Role of intramolecular disulfide bond formation in the assembly and secretion of apolipoprotein B-100-containing lipoproteins

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Abstract Apolipoprotein B-100 (apoB) is essential for the hepatic assembly and secretion of triglyceride-rich very low density lipoprotein (VLDL). The mechanism of VLDL assembly was explored by perturbing apoB folding in HepG2 cells with the thiol reducing agent dithiothreitol (DTT). Although apoB contains eight known disulfide bonds, seven of which are positioned in the amino-terminal 21% of the protein, its assembly and secretion was only partially blocked in cells treated with 2 mM DTT, a condition that fully blocks the secretion of other disulfide-bonded proteins. Nonreducing gel electrophoresis of an apoB-derived proteolytic peptide revealed that apoB escapes the secretory block normally caused by DTT because its amino-terminal disulfide bonds undergo maturation to a DTT-resistant form after completing synthesis of only the first ~20–25% of the protein. If, however, DTT was used under conditions that prevented the initial formation of amino-terminal disulfide bonds, lipoprotein secretion was blocked. Reduced forms of apoB were extremely labile and, unlike other disulfide-bonded proteins, incapable of achieving secretion competence posttranslationally. These results indicate that disulfide bond formation within the amino-terminus of apoB is essential for the proper folding and assembly of its downstream lipophilic sequences. The onset of DTT resistance while still a nascent polypeptide chain is consistent with a model in which the amino-terminal domain of apoB undergoes an independent folding and maturation process, the completion of which may represent an initiation phase of triglyceride-rich lipoprotein assembly.—**Shelness, G. S., and J. T. Thornburg.** Role of intramolecular disulfide bond formation in the assembly and secretion of apolipoprotein B-100-containing lipoproteins. *J. Lipid Res.* 1996. **37:** 408–419.

Supplementary key words protein synthesis • protein folding • very low density lipoprotein • HepG2 cells • dithiothreitol

The folding and assembly of apolipoprotein B-100 (apoB) represents a novel form of secretory protein biogenesis. In addition to steps common to all secretory precursor proteins such as membrane translocation, signal peptide cleavage, covalent modification, and folding, apoB must undergo assembly with lipids to achieve secretion competence in the form of a triglyceride-rich very low density lipoprotein (VLDL) (for reviews see refs. 1–3). As with many other forms of protein maturation, the initial assembly of apoB with lipids is believed to occur cotranslationally (4, 5). In a cotranslational model of lipoprotein assembly, it would be expected that at least some aspects of the maturation of the amino-terminus of apoB would precede its lipidation (6). This might be necessary for apoB to achieve a conformation in which it is competent to begin sequestering lipids, or may be related to the need of apoB to be recognized as an acceptor for lipid transfer by the microsomal triglyceride transfer protein (MTP) (6, 7). Indeed, both structural modeling and experimental analyses have revealed that the amino-terminus of apoB is atypical relative to the rest of the protein. While much of apoB is composed of amphipathic α-helices and β-sheets, which are thought to be responsible for its avid association with the surface and perhaps the interior of the lipoprotein particle, the amino-terminal ~20% is predicted to be more globular and to display relatively weak and reversible interactions with neutral lipid-rich emulsion particles (8–11). Furthermore, while intramolecular disulfide bonds are a common feature of most secretory proteins, they are absent from all known apolipoproteins with the exception of apoB. Of the eight known disulfide bonds in apoB, seven are clustered in the amino-terminal 21% of the protein (12).

To investigate the function of the amino-terminal

Abbreviations: ALLN, N-acetyl-leucyl-leucyl-norleucinal; apoB, apolipoprotein B-100; DTT, dithiothreitol; ER, endoplasmic reticulum; FBS, fetal bovine serum; IAA, iodoacetamide; MEM, minimal essential medium; MTP, microsomal triglyceride transfer protein; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; VLDL, very low density lipoprotein.

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disulfide-bonded region of apoB we examined the effects of the membrane permeable thiol reducing agent, dithiothreitol (DTT), on the assembly and secretion of apoB-containing lipoproteins in the human hepatoma-derived cell line, HepG2. The use of DTT to perturb disulfide bonding in living cells has been extensively characterized and has provided useful insights into the process of protein folding and assembly in the ER (13–16). DTT equilibrates rapidly across intracellular membranes causing a rapid alteration in the mildly oxidizing environment within the ER (17). As a result, disulfide bonding that would normally occur during or shortly after translation is blocked and disulfide bonds formed prior to the addition of DTT are readily reduced. Reduced secretory precursor proteins are retained in the ER, many as homo- and heterotypic aggregates (18, 19). Remarkably, however, if DTT is removed from cells, reduced and aggregated proteins undergo posttranslational folding followed by virtually normal rates of transport (14–16). While all disulfide-bonded proteins are sensitive to DTT-mediated reduction in the ER, their sensitivity is transient. In the case of two well-characterized viral glycoproteins, vesicular stomatitis virus G protein and the hemagglutinin of influenza virus, the onset of DTT resistance occurs with a half-time of ~5 min (15). The basis for the early susceptibility of these protein folding intermediates to DTT may be related to their incompletely folded state as the kinetics of onset of DTT resistance corresponds roughly to the amount of time necessary for the viral glycoprotein monomers to assemble into native trimers, an event that occurs in the ER (15, 20, 21).

Analysis of apoB secretion in DTT-treated HepG2 cells revealed that its behavior was atypical. Unlike other disulfide bonded proteins, whose folding and secretion remain sensitive to DTT for a characteristic period of time after the completion of the polypeptide chain, the assembly and secretion of apoB becomes resistant to DTT after completing synthesis of the only the first ~20–25% of the protein. If, however, the amino-terminus of apoB was synthesized in the presence of DTT, thereby preventing initial disulfide bond formation, apoB secretion was blocked. Reduced forms of apoB were subjected to rapid intracellular degradation and were incapable of achieving secretion competence post-translationally. These results indicate that the maturation of the amino-terminal globular domain of apoB precedes entry of internal lipophilic portions of apoB into the ER. This system of modular protein folding may function to ensure that domains of apoB whose folding depends upon interaction with a lipid surface, encounter a previously initiated lipoprotein particle shortly after their synthesis and translocation into the ER. Models are discussed in which the amino-terminus of apoB functions to initiate triglyceride-rich lipoprotein assembly by recruiting a phospholipid surface, to which MTP subsequently transfers neutral lipids and phospholipids, or by acting as direct proteinaceous acceptor for MTP-mediated lipid transfer.

MATERIALS AND METHODS

Reagents

Tissue culture media and supplements were obtained from JRH Biosciences (Lenexa, KS) or ICN Biomedicals (Irvine, CA). Sodium oleate, fatty acid-free bovine serum albumin, porcine thrombin, and iodoacetamide (IAA) were from Sigma Chemical Co. (St. Louis, MO). Antibodies to apoB, α_2-macroglobulin, and albumin were from Boehringer Mannheim Corp. (Indianapolis, IN). An anti-peptide antibody directed against amino acids 259–279 of apoB was provided by Dr. Thomas Innerarity, Gladstone Institute of Cardiovascular Disease. Protein G-Sepharose was obtained from Pharmacia Biotech, Inc. (Piscataway, NJ). Trans^{35}S-label (an approximately 5:1 mixture of [^{35}S]methionine and cysteine) was from ICN Biomedicals.

Tissue culture and metabolic labeling

HepG2 cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in MEM containing 2 × vitamins, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FBS (complete MEM) at 37°C in an atmosphere of 5% CO_2. Prelabeling medium was complete MEM lacking FBS; labeling medium was complete MEM lacking FBS, methionine, and cystine; chase medium was complete MEM lacking FBS and containing an additional 1 mM cysteine and methionine. For metabolic labeling experiments, a confluent 75-cm^2 flask was trypsinized, seeded at a 1:10 dilution into 100-mm dishes, and grown for 5–6 days in complete MEM to ~90% confluence. All media used for prelabeling, labeling, and chase protocols contained 0.8 mM sodium oleate complexed to 1.5% fatty acid bovine serum albumin to induce apoB secretion (22, 23). Experiments were initiated by washing cells once with PBS and then incubating for 2 h with 4 ml of prelabeling medium. After removal of prelabeling medium and washing once with PBS, 2 ml of labeling medium was added and cells were incubated for 20–30 min. Labeling medium was removed and replaced with 2 ml of fresh labeling medium. After an additional 5 min incubation, 200 μCi Trans ^{35}S-label was added. Cells to be pulsed in the presence of 2 mM DTT received 20 μl of a 200 mM stock of freshly prepared DTT prior to addition of label. The time of DTT preincubation prior to addition of label was 1 min unless otherwise indicated. Pulse label-

ing was terminated by addition of 5 ml of chase medium with or without 2 mM DTT depending on the chase condition. Medium was then removed and replaced with 2 ml of fresh chase medium with or without 2 mM DTT as required. At the end of the chase period cells were placed on ice and adjusted to 100 mM IAA by addition of 200 µl of a 1 M stock prepared in 500 mM Tris HCl, pH 8.7 (16). After a minimum 5-min incubation with IAA, the media were removed and the cells were washed and scraped in PBS containing 100 mM IAA. Cell pellets were recovered by centrifugation.

Immunoprecipitations

Cell pellets were resuspended in 1 ml of lysis buffer (25 mM Tris HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM PMSF, 10 µg/ml leupeptin, 10 µg/ml pepstatin) and incubated for 30 min on ice. Samples were centrifuged for 5 min at 14,000 rpm in an Eppendorf micro centrifuge. Supernatants were aliquoted into tubes containing an excess (10 µl) of the appropriate antiserum, 25 µl of bovine serum albumin (50 mg/ml), and a volume of lysis buffer necessary to achieve a final volume of 500 µl. After a 14–18 h incubation at 4°C with gentle inversion, 35 µl of a 50% (v:v) slurry of protein G-Sepharose was added and incubation was continued for an additional 2 h. Immune complexes were pelleted and washed 3 times with lysis buffer followed by a single wash with 10 mM Tris HCl, pH 7.4, 0.1% SDS. Media samples were adjusted to lysis buffer conditions and subjected to the same immunoprecipitation and wash procedures described above. Control experiments confirmed that both the anti-apoB polyclonal antibody and the anti-apoB peptide antibody reacted uniformly with both reduced and disulfide bonded forms of apoB (data not shown).

Thrombin digestion

Two ml media samples were concentrated to 100 µl using Centricon-100 centrifugal concentrators (Amicon, Inc., Beverly, MA). The concentrated samples were diluted to 2 ml with 25 mM Tris HCl, pH 7.4, 140 mM NaCl and again concentrated to 100 µl. After adjusting to 1% Triton X-100, the indicated amount of porcine thrombin was added and samples were incubated for 4 h at room temperature. After addition of protease inhibitors, samples were subjected to immunoprecipitation as described above.

SDS-PAGE and fluorography

Immune pellets were suspended in SDS-PAGE sample buffer (50 mM Tris base, 4% SDS, 12% glycerol, 1 mM EDTA) with or without 50 or 100 mM DTT, as indicated, and boiled for 5 min. Protein G-Sepharose beads were removed by centrifugation and the supernatants were fractionated by SDS-PAGE (24, 25). Unless otherwise indicated, 4–20% polyacrylamide gradient gels were used to resolve apoB and α_2-macroglobulin and 8% polyacrylamide gels were used to resolve albumin. Gels were soaked for 30 min in 1 M sodium salicylate prior to drying (26). Dried gels were exposed to Kodak XAR or Bio-Max MR film at -70°C. For quantitation by densitometry, XAR film was preflashed prior to exposure to gels (27). Films were scanned using a pdi 325oe scanning densitometer (Protein and DNA Imageware Systems, Inc., Huntington Station, NY) and quantitated using ImageMaster software (Pharmacia Biotech, Inc.). For data in Fig. 6, gel bands were quantitated using a Molecular Dynamics model 445 SI Phosphorimager (Molecular Dynamics, Sunnyvale, CA).

RESULTS

DTT treatment of HepG2 cells partially blocks the secretion of apoB

To investigate the folding and assembly of apoB, we examined its behavior in DTT-treated HepG2 cells. HepG2 cells were subjected to a 10-min pulse with Trans^{35}S-label, followed by a 0 or 60 min chase as described by Lodish and Kong (16). Pulse and chase media contained 2 mM DTT as indicated in **Fig. 1.** At the completion of the experimental procedure, cells

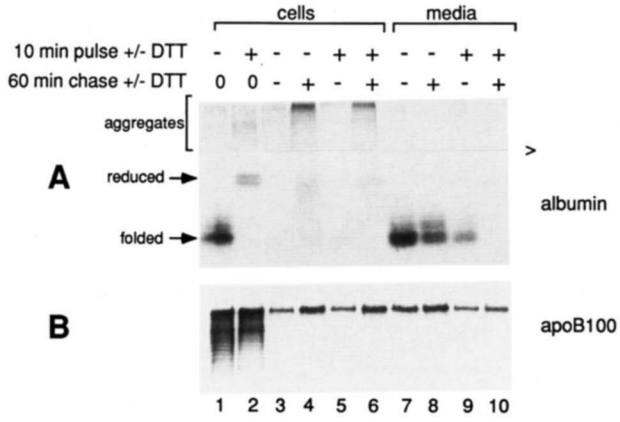

Fig. 1. The effects of DTT on apoB secretion and intracellular turnover. HepG2 cells were pulse-labeled for 10 min and chased for 60 min in the presence and absence of 2 mM DTT as indicated. After incubation of cells with IAA, cell pellets (lanes 1–6) and media (7–10) were subjected to immunoprecipitation with antibodies to albumin (A), or apoB (apoB100) (B). Albumin samples were resolved by SDS-PAGE without prior reduction. ApoB immunoprecipitates were reduced by heating in 100 mM DTT prior to SDS-PAGE. The notch on the right hand side indicates where a middle section of the gel was removed so that only areas containing appreciable radioactive signal are displayed. Arrows and bracket indicate positions of disulfide bonded (folded), reduced, and aggregated forms of albumin. The spectrum of bands under full-length apoB in panel B, lanes 1 and 2, are nascent polypeptide chains.

were placed on ice and treated with 100 mM IAA to derivatize free sulfhydryls. Aliquots of media and cell detergent lysates were immunoprecipitated with antibodies to albumin and apoB and analyzed by SDS-PAGE and fluorography. As reported previously (16) albumin synthesized in the presence of DTT migrated more slowly during nonreducing SDS-PAGE than albumin synthesized in control cells (Fig. 1A, compare bands labeled "reduced" and "folded" in lanes 1 and 2). This is consistent with the ability of DTT to prevent disulfide bonding and to reduce disulfide bonds formed during or shortly after synthesis. After a 60-min chase in the absence of DTT, most of the reduced albumin underwent posttranslational folding and was recovered in the medium (Fig. 1A, compare lanes 2 and 9). In contrast, when DTT was present throughout the experimental period, albumin was recovered from the cells in reduced and aggregated form (lane 6) and virtually none was detected in the cell medium (lane 10). The secretion of α_1-antitrypsin (a protein devoid of disulfide bonds) from cells in continuous contact with DTT confirmed that the inhibition of albumin secretion caused by DTT was specific for disulfide-bonded proteins (data not shown) (16).

Although apoB contains at least eight disulfide bonds, striking differences were observed between the behavior of apoB and the disulfide-bonded control, albumin. First, while inhibition of protein synthesis has been reported as a general effect of DTT in HepG2 cells (16, 19, 28), the synthesis of apoB was relatively unaffected by DTT as compared to the ~70% inhibition observed for albumin (Fig. 1A and B, compare lanes 1 and 2). Also, while the presence of DTT in the pulse reduced the amount of apoB secreted during the 60-min chase by ~40% (Fig. 1B, compare lanes 7 and 9), the presence of DTT in the chase had no dramatic effect on apoB secretion as it did with albumin (Fig. 1A and B, compare lanes 9 and 10). In these experiments albumin was used as a control disulfide-bonded protein because reduced and disulfide-bonded forms can be readily resolved by nonreducing SDS-PAGE. We have also analyzed the behavior of α_2-macroglobulin, a protein that is 2.5 times longer than albumin and which contains both intra- and intermolecular disulfide bonds (29, 30). As with albumin, and all other disulfide-bonded proteins analyzed to date, the secretion of α_2-macroglobulin from cells pulsed in the presence of DTT is completely dependent upon DTT removal during the chase (data not shown and Fig. 2). Also, α_2-macroglobulin remai ıs sensitive to DTT for a considerably longer period of time than albumin. After a 10-min pulse in the absence of DTT, addition of DTT during the chase does not appreciably affect the secretion of albumin or apoB (Fig. 1, compare lanes 7 and 8). In contrast, under the same conditions, α_2-macroglobulin secretion is inhibited by ~95% (data not shown). Thus, the resistance to DTT-mediated inhibition of secretion displayed by apoB is not a simple consequence of its size.

Kinetics of apoB secretion are identical in control and DTT-treated cells

The results displayed in Fig. 1 indicate that, in comparison to albumin, the secretion of apoB was relatively unaffected by DTT. To further characterize the effects of DTT, we examined whether the kinetics of apoB secretion were perhaps different in DTT-treated and control cells. Groups of HepG2 cells were pulsed for 10 min and chased for periods between 0 and 90 min. Aliquots of media were immunoprecipitated with antibodies to albumin, α_2-macroglobulin, and apoB. As

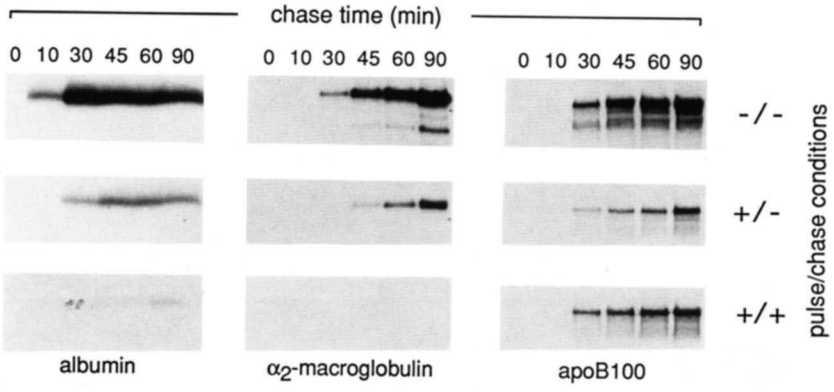

Fig. 2. Kinetics of apoB secretion from DTT-treated HepG2 cells. HepG2 cells were pulsed for 10 min and chased for various periods of time in the presence or absence of 2 mM DTT as described for Fig. 1. After the indicated chase times, aliquots of media were immunoprecipitated with antibodies to albumin, α_2-macroglobulin, and apoB as indicated. Samples were reduced prior to SDS-PAGE. (-/-), cells pulsed and chased in the absence of DTT; (+/-) cells pulsed in the presence and chased in the absence of DTT; (+/+) cells pulsed and chased in the presence of DTT.

shown in **Fig. 2,** the secretion of albumin and α_2-macroglobulin was delayed in cells pulsed in the presence and chased in the absence of DTT (+/-) as compared to control (-/-) cells (compare top and middle panels for albumin and α_2-macroglobulin). This is consistent with the inhibition of secretion by DTT and the fact that when DTT is removed, the protein undergoes a time-dependent posttranslational folding and assembly process. As would be expected, virtually no secretion of either albumin or α_2-macroglobulin was observed in cells treated with DTT during both the pulse and the chase (+/+). In contrast to the results observed for albumin and α_2-macroglobulin, the kinetics of secretion of apoB were virtually identical in -/- and +/- cells, providing another indication that little if any apoB secretion observed in +/- cells arose from posttranslational folding and assembly. The secretion of apoB from cells in continuous contact with DTT (+/+) also occurred with the same kinetics observed in controls cells, confirming that there is a population of nascent apoB in HepG2 cells whose ultimate secretion is fully resistant to the inhibitory effects of DTT.

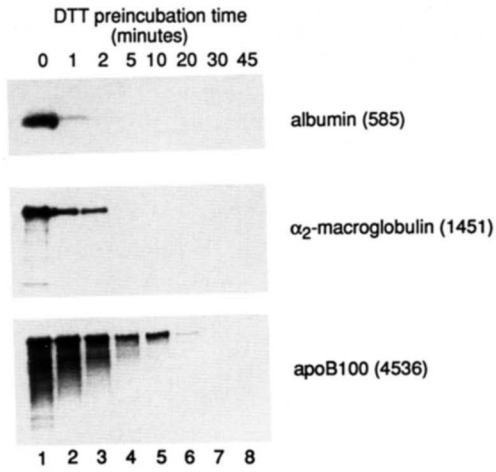

Fig. 3. DTT inhibits the initiation of protein synthesis. HepG2 cells were preincubated with 2 mM DTT for the indicated times and then pulse labeled for 10 min, also in the presence of 2 mM DTT. After each pulse, aliquots of cell lysate were immunoprecipitated with antibodies to albumin, α_2-macroglobulin, and apoB as indicated. Samples were reduced prior to SDS-PAGE. The numbers in parentheses indicate the length, in amino acid residues, of the mature form of each protein.

DTT inhibits the initiation of protein synthesis in HepG2 cells

Although some apoB polypeptide chains are resistant to DTT treatment, an ~40% reduction in apoB secretion was consistently observed in cells pulsed for 10 min in the presence of DTT (Fig. 1B, compare lanes 7 and 8 with lanes 9 and 10). In the case of albumin, reduced secretion from cells pulsed in the presence of DTT was attributable to reduced synthesis; however, as mentioned above, the synthesis of apoB appeared to be relatively unaffected by DTT. To better characterize the possible differential effects of DTT on protein synthesis, HepG2 cells were preincubated with DTT for 0–45 min and then pulse-labeled for 10 min in the presence of DTT. Aliquots of cell lysate were immunoprecipitated with antibodies to albumin, α_2-macroglobulin, and apoB and analyzed by SDS-PAGE and fluorography. As observed in **Fig. 3,** the kinetics of inhibition of the three proteins varied considerably. While almost complete inhibition of albumin synthesis was achieved with a 2-min preincubation, α_2-macroglobulin synthesis was not similarly affected until the 5-min time point. The rate of inhibition of apoB synthesis was clearly less than either of the two controls. Increasing the time of DTT preincubation also reduced the relative abundance of apoB nascent polypeptide chains and increased their average length. Based on these two criteria (the inverse relationship between protein size and sensitivity to inhibition, and the gradual lengthening and eventual disappearance of apoB nascent chains), we concluded that, in HepG2 cells, DTT is predominantly an inhibitor of protein synthesis initiation but not elongation. This inhibition was not confined to secretory proteins as incorporation of radioactivity into total trichloroacetic acid precipitable protein was reduced to ~10% of control values within 10 min of preincubation (data not shown).

Labeling HepG2 cells in the presence of DTT creates two forms of apoB with different intracellular fates

The results presented above illustrate that the secretion of a large percentage of newly synthesized apoB is resistant to the effects of DTT. However, DTT caused an ~40% decrease in apoB secretion and this decrease was only partially due to a decrease in apoB synthesis (in Figs. 1 and 3, a 1-min preincubation with DTT resulted in an ~5% decrease in apoB synthesis). How two populations of nascent apoB with different sensitivities to DTT may be formed during labeling protocols utilizing DTT is depicted in **Fig. 4.** When DTT is added to cells, nascent polypeptide chains exist at various stages of completion (solid horizontal lines). The DTT sensitive forms of apoB (form I) may arise from relatively short nascent chains that have yet to be translated beyond a critical coordinate within the protein (double vertical line). As a result, when DTT is added to this population of nascent apoB, its ability to form disulfide bonds in this region would be blocked. In addition, disulfide bonds formed prior to addition of DTT may also be sensitive to DTT-mediated reduction (although this is not depicted in Fig. 4). At the same time, some nascent chains may have been translated beyond the critical

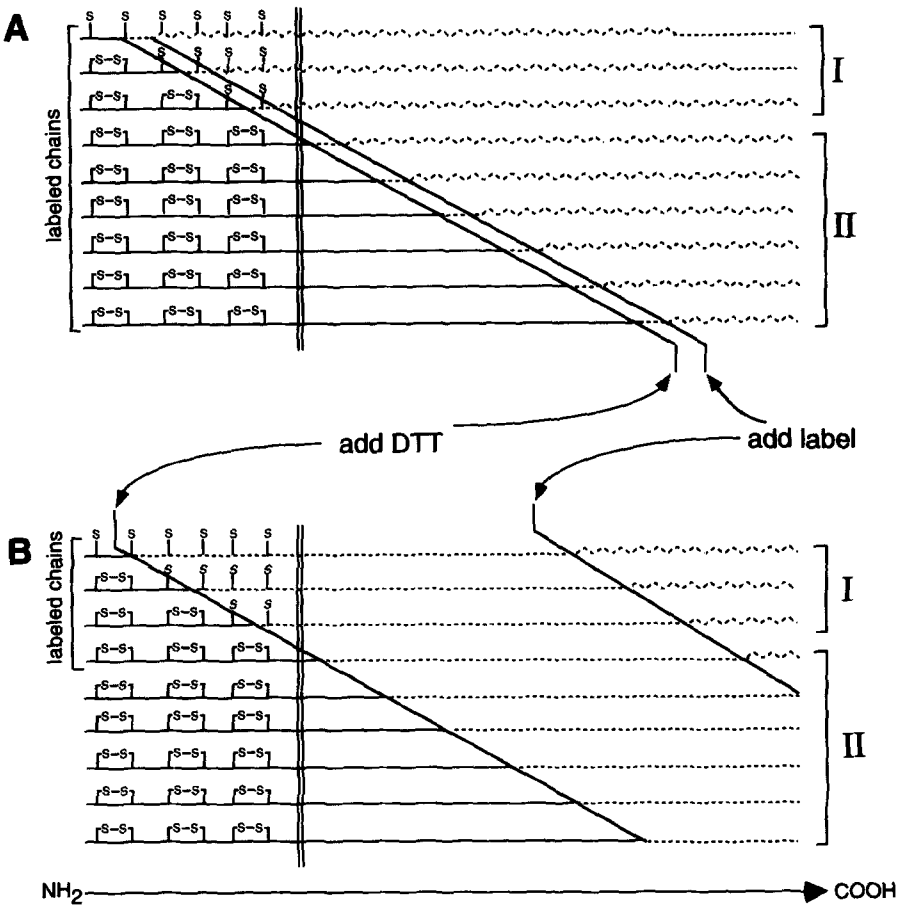

Fig. 4. Model for the generation of two forms of apoB during labeling in the presence of DTT. Solid horizontal lines represent the length of the various apoB nascent chains at the start of the experiment. Dashed lines are portions of apoB synthesized in the presence of DTT; jagged dashed lines are portions of apoB labeled during a 10-min pulse with radioactive amino acids. The two diagonal lines indicate the times at which DTT and label are added. The double vertical lines indicate a putative length of apoB that must be synthesized in order to escape the secretory block caused by DTT. Synthesis of this critical length of apoB prior to addition of DTT may determine whether it is secretion competent (form II) or unable to form a secretable lipoprotein (form I). The effects of a short (A) and long (B) DTT preincubation time are shown on the relative proportion of labeled form I and form II apoB. As DTT preincubation time is increased (B), the form I:form II ratio is also increased, thereby reducing the secretion efficiency of apoB. The diagram is not drawn to scale.

coordinate to form a second population of apoB (form II) that is resistant to the effects of DTT. Because form II apoB nascent chains would have already completed folding in their amino-terminal domain, DTT addition would have no effect on their subsequent assembly and secretion.

If our assumptions about the behavior of apoB in DTT-treated cells (Fig. 4) are correct, then predictions can be made concerning the proportion of form I and form II polypeptide chains generated during various labeling protocols. During the standard labeling procedure used in Figs. 1 and 2, DTT was added 1 min prior to addition of label (16). Under these conditions the majority of labeled apoB would contain amino-termini translated prior to the addition of DTT (Fig. 4A, form II). As DTT blocks translation initiation but not elongation (Fig. 3), lengthening DTT preincubation time would increase the form I:form II ratio and thereby decrease the secretion efficiency of labeled apoB (compare ratio of labeled form I:form II in Fig. 4A and B). To test this hypothesis the experiment shown in Fig. 1 was repeated using a prolonged preincubation with DTT (**Fig. 5**). When the preincubation time was increased from 1 to 15 min, the secretion efficiency of apoB was reduced from 22% to 5% (Figs. 1B and 5, ratio of lane 9 or 10 to lane 2). To ensure that this decreased secretion efficiency was specific for apoB and not a general effect of prolonged preincubation with DTT, the secretion efficiencies of apoB and α_2-macroglobulin were compared as a function of DTT preincubation time. Pairs of tissue culture dishes were preincubated with 2 mM DTT for 2.5, 5, 7.5, and 10 min, pulsed for 10 min in the presence of DTT, and chased for either 0 or 60 min in the absence of DTT. Cell lysates from the

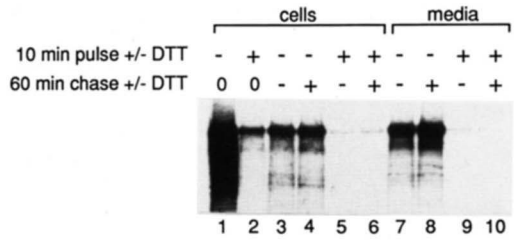

Fig. 5. Effect of DTT preincubation time on apoB secretion efficiency and intracellular turnover. The protocol described for Fig. 1 was repeated, except that a 15-min preincubation with DTT was used prior to addition of label.

0-min chase and media samples from the 60-min chase were immunoprecipitated and subjected to SDS-PAGE. Secretion efficiency was calculated using data obtained by phosphorimager analysis. As shown previously (Fig. 3), the amount of α_2-macroglobulin and apoB synthesized during a 10-min pulse was severely reduced by increasing the time of DTT preincubation. In this experiment, preincubation with DTT for 10 min reduced pulse-labeled cellular α_2-macroglobulin by 97% relative to the 2.5-min time point (data not shown). Nonetheless, the secretion efficiency of α_2-macroglobulin was the same irrespective of preincubation time (**Fig. 6**). In contrast, apoB's secretion efficiency was reduced by ~3-fold as the preincubation time was increased from 2.5 to 10 min.

Reduced forms of apoB are subjected to rapid intracellular degradation

As shown in Fig. 1B (1 min preincubation), the presence of DTT during a 60-min chase protected apoB from intracellular degradation (compare lanes 5 and 6). In contrast, after prolonged (15 min) preincubation, the intracellular degradation of apoB appeared to be enhanced, and the presence of DTT failed to exert any protective effect (Fig. 5, compare lanes 5 and 6). In +/- cells subjected to a 1-min preincubation with DTT (Fig. 1B), the total apoB in media and cells after the 60-min chase was 42% of that made during the 10-min pulse. This value was 64% in the +/+ cells, reflecting decreased intracellular turnover. For cells preincubated for 15 min (Fig. 5), these values were 17% in +/- cells and 16% in +/+ cells. These results further support the hypothesis that two forms of apoB are produced in DTT-treated cells with different intracellular fates. ApoB in which amino-terminal disulfide bonding is prevented (form I), is rapidly degraded and is incapable of posttranslational folding into a secretion competent form. Unlike form II apoB (in which disulfide bonds are intact), the degradation of form I apoB cannot be inhibited by DTT. In a separate experiment we demonstrated that ALLN, a protease inhibitor that can protect newly synthesized apoB against intracellular degradation (31, 32), was also incapable of preventing the enhanced degradation of reduced forms of apoB (data not shown).

Disulfide bonds in the amino-terminal domain of apoB undergo rapid resistance to DTT-mediated reduction

The results presented above are consistent with one of two alternative explanations. *i)* Disulfide bonding in the amino-terminus of apoB is necessary to initiate lipoprotein assembly; however, once this initiation phase is complete the disulfide bonds can undergo DTT-mediated reduction without affecting subsequent steps in particle maturation and secretion. *ii)* Disulfide bonding in the amino-terminus of apoB is necessary to initiate lipoprotein assembly and this domain achieves resistance to DTT shortly after its translation. To distinguish between these two possibilities we attempted to establish whether the amino-terminal cysteine residues in apoB secreted from DTT-treated cells were in reduced or disulfide-bonded form. Because apoB is too large to electrophoretically resolve conformational differences based on disulfide bonding (data not shown), we utilized the capacity of thrombin to cleave apoB at specific sites within the polypeptide (33). We found that cleavage of apoB in HepG2 cell media in the presence of 1% Triton X-100 gave fragments that were immunoreactive with a peptide antibody directed against the

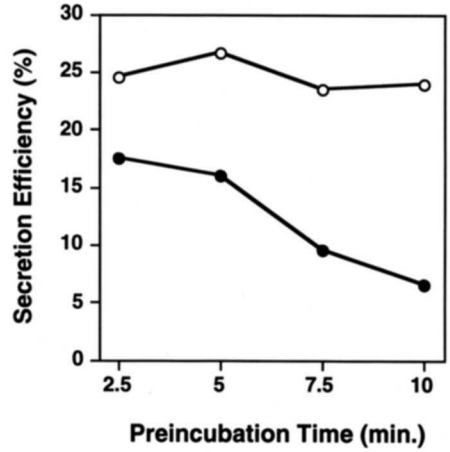

Fig. 6. Effect of DTT preincubation time on the secretion efficiency of apoB and α_2-macroglobulin. Pairs of tissue culture dishes were preincubated for the indicated times with 2 mM DTT and then pulse-labeled for 10 min, also in the presence of DTT. One dish was chased for 0 min and the other for 60 min in the absence of DTT. Aliquots of cell lysate from the 0-min chase and media samples from the paired 60-min chase were normalized to total cellular protein and immunoprecipitated with antibodies to either apoB or α_2-macroglobulin. Samples were resolved by reducing SDS-PAGE and bands were quantitated using a phosphorimager. Secretion efficiency was calculated as the immunoprecipitable-labeled protein in the media after a 60-min chase divided by the amount of labeled protein in cells after the 10-min pulse multiplied by 100. Filled circles apoB; open circles,

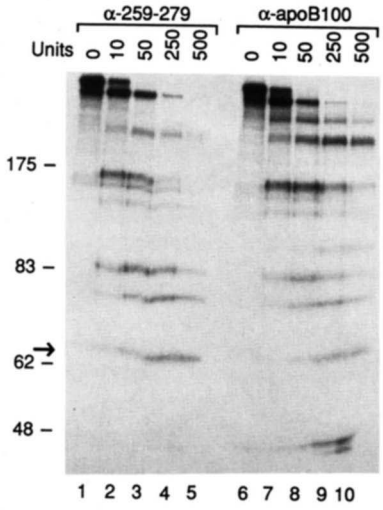

Fig. 7. Thrombin digestion of apoB. HepG2 cells were labeled continuously for 2 h. After centrifugal concentration, samples were adjusted to 1% Triton X-100, and aliquots were digested with the indicated amount (U) of porcine thrombin. The reaction was stopped by addition of protease inhibitors and an aliquot of each digestion was immunoprecipitated with polyclonal antibody to apoB (α-apoB-100) or a peptide antibody specific for amino acids 259–279 of apoB (α-259–279). Immunoprecipitates were reduced prior to fractionation by 6% SDS-PAGE. The arrow indicates the position of a ~65 kDa fragment referred to under Results and further characterized in Fig. 9.

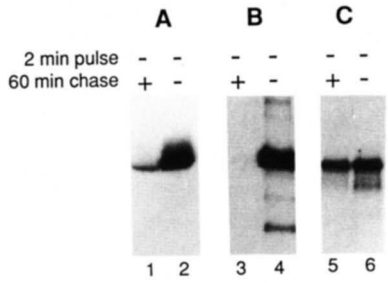

Fig. 8. Differential sensitivities of albumin, α_2-macroglobulin, and apoB secretion to DTT. Cells were pulse-labeled for 2 min in the absence of DTT and chased for 60 min in the presence or absence of 2 mM DTT as indicated. Aliquots of media were immunoprecipitated with antibodies to albumin (A), α_2-macroglobulin (B), or apoB (C).

amino-terminal end (amino acid residues 259–279) of apoB (34). One such band that predominated in extensively degraded samples migrated with a molecular mass of ~65 kDa (**Fig. 7,** arrow). Assuming that the 65 kDa peptide is 590 amino acids long, this fragment lies somewhere within the amino-terminal 19% of apoB.

To determine whether amino-terminal disulfide bonds undergo rapid resistance to DTT-mediated reduction we examined amino-terminal disulfide bonds in apoB secreted from cells that were pulsed for 2 min in the absence of DTT and then chased for 60 min in the presence of DTT. Under these labeling conditions the secretion of disulfide-bonded proteins is severely inhibited because 2 min is insufficient time to achieve DTT resistance. Indeed, under these conditions, albumin secretion was inhibited by ~95% and α_2-macroglobulin secretion was blocked completely. In the same cells, however, apoB secretion was inhibited by only ~20% (**Fig. 8**). Because label was added to the cells 2 min prior to DTT, the amino-termini of many of the apoB chains secreted under these conditions will contain radioactive amino acid residues. To examine whether the apoB secreted from cells treated under these condition contained intact disulfide bonds, media samples were subjected to thrombin digestion and immunoprecipitation with antibodies to the amino-terminus of apoB. Each immunoprecipitate was divided into equal aliquots, one of which was heated in SDS-PAGE sample buffer alone and the other was heated in sample buffer containing 50 mM DTT. After cooling to room temperature, 100 mM IAA was added to each and samples were analyzed by SDS-PAGE and fluorography. The thrombin-derived amino-terminal 65 kDa peptide of apoB obtained from both -/- and -/+ cells displayed reduced SDS-PAGE mobility upon heating in the presence of DTT (**Fig. 9,** compare lanes 1 and 2; 3 and 4). In addition, the nonreduced samples from control cells (-/-) and cells chased in the presence of 2 mM DTT (-/+) migrated identically (compare lanes 2 and 4). The lower amount of labeled amino-terminal 65 kDa fragment in the -/+ versus the -/- samples is to be expected because many of the labeled nascent chains will not have been extended beyond the critical coordinate necessary to achieve DTT resistance. Hence, when the chase is initiated in the presence of DTT, these nascent chains (form I) will be not be able to complete disulfide bonding and will, therefore, not be secreted into the medium. On the basis of this and previous experiments, it can be concluded that most, if not all, of the disulfide bonds in the

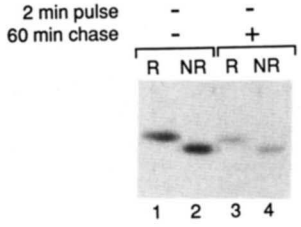

Fig. 9. ApoB secreted from DTT-treated HepG2 cells contains intact disulfide bonds. Cells were pulse-labeled for 2 min in the absence of DTT and then chased for 60 min in the presence or absence of DTT as indicated. Media samples were recovered, subjected to centrifugal concentration, and treated with 50 U porcine thrombin. Reactions were immunoprecipitated with the same amino-terminal anti-peptide antibody used in Fig. 7. Immunoprecipitates were split into equal aliquots. One aliquot was boiled in SDS-PAGE sample buffer (NR) and the other in SDS-PAGE sample buffer containing 50 mM DTT (R). After cooling to room temperature, samples were adjusted to 100 mM IAA, incubated at 37° for 30 min, and resolved by 6% SDS-PAGE.

amino-terminus of apoB become resistant to DTT-mediated reduction while apoB is still a nascent chain. The amount of apoB that needs to be synthesized to progress to a DTT-resistant form was estimated to be ~20–25%. This estimate was based on the presence of radioactive amino acids in the 65 kDa thrombin fragment and the relative decrease in apoB secretion observed in DTT-treated cells (Discussion).

DISCUSSION

Current evidence indicates that the association of apoB with lipids occurs cotranslationally (4), although the ultimate maturation of apoB-containing lipoproteins may also require posttranslational lipid addition (5, 35). In a cotranslational model of lipoprotein formation it would be expected that the amino-terminus of apoB may participate in a critical event necessary to initiate lipoprotein formation (6). Because the amino-terminal 21% of apoB is rich in intramolecular disulfide bonds, we were able to perturb its folding with DTT and assess the resulting effects on lipoprotein secretion. The behavior of apoB in DTT-treated HepG2 cells was unique relative to other disulfide-bonded proteins. Characterization of this novel behavior revealed that folding of the amino-terminal disulfide-bonded domain of apoB is indeed essential for the initiation and also perhaps later steps in apoB-containing lipoprotein assembly in the ER.

When DTT was added to HepG2 cells 1 min prior to addition of label, two populations of labeled apoB were generated. The secretion of one form was resistant to the inhibitory effects of DTT. The secretion of a second population of apoB, however, appeared to be irreversibly blocked by DTT addition. As diagrammed in Fig. 4, lengthening DTT preincubation time increased the percentage of labeled apoB polypeptide chains with amino-termini synthesized in the presence of DTT. As the percentage of apoB synthesized under these conditions increased, the secretion efficiency of apoB was correspondingly decreased. This result indicates that when the folding of the amino-terminal domain of apoB is perturbed by the presence of DTT, the ability of apoB to form a secretable lipoprotein is irreversibly blocked. Conversely, when this domain is allowed to fold unperturbed, apoB assembly and secretion progresses to a stage that is unaffected by subsequent DTT addition. Analysis of an amino-terminal thrombolytic fragment of apoB secreted from DTT-treated cells revealed that the amino-terminus of apoB progresses to a DTT-resistant form well before apoB translation has been completed. For many proteins, progression to DTT resistance is associated with the formation of a native higher order structure (15). In the case of apoB it is not known whether the rapid onset of DTT resistance is related to protein-mediated folding, and/or its presumed cotranslational association with lipids. While DTT resistance alone is not informative as to the detailed nature of a particular stage of apoB assembly, its onset appears to signal the completion of a discrete and early stage in the process of lipoprotein formation. Clearly, when disulfide bonding is perturbed prior to the completion of this stage, later steps in lipoprotein assembly are irreversibly blocked.

Two criteria were used to estimate the amount of apoB that must be synthesized in order to become DTT resistant. Based on its predicted length of 590 amino acids and its reactivity with an antibody directed against amino acids 259–279, the carboxyl-terminal boundary of the 65 kDa thrombin fragment (Figs. 7 and 9) is predicted to lie between amino acids 590–849, depending on the location of thrombin cleavage site(s). As about 325 amino acids are incorporated into apoB per min (36), an additional 650 amino acids could be added during the 2-min pulse. Assuming that addition of label was initiated between amino acids 590–849, the upper limit for the length of nascent chain that becomes DTT resistant is 1240–1499 amino acids (apoB27–apoB33).

The second criterion for judging how much apoB must be translated to achieve DTT resistance is based on the inhibition of apoB secretion caused by DTT. Assuming a uniform rate of apoB translation (and the fact that methionine residues display a relatively random distribution in apoB), the percentage decrease in secretion caused by DTT will be roughly equal to the length of apoB that must be translated to become DTT resistant. In Fig. 1 and related experiments, DTT reduced the secretion of apoB radioactivity by ~40%. However, use of a long (10 min) pulse in the presence of DTT gave rise to a series of labeled apoB polypeptide chains with very different amounts of radioactive amino acid incorporation (Fig. 4A, compare lengths of jagged lines in apoB polypeptides). Because, under these labeling conditions, form I apoB contained on average more label than form II, use of the 10-min pulse tended to overestimate the percentage of apoB polypeptide chains whose secretion was inhibited by DTT. The results shown in Fig. 8, however, are a better reflection of the percentage of apoB polypeptide chains that were susceptible to DTT-mediated inhibition of secretion. First, as a 2-min pulse was performed in the absence of DTT, little of the inhibition of secretion could be attributed to decreased synthesis. Second, the use of a short pulse tended to create more uniform incorporation of label into apoB polypeptides. Under these conditions, the inhibition of apoB secretion was approximately 20%. Combining the two criteria discussed above, we estimated that ~20–25% of apoB must be synthesized to become DTT resistant.

As 7 of the 8 disulfide bonds in apoB are localized to the amino terminal 21% of the protein, it would appear that the onset of resistance to DTT is essentially cotranslational. This finding also indicates that the disulfide bond formed between cysteine residues 3167 and 3297 in the carboxyl-terminal half of apoB (12) is not essential for the assembly and secretion of apoB-containing lipoproteins.

While nascent forms of apoB whose translation proceeded beyond the amino-terminal 20–25% were resistant to DTT addition, DTT added prior to this point caused production of aberrant forms of apoB that were subjected to irreversible ER retention and rapid intracellular turnover. The behavior of these forms of apoB was consistent with their inability to undergo appreciable assembly with lipids. However, as we could not selectively monitor the density of this intracellular population of apoB, it could be argued that even under reducing conditions, apoB was capable of undergoing intracellular lipidation but was unable to achieve secretion competence for other reasons. We find this possibility unlikely considering that if the basic architecture of a lipoprotein particle could be formed under reducing conditions, removal of DTT would promote posttranslational disulfide bonding and secretion, as is observed for other disulfide-bonded proteins (14–16, 37–40). As shown in Figs. 1, 2, and 5, however, we observed no evidence of posttranslational formation of secretion competent apoB. We believe, therefore, that cotranslational lipidation of apoB cannot be achieved unless the amino-terminus is first allowed to complete its normal biosynthetic pathway.

How the amino-terminus of apoB functions to initiate lipoprotein assembly cannot be established based on the current results; however, we envision two alternative hypotheses. *i)* The initiation phase of lipoprotein assembly provided by the amino-terminus of apoB could involve the recruitment of a phospholipid surface which would then act as an acceptor for both neutral lipid and phospholipid transfer by MTP; or *ii)* the amino-terminus of apoB may function directly as a proteinaceous acceptor for MTP-mediated lipid transfer. In support of the former hypothesis, a number of reports indicate a relationship between the amino-terminal domain of apoB and structural and functional interactions with phospholipid. Herscovitz et al. (41) demonstrated that the amino-terminal 17% of apoB can spontaneously organize dimyristoylphosphatidylcholine vesicles into discoidal particles in vitro. It has also been reported that apoB-15 translated in vitro in the presence of rat liver microsomes incorporates phosphatidylcholine (42). Experiments in which cellular phospholipid membrane composition was perturbed by incubation of hepatocytes and hepatoma cells with the ethanolamine/choline analog, monomethylethanolamine, selectively reduced the secretion of both full-length apoB (43, 44) and transfected forms of apoB as small as apoB-18 (42). The hypothesis that the amino-terminus of apoB may recruit phospholipid to initiate lipoprotein assembly is also consistent with the fact that MTP, in vitro, achieves lipid transfer between phospholipid vesicles in the absence of other protein factors (45, 46). While the existence of apoB on a nascent lipoprotein particle could enhance the rate of MTP-dependent lipid transfer or improve its specificity, MTP can clearly function independently of apoB provided an appropriate phospholipid acceptor surface is present.

Overall, our results are consistent with a model in which the amino-terminal disulfide-bonded domain of apoB initiates the process of lipoprotein formation. After the successful assembly of the amino-terminal domain, downstream sequences are translocated into the ER. These domains are predicted to be rich in amphipathic β-sheet structures (8, 11) and, because of their predicted avid lipid binding properties, may require cotranslational association with a previously initiated lipoprotein for their proper folding. This is illustrated by the fact that when apoB translation is allowed to continue without first completing this initiation phase of assembly, apoB becomes extremely labile and is a target for one or more protease that does not normally degrade intracellular apoB (i.e., a non ALLN- and DTT-inhibitable protease). Furthermore, unlike other disulfide-bonded proteins, reduced forms of apoB display no capacity for posttranslational assembly. While a molecular description of the initiation event achieved by the amino-terminus of apoB is not yet defined, its autonomous folding indicates that its structure and function can be examined independently of the rest of the protein. Studies of the biosynthetic behavior of this domain in transfected cells, as well as analyses of its structural and functional properties in vitro, will likely further clarify the complex process by which apoB, MTP, lipids, and other factors interact to achieve lipoprotein assembly in the ER.

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