Identification of Cysteine Pairs within the Amino-terminal 5% of Apolipoprotein B Essential for Hepatic Lipoprotein Assembly and Secretion*

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There is growing evidence that the amino-terminal globular domain of apolipoprotein B (apoB) is essential for lipoprotein particle formation in the hepatic endoplasmic reticulum. To identify the structural requirements for its function in lipoprotein assembly, cysteine (Cys) pairs required to form the seven disulfide bonds within the amino-terminal 21% of apoB were replaced in groups or individually by serine. Substitution of Cys pairs required for formation of disulfide bonds 1–3 or 4–7 (numbered from amino to carboxyl terminus) completely blocked the secretion of apoB28 in transfected HepG2 cells. To identify the specific disulfide bonds required for secretion, Cys pairs were mutated individually. Substitution of Cys pairs required for disulfide bonds 1, 3, 5, 6, or 7 had little or no impact on apoB28 secretion or buoyant density. In contrast, individual substitution of Cys pair 2 (amino acid residues 51 and 70) or 4 (218 and 234) severely inhibited apoB28 secretion and its capacity to undergo intracellular assembly with lipid. The same assembly and secretion defects were observed when these mutations were expressed as part of apoB50. These studies provide direct evidence that the ability of the internal lipophilic regions of apoB to engage in the recruitment and sequestration of lipid during translation is critically dependent upon a structural configuration contained within or affected by the amino-terminal 5% of the protein.

Little is known about how the initial folding of apolipoprotein B (apoB) influences its interactions with microsomal triglyceride transfer proteins (MTP) and other factors to initiate the process of hepatic apoB-containing lipoprotein assembly (1–3). Although incapable of assembling with appreciable lipid on its own (4–7), the amino-terminal disulfide-bonded region of apoB may, nonetheless, be essential for lipoprotein assembly. Based on the use of dithiothreitol (DTT) to probe protein folding in vivo, the amino-terminal domain of apoB was shown to undergo a folding reaction that is completed before the translation and translocation of the internal lipophilic regions of apoB. If DTT was added to cells during the translation of this domain, thereby preventing its proper folding, apoB was incapable of engaging in lipoprotein assembly (8, 9). Although these and other studies (7) are consistent with an essential role in the initiation of lipoprotein assembly, it is not known which, among the seven disulfide bonds present in the amino-terminal 21% of apoB (10), are required for this function. Furthermore, it could not be established whether the block in lipoprotein assembly caused by DTT was caused only by perturbed apoB amino-terminal folding or whether it also resulted from DTT-mediated inhibition of one or more assembly factors.

In the present study, the relationship between the structure of the amino-terminal domain of apoB and its capacity to initiate lipoprotein assembly in vivo was examined by performing systematic mutagenesis to disrupt its normal pattern of disulfide bonding (10). Individual substitution of five of the seven Cys pairs within the amino-terminal domain had little or no impact on apoB assembly or secretion. However, substitution of Cys pairs required for formation of disulfide bonds 2 or 4 virtually abolished the capacity of both apoB28 and apoB50 to engage in lipoprotein particle assembly and secretion. These studies indicate that the ability of the lipophilic regions of apoB (11) to engage in lipid recruitment and sequestration during translation is critically dependent upon a structural configuration within or affected by the amino-terminal 5% of the protein. It is possible that this domain serves as part of a recognition site for MTP or engages in an initial lipid recruitment step required to recruit nascent apoB polypeptides into acceptor particles for the cotranslational phase of MTP-dependent lipid transfer.

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.). Trans35S-label ([35S]Met and Cys) was from ICN. Anti-FLAG M2 monoclonal antibody (an antibody that recognizes the 8-amino acid FLAG epitope (DYKDDDDK) positioned at the carboxy-terminal end of apoB28F and apoB50F) and anti-FLAG M2-agarose were from Eastman Kodak Scientific Imaging Systems (New Haven, CT). FLAG peptide was synthesized and purified by the Protein Analysis Core Laboratory of the Comprehensive Cancer Center of Wake Forest University. Centricon-10 and Centriprep-10 centrifugal concentrators were from Amicon (Beverly, MA).

Mutagenesis of ApoB28F and ApoB50F cDNA—Pairwise Cys to Ser mutations were made in apoB28F (9) to disrupt the seven disulfide linkages reported in human low density lipoprotein apoB (10) (C1–C7; Fig. 1A). Mutations were introduced by overlap PCR extension (12) using primers containing T to A substitutions in the first position of the appropriate Cys codons (13). PCR amplification products were digested with restriction enzymes, purified by agarose gel electrophoresis, and used to replace the corresponding restriction fragment of wild type apoB28F cloned into pBluescript II SK+ (9). The following restriction

protein(s); DTT, dithiothreitol; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; ER, endoplasmic reticulum.

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1 The abbreviations used are: apoB, apolipoprotein B; MTP, microsomal triglyceride transfer protein(s); DTT, dithiothreitol; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; ER, endoplasmic reticulum.
expression vector (14). This was accomplished by digesting wild type mid, the entire apoB28 coding region was transferred to the pCMV5 the recipient for the mutant PCR restriction fragments. Once the ap- by using mutant apoB28F as a template for subsequent PCRs and as

conjugated to agarose beads (50-

with gentle inversion at 4 °C with anti-FLAG M2 monoclonal antibody

centrifugation to remove nuclei and cell debris, supernatants were

two rounds of batch elution with 125

centration, 150-mm dishes were employed. In both cases, cells were tran-

was analyzed by density gradient centrifugation or proteolytic diges-

rum (8). For studies involving apoB secretion 35-mm dishes in six-well

site) and

apoB28F (3-

was transferred to the pCMV5 expression vector (14). This was accomplished by digesting wild type apoB28F contained in pCMV5 with EcoRI (5- flanking site) and XhoI (3- end of the apoB28 coding sequence) and replacing it with the same restriction fragment generated by digestion of the mutant apoB28F pBluescript clone. All mutations were verified by sequencing the entire area within each clone that was created by PCR. Mutant forms of apoB50F were made by digesting the appropriate apoB28F mutant plasmid with EcoRV (amino acid 580) and KpnI (3- flanking site) and replacing it with the EcoRV and KpnI fragment from wild type apoB50F (15).

Tissue Culture and Metabolic Labeling—HepG2 cells were cultured in Eagle’s minimal essential medium containing 10% fetal bovine se-

Immuno precipitation and Gel Electrophoresis—After metabolic la-

Proteolytic Digestion— transiently transfected HepG2 cells (150-mm

FIG. 1. Effect of sequential pairwise Cys to Ser substitution on apoB28F secretion. Panel A, the bold horizontal line depicts the apoB28 polypeptide backbone. The vertical lines show the relative position of Cys residues; numbers indicate amino acid coordinates relative to the amino-terminal amino acid of the mature protein. Bracketed Cys residues exist in disulfide-bonded form in human low density lipoprotein apoB (10). Cys pairs (C1–C7) are numbered from amino to carboxyl terminus. Panel B, HepG2 cells were transfected with wild type (wt) and mutant forms of apoB. After metabolic labeling for 3 h with [35S]Met/Cys, apoB28F in cells (C) and media (M) was immunoprecipitated and analyzed by SDS-PAGE and fluorography. C1, substitution of two Cys residues required for disulfide bond 1; C1–2, substitution of four Cys residues required for disulfide bonds 1 and 2, etc. In lanes 1 and 2 (mock), cells were subjected to transfection procedure in the absence of DNA.

Effects of Sequential Cys to Ser Substitutions on ApoB28F Secretion—Seven of the eight disulfide bonds present in apoB100 are positioned within the amino-terminal 21% of the protein (10). The effects of disrupting these disulfide bonds were assessed using a polypeptide containing the amino-termi-

nally 28% of apoB100 to which the 8-amino acid FLAG epitope was attached at the carboxyl-terminal end (apoB28F) (9, 17). Sequential disruption of disulfide bonds from the amino- and carboxyl-terminal ends of apoB28F (Fig. 1A) was achieved by making pairwise Cys to Ser substitutions as described under “Experimental Procedures.” Transiently transfected HepG2 cells expressing the wild type and mutant forms of apoB28F were metabolically labeled for 3 h with [35S]Met/Cys, and the amount of radioactive protein present in media and cells was assessed by immunoprecipitation followed by SDS-PAGE and fluorography. When the 2 Cys residues required to form disul-

fraction was pooled and incubated with trypsin (0.5 μg/ml; Boehr-

inhibitor (final concentration of 500 μg/ml). Samples were subsequently adjusted to 5 mM phenylmethylsul-

fruits were analyzed by 8% SDS-PAGE. Gels with the 2 Cys residues required to form disul-

firmed. Finally, when Cys pairs 6 and 7 (C6–7) or 5, 6, and 7 (C5–7) were re-

No inhibition of secretion was observed upon substitution of

“Disulfide Bonds 2 a means Are inhibitors for ApoB28F Secretion—The analysis above suggests that Cys pairs 2 and 4 may individually be critical for apoB secretion. To confirm this, individual Cys pair mutations were made in apoB28F, and the effects on secretion were assessed by pulse-chase analyses. Transiently transfected HepG2 cells were pulsed for 10 min with [35S]Met/Cys and chased for the times indicated with medium containing an excess of unlabeled amino acids (Fig. 2). ApoB28F bearing mutations in Cys pairs 1, 3, 5, and 7 (C1, C3,
C5, C7) displayed little or no effect on secretion kinetics or efficiency relative to wild type. In each case ∼40–55% of the peak apoB28F radioactivity present in cells after the 10-min pulse was recovered in the medium after 3 h of chase. Substitution of Cys pair 6 (C6) caused a partial secretion defect in which only 15% of the apoB28F synthesized during the 10-min pulse was recovered in the medium. As predicted from the sequential deletion analysis (Fig. 1B), individual pairwise Cys to Ser substitutions that block formation of disulfide bonds 2 or 4 (C1, C2) virtually abolished apoB28F secretion. In both cases less than 3% of the peak apoB28F radioactivity present in cells after the pulse was recovered in the medium after 3 h of chase. Although these mutations inhibited secretion, they did not affect intracellular degradation dramatically. For mutants C2 and C4, ∼60–80% of the peak radioactivity present in cells after the pulse was recovered in cells and medium after the 3-h chase, a value similar to that observed for the wild type.

Cys Pairs 2 and 4 Are Essential for ApoB28F Lipid Recruitment in the Endoplasmic Reticulum (ER)—The requirement for disulfide bonds 2 and 4 in the assembly of apoB was explored further by examining the effects of Cys mutations on the capacity of apoB28 to recruit lipid intracellularly. For these studies the buoyant density of wild type apoB28F from intracellular pools was compared with that of mutant forms that had either no impact on apoB secretion (C1) or inhibited secretion strongly (C2 and C4). Transiently transfected HepG2 cells were labeled for 3 h with [35S]Met/Cys, and postnuclear membrane fractions were prepared and extracted with sodium carbonate, pH 11.5, to obtain lumenal contents (15). After centrifugation, pellet fractions were subjected directly to immunoprecipitation. Supernatant was neutralized, adjusted to 1.25 g/ml with KBr (total volume, 3 ml), and analyzed by equilibrium density gradient centrifugation as described (9). ApoB28F was recovered from the top 1-ml (T) and bottom 2-ml (B) fractions by immunoprecipitation.

Because apoB50F contains more extensive lipid binding sequences than apoB28F (11) we explored the possibility that, although incapable of secretion, mutant forms were able to recruit an appreciable amount of lipid intracellularly. As displayed in Fig. 5, most of the carbonate-extractable apoB50F wild type and C1 mutant floated in the top, d < 1.25 g/ml gradient fraction. For the C2 and C4 mutations, a small amount of buoyant apoB50F was observed. However, the ratio of buoyant to lipid poor apoB50F was much lower for disulfide bond mutants C2 and C4 (−0.5) compared with the wild type and C1 mutant (−6). Furthermore, relative to the wild type and C1 mutant, a smaller percentage of the C2 and C4 forms of apoB50F was extracted by sodium carbonate. This behavior would be expected for apoB that was unable to engage in lipoprotein assembly and, therefore, may engage in binding to the inner leaflet of the ER membrane or aggregation. We conclude, therefore, that Cys pairs 2 and 4 are essential for the assembly of native forms of apoB.
Disulfide Bonds Essential for ApoB Lipoprotein Assembly

FIG. 4. Effects of Cys to Ser substitutions on the secretion of apoB28F. Wild type apoB50F and apoB50F bearing Cys pair mutations to disrupt disulfide bonds C1, C2, and C4 were transfected into HepG2 cells. After labeling with [35S]Met/Cys for 3 h, apoB50F in cells (C) and media (M) was immunoprecipitated. The asterisk shows the position of an endogenous background band that immunoprecipitates nonspecifically.

FIG. 5. Disruption of disulfide bonds 2 and 4 inhibits apoB28F-containing lipoprotein assembly. Wild type (wt) apoB50F and apoB50F bearing mutations affecting disulfide bonds C1, C2, and C4 were transfected into HepG2 cells. After labeling with [35S]Met/Cys for 3 h, a postnuclear membrane fraction was obtained and extracted with sodium carbonate, pH 11.5. After centrifugation the pellet (P) was solubilized and subjected to direct immunoprecipitation. The carbonate supernatant was subjected to density gradient centrifugation as described in Fig. 3. The top 1-ml (T) and bottom 2-ml (B) fractions were subjected to immunoprecipitation. T/B is the ratio of apoB50F in the top and bottom fractions.

Critical events that occur almost immediately after entry of apoB into the ER are critically important to promote hepatic lipoprotein assembly.

FIG. 6. Assembly-defective forms of apoB28F recovered from cells display an altered structure. HepG2 cells expressing wild type apoB28F and the C1, C2, and C4 mutants were labeled for 2 h with [35S]Met/Cys and subjected to lysis in non-ionic detergent. After affinity purification, aliquots were digested with trypsin for the times indicated and analyzed by SDS-PAGE and fluorography. Arrows on the left indicate two predominant peptides generated by cleavage of assembly-competent forms of apoB28F (wild type and C1). The arrow on the right indicates the position of a peptide generated by digestion of assembly-defective forms of apoB28F (C2 and C4). Migration of molecular mass standards is indicated in kDa. Non–full-length bands present in the untreated (0 min) lanes are nonspecific polypeptides that copurify with apoB28F during immunoprecipitation.

DISCUSSION

Previous studies in which DTT was used to perturb disulfide bonding in apoB28 (9) and apoB100 (8) indicated that the amino-terminal disulfide-bonded domain of apoB may be critically important for the initiation of apoB-containing lipoprotein assembly. However, because of the relatively wide distribution of disulfide bonds within the amino-terminal 950 amino acids of apoB it was not possible to identify which of the seven disulfide bonds are essential. In the current report Cys pairs involved in disulfide bond formation within the amino-terminal 21% of apoB were systematically replaced in groups or individually by Ser, and the effects on apoB-containing lipoprotein assembly and secretion were assessed. Remarkably, it was found that disruption of two of the four Cys pairs located within the amino-terminal 5% of apoB severely limited the ability of apoB28 and apoB50 to engage in lipoprotein particle assembly. Because we have shown previously that the disulfide bonds in the amino-terminal domain of apoB form during the process of translation (8, 9) it can be concluded that folding events that occur almost immediately after entry of apoB into the ER are

for each mutant. Intracellular forms of wild type and mutant apoB28F were also digested with proteases to assess possible conformational differences caused by mutagenesis (19). As observed in Fig. 6, the overall susceptibilities of both wild type and mutant forms of apoB28F to proteolytic degradation are similar. However, the wild type and C1 mutants, both of which are assembly- and secretion-competent, were cleaved internally giving rise to two predominant peptides of ~65 and 75 kDa. In contrast, the assembly-defective mutants, C2 and C4, displayed only limited formation of these peptides and instead produced a larger peptide of ~120 kDa. These results indicate that disruption of either the C2 or C4 disulfide bond affects the capacity of apoB28 to fold into a conformation capable of engaging in lipoprotein formation in the ER.

Although the data presented here provide compelling evidence as to the importance of the amino-terminal region of apoB in lipoprotein assembly, it is not known how this domain functions. Previous studies have demonstrated a physical interaction between MTP and the amino-terminal domain of apoB (20, 21). It is possible therefore that discrete disulfide-bonded loops within this domain form a critical recognition site for the initial interaction of MTP with the nascent apoB polypeptide chain. In addition, the amino-terminal domain of apoB has been shown to recruit phospholipid spontaneously upon incubation in vitro with vesicles composed of dimyristoylphosphatidylcholine (22) or during cell-free translation in the presence of rat liver microsomes (23). Hence, an alternative or additional function provided by the amino-terminal domain of apoB may involve recruitment of a phospholipid surface that serves as an acceptor site for MTP-dependent transfer of neutral and polar lipids. This recruitment step could be achieved in a number of ways including formation of a membranous bleb or bud on the luminal side of the ER membrane (4, 24, 25). The requirement of the amino-terminal domain to initiate or achieve this budding process is supported in part by studies demonstrating that forms of apoB containing as little as the amino-terminal 15% of the protein are capable of inserting into the inner leaflet of the ER membrane in a carbonate-inextractable form (26).

In large part, the various hypothetical functions of the amino-terminal domain of apoB discussed above are based on cell-free assays. However, no experimental relationship has been established to link the capacity of the amino-terminal domain to interact with MTP, lipids, and membranes in vitro and its
ability to initiate lipoprotein particle assembly in vivo. It will be of interest to assess how the same structural alterations produced by mutagenesis which affect lipoprotein assembly in cells will affect the capacity of this domain to associate with MTP, lipids, and membranes in cell-free assays. The critical function of the amino-terminal domain may emerge by identifying which cell-free assay(s) display a structure-function profile similar to that observed for the capacity of apoB to initiate lipoprotein assembly in vivo.

Although disulfide bonds 2 and 4 appear necessary for apoB assembly and secretion it is interesting that removal of five of the seven disulfide bonds within the amino-terminal domain of apoB had little or no impact on secretion (Figs. 1 and 2) or buoyant density (data not shown). It is not uncommon that mutagenic removal of disulfide bonds fails to affect and can even enhance folding and secretion rates (27, 28). However, it is also possible that the insensitivity of apoB assembly to substitution of particular Cys pairs reflects the multifunctional nature of the amino-terminal domain. For example, disulfide bond mutations that fail to affect the first cotranslational phase of lipoprotein assembly, which predominates in HepG2 cells (4), could nonetheless have an impact on the posttranslational ability of apoB48 to form very low density lipoprotein particles in hepatocytes or olate stimulated rat hepatoma cells (29, 30). It is also conceivable that although competent for secretion, these disulfide bond mutations will affect intravascular aspects of apoB metabolism such as its ability to undergo lipoprotein lipase-mediated lipolysis (31, 32) or its ability to undergo the conformational changes that are required for it to function as a ligand for the low density lipoprotein receptor (33).

One of the most common genetic disorders involving apoB (1/500–1/1000 in Western populations) is heterozygous hypobetalipoproteinemia (34). In these individuals frameshift and nonsense mutations result in the production of truncated forms of the apoB protein. Invariably, these mutations result in low plasma low density lipoprotein cholesterol either because of reduced expression of the mutant allele or an enhanced rate of intravascular clearance. However, in cases where mutations were predicted to form very short apoB proteins (smaller than apoB30), no evidence of truncated apoB was detected in plasma (35, 36). Based on the results presented here, it is a formal possibility that missense mutations at one of the 4 Cys residues required for the formation of disulfide bonds 2 or 4 or at other residues that participate in the formation of this critical amino-terminal structural domain could underlie some cases of this lipid disorder.

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REFERENCES

34. Linton, M. F., Farese, R. V., Jr., and Young, S. G. (1993) J. Lipid Res. 34, 521–541
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