-translational folding was essentially complete within about 4 min of DTT removal (Fig. 5), this folding was not accompanied by appreciable post-translational lipidation as the percentage of d < 1.25 g/ml apoB28F was not significantly changed after the 5-min chase (compare ratio of apoB28F in lanes 2 and 3 versus lanes 5 and 6). During this time frame, a greater proportion of apoB28F became carbonate extractable. Only ~35% of apoB28F was extracted after the 0-min chase whereas ~65% was extracted after the 5-min chase (compare ratio of apoB28F in supernatant and pellet fractions in lanes 1–3 and 4–6). This result has been consistently observed and indicates that, as apoB28F undergoes post-translational folding (Fig. 5B), some of it is converted from carbonate-resistant to carbonate-extractable form, perhaps reflecting disaggregation and/or release from the ER membrane. However, virtually none of the carbonate-resistant apoB28F that was released into the supernatant after 5 min of chase was lipidated. After 15 min of chase, the proportion of carbonate-extractable apoB28F was unchanged. During this same time frame, however, the proportion of the apoB28F in the d < 1.25 g/ml fraction was increased by ~4-fold (compare lanes 2 and 3 with 8 and 9). This increase in the percentage of buoyant apoB28F formed post-translationally was dependent upon amino-terminal folding as it was not observed if DTT was present throughout the 15-min chase period (lanes 10–12).

To quantitate the extent of post-translational lipidation of apoB28F, cells were pulse-labeled in the presence of DTT for 10 min and then chased for 0, 5, or 15 min in the presence of DTT. After each chase period, cells were placed on ice, treated with IAA, and prepared for immunoprecipitation. As observed in Fig. 6A, lanes 2 and 3, cells labeled for 10 min in the presence of DTT contained predominantly lipid-poor apoB28F in the carbonate supernatants (i.e. most was recovered in the bottom fraction of the gradient). Although it was previously demonstrated that post-translational folding was essentially complete within about 4 min of DTT removal (Fig. 5), this folding was not accompanied by appreciable post-translational lipidation as the percentage of d < 1.25 g/ml apoB28F was not significantly changed after the 5-min chase (compare ratio of apoB28F in lanes 2 and 3 versus lanes 5 and 6). During this time frame, a greater proportion of apoB28F became carbonate extractable. Only ~35% of apoB28F was extracted after the 0-min chase whereas ~65% was extracted after the 5-min chase (compare ratio of apoB28F in supernatant and pellet fractions in lanes 1–3 and 4–6). This result has been consistently observed and indicates that, as apoB28F undergoes post-translational folding (Fig. 5B), some of it is converted from carbonate-resistant to carbonate-extractable form, perhaps reflecting disaggregation and/or release from the ER membrane. However, virtually none of the carbonate-resistant apoB28F that was released into the supernatant after 5 min of chase was lipidated. After 15 min of chase, the proportion of carbonate-extractable apoB28F was unchanged. During this same time frame, however, the proportion of the apoB28F in the d < 1.25 g/ml fraction was increased by ~4-fold (compare lanes 2 and 3 with 8 and 9). This increase in the percentage of buoyant apoB28F formed post-translationally was dependent upon amino-terminal folding as it was not observed if DTT was present throughout the 15-min chase period (lanes 10–12).

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To further characterize the nature of the apoB28F-containing lipoproteins formed post-translationally, their buoyant density profile was examined. Transiently transfected HepG2 cells were pulse-labeled for 10 min in the presence of DTT and chased in the absence of DTT. After the chase, media was recovered, concentrated, and subjected to density gradient centrifugation as described for Fig. 1A. Although approximately 80% of the labeled apoB28F in media from such an experiment is due to post-translational folding and assembly (Fig. 7A), no evidence was obtained to indicate that the buoyant density profile of this lipoprotein population differed from that observed in control cells (Fig. 1A). This result, along with the secretion data (Fig. 7A), indicates that, although the usual pathway for apoB assembly occurs cotranslationally, apoB28F is capable of undergoing a post-translational folding and assembly process which gives rise to a native, secretion-competent lipoprotein particle. As is the case for normal cotranslational assembly (Fig. 4), the post-translational pathway also demonstrates that amino-terminal folding is a prerequisite for apoB’s capacity to undergo MTP-dependent assembly with lipid.

DISCUSSION

Recent studies in hepatoma cells indicate that translation of the amino-terminal ~25% of apoB is sufficient to produce a small lipid core-containing lipoprotein particle (14, 19). Once formed, this small dense particle appears to undergo further MTP-dependent enlargement and maturation concomitant with the ongoing translation of apoB (8). Additional lipid may also be added to nascent lipoprotein particles post-translationally (48–50). While a conceptual framework exists for understanding how a small dense lipoprotein formed by the amino-terminal ~25% of apoB can be enlarged by both co- and post-translational mechanisms, little is known about how the process of lipoprotein formation is initiated. Considering the cotranslational nature of lipoprotein assembly we assumed that steps required to initiate the formation of a small dense lipoprotein particle (e.g. that formed by apoB28) should be the same as those required for native forms of apoB48 and -100. We, therefore, examined the biogenesis of apoB28F with a particular focus on events that precede and, therefore, may be responsible for its capacity to function as an acceptor for MTP-mediated lipid transfer in the hepatic ER.

Using both electrophoretic mobility changes during nonreducing SDS-PAGE and the onset of DTT resistance as criteria for protein folding in vivo, as used extensively by others (33–36, 39, 47), the rate and extent of folding of the amino-terminal domain of apoB28F was examined in both hepatoma and CHO cells. As with many disulfide bonded proteins, these studies revealed that apoB28F and, we presume by analogy apoB100, forms the bulk of its amino-terminal disulfide bonds during the process of translation. Unlike other proteins, however, apoB28F did not display a distinct temporal window in which its disulfide bonds were sensitive to DTT-mediated reduction in the ER (9, 34, 36, 39, 40). Interestingly, this uniquely rapid onset of DTT resistance was not dependent upon MTP or lipoprotein formation, as the rate and extent of folding was similar in CHO cells, a cell line that can neither lipidate nor secrete apoB28F (Fig. 3). Hence, the rapid folding of the amino-terminal domain is an autonomous property of the apoB protein and not a consequence of its extensive assembly with lipids.

The underlying basis for the rapid onset of DTT resistance of apoB28F is not fully understood. The ability of DTT to reduce newly disulfide bonded proteins in the ER is an ATP-requiring event suggesting the involvement of ER-localized chaperones (34, 35). That the amino-terminal domain of apoB28F...
progresses so rapidly to a form that resists DTT may reflect its rapid rate of folding and/or its unusually short-lived interactions with ER chaperones. Irrespective of its underlying basis, it is clear that the disulfide bonds in the amino-terminal domain of apoB achieve DTT resistance cotranslationally. The amino terminus of apoB may, therefore, represent a modular domain whose folding occurs during its translation and whose function is essential for subsequent stages of lipoprotein assembly. This latter possibility was explored by examining the intracellular fate of apoB28F translated under conditions that blocked folding of its amino-terminal domain.

When folding of the amino-terminal domain was disrupted by preincubation of transfected HepG2 cells with 2 mM DTT, apoB28F was unable to recruit sufficient lipid to float at d < 1.25 g/ml. In contrast, ∼50% of apoB28 was present in d < 1.25 g/ml fraction under control labeling conditions. This implies that, the strong lipid binding sequences in the form of predicted 


d

b

in vivo

and/or refolding of apoB's lipophilic domains, once translocated into the ER in unlipidated or underlipidated form, may be inversely related to the length of these domains and hence their degree of aggregation.

Although the present results demonstrate a distinct stage in apoB folding which precedes and appears to be essential for its MTP-dependent lipidation, the results do not directly demonstrate the specific role played by the amino-terminal domain of apoB. We had previously hypothesized that folding of the amino-terminal domain of apoB may be accompanied by an autonomous phospholipid recruitment step which would provide a luminal lipid surface for MTP-mediated lipid transfer in the ER (9). This prediction is based, in part, on the fact that the amino-terminal domain of apoB can solubilize dimyristoylphosphatidylcholine vesicles in vitro (16) and that enrichment of hepatic membranes with the phosphatidylcholine/ethanolamine analog, phosphatidylmonomethylethanolamine, reduces the secretion of even small amino-terminal forms of apoB (52). In the present studies, folding of the amino-terminal domain of apoB was not accompanied by substantial lipid recruitment. However, depending upon the amount of lipid associated with amino-terminal folding, density gradient centrifugation may not be a sufficiently sensitive means of detecting such protein-lipid interactions. Recent results of Wu et al. (53) indicate that during the assembly of apoB100 in HepG2 cells, MTP and apoB100 form a complex which can be communoprecipitated. It has since been shown that apoB-MTP interactions occur for forms of apoB containing as little as the amino-terminal 13% of the protein (54). These results indicate that a direct apoB-MTP interaction may be necessary for the selective transfer of lipid from the ER membrane or some other donor site to nascent apoB. The strict dependence of amino-terminal folding on the capacity of apoB to initiate lipid sequestration indicates that this domain may either directly (via a protein-protein interaction) or indirectly (via a protein-lipid interaction) mediate a critical apoB-MTP interaction necessary for lipoprotein formation.

In conclusion, it appears that while much of apoB is composed of domains with avid and in some cases irreversible lipid binding properties, these sequences alone may not be sufficient

\[ X. F. Huang and G. S. Shelnas, unpublished observations. \]
to drive the normal process of lipoprotein assembly. In addition to apoB’s lipophilic sequences, a relatively soluble amino-terminal domain appears to play an essential role in the initiation and perhaps subsequent stages of lipoprotein particle formation. Future studies of the structure and function of this domain will likely reveal important mechanistic insights into the process of apoB-containing lipoprotein assembly. Finally, the development of the post-translational assay reported here, which uncouples the assembly of apoB with lipids from the process of translation, may provide an additional valuable system to further dissect steps required for apoB’s unique form of assembly in the hepatic ER.

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