Vesicle-binding properties of wild-type and cysteine mutant forms of α_1 domain of apolipoprotein B

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Abstract **Previous studies demonstrated that structural perturbation of the α_1 domain of apolipoprotein B (apoB) blocked the initiation of lipoprotein assembly. We explored the hypothesis that this domain may interact with the inner leaflet of the endoplasmic reticulum membrane in a manner that may nucleate microsomal triglyceride transfer protein-dependent lipid sequestration. ApoB-17 (amino-terminal 17% of apoB), which contains most of the α_1 domain, was expressed stably in rat hepatoma cells and recovered from medium in lipid-poor form. On incubation with phospholipid vesicles composed of 1-myristol-2-myristoyl-*sn*-glycero-3-phosphocholine or 1-palmitoyl-2-oleoyl-*sn*-gylycero-3-phosphocholine, apoB-17 underwent vesicle binding and was recovered in the d $<$ 1.25 g/ml gradient fraction. To determine whether vesicle binding is disrupted by the same structural perturbations that block lipoprotein assembly in vivo, apoB-17 was subjected to partial and complete chemical reduction. Although normally a soluble peptide, mild reduction of apoB-17 caused its precipitation, suggesting that hydrophobic, solvent-inaccessible domains within the α_1 domain of apoB are stabilized by intramolecular disulfide bonds. In contrast to apoB-17 chemically reduced in vitro, forms of apoB-17 bearing pairwise cysteine-to-serine substitutions were recovered in soluble form from transiently transfected COS-1 cell extracts. Although individual disruption of disulfide bond 2 or 4 in apoB-28 and apoB-50 was previously shown to block lipoprotein assembly in vivo, these alterations had no impact on the ability of apoB-17 to bind to phospholipid vesicles in vitro or on its capacity to form recombinant lipoprotein particles. These results suggest that while the vesicle/lipid-binding property of the α_1 domain may reflect an essential role required for the initiation of lipoprotein formation, some other aspect of α_1 domain function is perturbed by disruption of native disulfide bonds.**—DeLozier, J. A., J. S. Parks, and G. S. Shelness. **Vesicle-binding properties of wild-type and cysteine mutant forms of α_1 domain of apolipoprotein B.** ***J. Lipid Res.*** **2001.** 42: **399–406.**

Supplementary key words lipoprotein assembly • recombinant lipoproteins • disulfide bonds • apoB-17 • vesicle-binding assay

Apolipoprotein B-100 (apoB) is an amphipathic glycoprotein that is required for the microsomal triglyceride transfer protein (MTP)-dependent assembly and secretion of triglyceride-rich very low density lipoproteins (1–3). The strong lipid-binding sites in apoB are predicted to reside within a series of amphipathic α-helical and β-sheet domains concentrated in the C-terminal ~80% of the polypeptide chain (4). In contrast, the N-terminal ~20% of apoB (α_1 domain) is highly disulfide bonded and is secreted from transfected hepatic and nonhepatic cells in soluble, lipid-poor form (4–6). Despite its inability to form a lipoprotein particle when expressed on its own, disruption of native disulfide bonds within the α_1 domain prevents the downstream lipophilic regions of apoB from engaging in cotranslational lipid recruitment (7–11). Hence, it appears that the α_1 domain of apoB plays a critical role in the initiation of apoB-containing lipoprotein assembly, although the specific mechanism underlying this process is unknown.

The α_1 domain of apoB displays amino acid sequence homology with the amino-terminal domain of MTP and members of the vitellogenin gene family (12–16). On the basis of the known crystal structure of lamprey lipovitellin (the processed form of vitellogenin), the α_1 domain of apoB and the corresponding domain of MTP may comprise an N-terminal β-barrel and an extended C-terminal α-helical domain. These domains, which serve as homodimerization surfaces in lipovitellin, may be responsible for the capacity of MTP to physically interact with apoB (15). The detailed structural requirements for apoB-MTP interactions, including the requirement for native disulfide bonding, are currently under study (17–19).

Another property of the α_1 domain of apoB that may be relevant to the initiation of apoB-containing lipoprotein assembly is its capacity to interact with phospholipid sur-

Abbreviations: apoB, apolipoprotein B; DMPC, 1-myristoyl-2-myristoyl-*sn*-glycero-3-phosphocholine; DTT, dithiothreitol; ER, endoplasmic reticulum; IAA, iodoacetamide; MLV, multilamellar vesicle; MTP, microsomal triglyceride transfer protein; PBS, phosphate-buffered saline; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SUV, small unilamellar vesicle; TG, triglyceride.

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faces. In one commonly cited hypothetical model of lipoprotein formation, apoB intercalates into the inner leaflet of the endoplasmic reticulum (ER) membrane during its translation and provides a hydrophobic nucleation site for the accumulation of triglyceride (TG) (20–22). That the α_1 domain may participate in this membrane-binding process is supported by cell-free studies demonstrating that during the cotranslational translocation of apoB-17 into the interior of dog pancreas microsomes, it interacts with the inner leaflet of the microsomal membrane, rendering it carbonate inextractable (23). Furthermore, it was shown that apoB-17, although secreted from transfected cells in lipid-poor form, efficiently binds to vesicles composed of 1-myristoyl-2-myristoyl-*sn*-glycero-3-phosphocholine (DMPC) and forms recombinant discoidal lipoproteins (24).

In the present study the relationship between disulfide bond-dependent structural properties of the α_1 domain and its capacity to interact with phospholipid vesicles in vitro was explored. We hypothesized that if the vesicle-binding behavior of the α_1 domain of apoB reflects a critical role in the initiation of lipoprotein assembly, this capacity may be disrupted by the same pattern of cysteine-to-serine substitutions that was previously shown to disrupt apoB-28 and apoB-50-containing lipoprotein assembly in vivo (9, 10). However, vesicle binding by apoB-17 was retained in all disulfide bond mutants tested. These results suggest that a function independent of membrane binding and phospholipid sequestration is perturbed by disruption of native disulfide bonds in the α_1 domain of apoB.

EXPERIMENTAL PROCEDURES

Reagents

FLAG peptide (DYKDDDDK) was synthesized and purified by the Protein Analysis Core Laboratory of the Comprehensive Cancer Center of Wake Forest University (Winston-Salem, NC). [^{35}S]Met/Cys (Easytag) was from New England Nuclear (Boston, MA). Anti-FLAG M2 monoclonal antibody, anti-FLAG M2-agarose, DMPC, and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) were obtained from Sigma (St. Louis, MO). FuGENE 6 transfection reagent was obtained from Roche Molecular Biochemicals (Indianapolis, IN). Human serum albumin cDNA plasmid pBAT14 was kindly provided by P. Arvan (Albert Einstein College of Medicine, Bronx, NY).

Plasmid construction and transfection

Pairwise cysteine-to-serine substitutions originally made in apoB-28F (9) were transferred to apoB-17F (25) as follows. For apoB-17F-C1 (Cys12Ser/Cys61Ser) the corresponding mutant form of apoB-28F (9) was digested with *Eco*RI (5′ flanking site) and *Hin*dIII (internal site corresponding to amino acid 691 of mature apoB). The insert fragment containing the amino-terminal ~15% of apoB was ligated to *Eco*RI and *Hin*dIII-digested apoB-17F to give rise to apoB-17F-C1. For apoB-17F-C2 (Cys51Ser/Cys70Ser), and apoB-17F-C4 (Cys218Ser/Cys234Ser), the corresponding apoB-28F plasmids (9) were subjected to partial cleavage with *Bam*HI (at the site corresponding to amino acid 782) and *Kpn*I (3′ flanking site). After gel purification the ~7-kb fragments containing the vector (pCMV5) and apoB-17 sequences were ligated to a double-stranded oligonucleotide containing a 5′ *Bam*HI site, the FLAG peptide sequence, a termination codon, and a 3′ *Kpn*I site. A C-terminally FLAG-tagged form of human serum albumin (hSA-F) was produced by polymerase chain reaction (PCR). The 5′ PCR primer included a flanking *Kpn*I restriction site; the 3′ antisense primer included sequences encoding the C-terminal FLAG peptide sequence, a termination codon, and *Bam*HI restriction site. The template used was pBAT14, a plasmid containing the complete coding region of human serum albumin. The PCR product was cloned into the *Bam*HI and *Kpn*I sites of pCMV5. Stably transfected McA-RH7777 cells expressing apoB-17F and hSA-F were produced by methods described previously (8). COS-1 cells grown in 150-mm dishes were transfected with 15 μg of plasmid DNA by either the DEAE transfection method as described (26), or the FuGENE 6 method as recommended by the manufacturer (Roche Molecular Biochemicals).

Radiolabeling of transfected recombinant proteins

Stably transfected McArdle-RH7777 cells were plated in 100- or 150-mm dishes at ~30% confluence. After 24 h (ca. 50% confluence) cells were washed with phosphate-buffered saline (PBS) and preincubated in a 9:1 mixture of Met/Cys-deficient serum-free Dulbecco's modified Eagle's medium (DMEM) and complete serum-free DMEM (labeling medium) for 20 min. Labeling medium was removed and replaced with 3 ml (100-mm dish) or 8 ml (150-mm dish) of fresh labeling medium containing [^{35}S]Met/Cys at 100 μCi/ml. COS-1 cells were radiolabeled 24 h after transfection by preincubation with Met/Cys-deficient DMEM for 20 min followed by addition of fresh Met/Cys-deficient DMEM containing [^{35}S]Met/Cys at 100 μCi/ml. Labeling was for 3 h.

Immunoaffinity purification of FLAG-tagged proteins

After metabolic labeling of transiently transfected COS cells, cell monolayers were washed once with cold PBS, scraped from the dish, and lysed by addition of 1 ml of ice-cold lysis buffer [25 mM Tris-HCl (pH 7.4), 300 mM NaCl, 1% Triton X-100] containing protease inhibitors [1 mM phenylmethylsulfonyl fluoride (PMSF), leupeptin (25 μg/ml), pepstatin (25 μg/ml), aprotinin (50 μg/ml)]. After a 15-min incubation on ice, samples were centrifuged at 16,000 *g* for 10 min at 4°C. The supernatants were transferred to a fresh microcentrifuge tube and adjusted to 2.5 mg/ml with bovine serum albumin. After addition of a 75-μl bed volume of anti-FLAG M2 monoclonal antibody conjugated to agarose beads, samples were incubated at 4°C for 18 h on a rotating wheel. M2 agarose beads were recovered by centrifugation at 16,000 *g* for 10 s. After washing five times with 1 ml of PBS, bound protein was displaced by resuspension in 75 μl of PBS containing FLAG peptide at 150 μg/ml and incubation at room temperature for 15 min. Batch elution was repeated a total of four times. For affinity purification of FLAG-tagged proteins from McArdle-RH7777 cell medium, samples were first adjusted to 25 mM Tris-HCl (pH 7.5), bovine serum albumin (2.5 mg/ml), 1 mM PMSF, leupeptin (25 μg/ml), pepstatin (25 μg/ml), aprotinin (50 μg/ml). Otherwise, binding, wash, and elution steps were performed as described above for cell lysates. Affinity-purified proteins were analyzed and quantitated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and phosphorimager analysis.

Preparation of multilamellar and small unilamellar vesicles

Multilamellar vesicles (MLV) were prepared by dissolving 32 mg of either DMPC or POPC in 5 ml of chloroform in a round-bottom flask. The phospholipids were dried in a rotary evaporator at 37°C, and lyophilized for 30 min. The lipids were resuspended in 4 ml of PBS buffer by vortexing vigorously for 4 min to obtain 8-mg/ml MLV dispersions. POPC small unilamellar vesicles (SUV) were prepared as described (27). The vesicles were stored at 4°C under an argon atmosphere for up to 2 weeks.

Phospholipid vesicle-binding assay

Media concentrated with Centricon-10 centrifugal concentrators (Millipore, Waltham, MA) or immunoaffinity-purified protein was incubated with a 4-mg/ml concentration of DMPC or POPC vesicles in a 300-μl total volume and inverted for 20 h at 24°C. Each sample was adjusted to a density of 1.25 g/ml with KBr and a final volume of 3 ml with PBS. The tubes were centrifuged at 100,000 rpm (412,000 *g*) for 16 h at 15°C in a Beckman (Fullerton, CA) TL100 tabletop ultracentrifuge equipped with the TLA 100.3 rotor. The top 1-ml ($d < 1.25$ g/ml) and bottom 2-ml ($d > 1.25$ g/ml) fractions were recovered with a tube slicer (Beckman). Protein in each fraction was precipitated with 10% trichloroacetic acid (TCA) and analyzed by SDS-PAGE.

Chemical reduction of apoB-17F

Two micrograms of apoB-17F purified from baculovirus-infected Sf9 cells (25, 28) or $\sim 3 \times 10^7$ phosphorimager units of [^{35}S]apoB-17F from McArdle-RH7777 cells was incubated for 10 min at the indicated temperatures in the presence or absence of dithiothreitol (DTT) (final volume of 20 μl). Free sulfhydryls were alkylated by adjusting the reaction to 100 mM iodoacetamide (IAA) and incubating at 37°C for 20 min. An equal volume of 2× concentrated SDS-PAGE sample buffer was added, with or without DTT as indicated. Samples were boiled for 5 min and analyzed by 8% SDS-PAGE.

Density gradient fractionation of apoB-17F vesicle-binding assays

Vesicle-binding assays were centrifuged at 16,000 *g* for 15 min. The supernatants were transferred to fresh tubes and centrifuged for an additional 15 min to ensure complete removal of MLV. The supernatants were adjusted to a density of 1.21 g/ml with KBr and a final volume of 2 ml and layered on top of 2 ml of 1.24-g/ml KBr in PBS in a Beckman SW41 polyallomer centrifuge tube. The 2-ml sample was then overlaid with 2 ml of 1.063-g/ml KBr/NaCl, 2.5 ml of 1.019-g/ml KBr/NaCl, and 2 ml of 1.006 g/ml NaCl. Tubes were centrifuged at 40,000 rpm (160,000 *g*) for 44 h at 15°C in an SW41 rotor. Each sample was fractionated from the top into 1-ml fractions, using an Autodensiflow gradient fractionator (Labconco, Kansas City, MO). The density of each fraction was determined gravitometrically. ApoB was recovered from each fraction by TCA precipitation and analyzed by SDS-PAGE and fluorography.

Gel electrophoresis

Proteins samples were incubated in SDS-PAGE sample buffer (50 mM Tris base, 4% SDS, 12% glycerol, 1 mM ethylenediaminetetraacetic acid, 100 mM DTT, 0.05% bromophenol blue) at 100°C for 5 min. Where indicated (Fig. 2) no DTT was included to the SDS-PAGE sample buffer. Proteins were fractionated by 8% SDS-PAGE, soaked for 30 min in 1 M sodium salicylate, dried, and exposed to Bio-Max MR film (Eastman Kodak, Rochester, NY) at −80°C (30). Alternatively, gels were dried without prior fluor impregnation and exposed to Kodak BioMax MS film, using a Kodak BioMax TransScreen LE intensifying screen. To quantitate the radioactive bands, dried gels were imaged with a Molecular Dynamics (Sunnyvale, CA) model 445 SI PhosphorImager.

RESULTS

ApoB-17F secreted from hepatoma cells associates with DMPC vesicles

McArdle-RH7777 cells were stably transfected with a plasmid encoding the amino-terminal 17% of apoB appended with a C-terminal eight-amino acid FLAG epitope tag (apoB-17F) (25). Cells were radiolabeled for 24 h with [^{35}S]Met/Cys and the concentrated medium was incubated with (+) or without (−) DMPC MLV for 20 h. After adjusting the density to 1.25 g/ml with KBr, samples were subjected to density gradient centrifugation. As shown in **Fig. 1**, in the absence of DMPC vesicles, virtually all apoB-17F was recovered in the bottom, lipid-poor fraction (lane 4). On incubation with DMPC MLV, ~90% of apoB-17F underwent vesicle binding and was recovered in the top, $d < 1.25$ g/ml density fraction (Fig. 1, lane 5). To verify that the ability of apoB to bind DMPC vesicles was specific, a C-terminally FLAG-tagged form of hSA-F secreted by stably transfected McArdle-RH7777 cells was incubated with DMPC vesicles under the same conditions. Virtually all hSA-F remained in the lipid-poor bottom fraction (compare lanes 1 and 2 in Fig. 1).

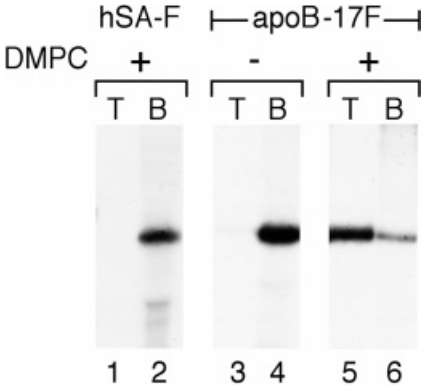

Fig. 1. Association of apoB-17F with DMPC MLV. McA-RH7777 cells stably transfected with C-terminal FLAG-tagged forms of human serum albumin (hSA-F) or apoB-17F were radiolabeled with [^{35}S]Met/Cys for 24 h. Concentrated medium was incubated in the presence (+) or absence (−) of MLV composed of DMPC (4 mg/ml). After adjusting the vesicle-binding assays mixtures to 1.25-g/ml KBr, samples were subjected to equilibrium density gradient centrifugation to obtain a $d < 1.25$ g/ml top (T) fraction and a $d > 1.25$ g/ml lipid-poor bottom (B) fraction. Samples were precipitated with trichloroacetic acid and analyzed by SDS-PAGE and fluorography.

Chemical reduction of apoB-17F requires thermal denaturation

To explore the role of native disulfide bonding in the binding of apoB-17F to DMPC vesicles, conditions were developed to achieve partial and complete chemical reduction. ApoB-17F immunoaffinity purified from recombinant baculovirus-infected Sf9 cell supernatant (25, 28) was incubated in the absence (−) or presence (+) of 50 mM DTT at the indicated temperatures (**Fig. 2**). Samples were treated with an excess of IAA to derivatize free sulfhydryls and analyzed by nonreducing SDS-PAGE. Incubation with 50 mM DTT at 22 or 30°C had no discernible effect on the gel mobility of apoB-17F (Fig. 2A, compare lanes 1 and 2 with lane 5). At 40°C, partial chemical reduction was achieved, as evidenced by the presence of bands migrating in both the reduced and folded position (Fig. 2, lane 3). At 50°C, apoB-17F appeared to be fully reduced (compare lanes 4 and 6 in Fig. 2). Virtually identi-

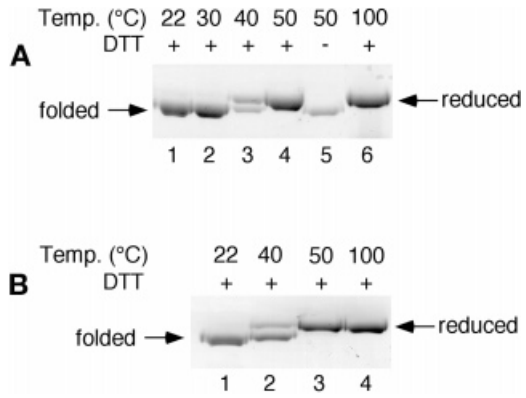

Fig. 2. Chemical reduction of apoB-17F with DTT requires thermal denaturation. A: Two micrograms of apoB-17F from baculovirus-infected Sf9 cells was incubated in the presence (+) or absence (−) of 50 mM DTT at the indicated temperatures. After alkylation with IAA, samples were adjusted to SDS-PAGE sample buffer conditions (without DTT), boiled, and subjected to nonreducing SDS-PAGE followed by staining with Coomassie blue. B: [^{35}S]Met/Cys-radiolabeled apoB-17F from stably transfected McA-RH7777 cells was subjected to treatment with 50 mM DTT at the indicated temperatures. Samples were processed as described above for (A), with the exception that bands were visualized by fluorography. The positions of folded and reduced forms of apoB-17F are indicated.

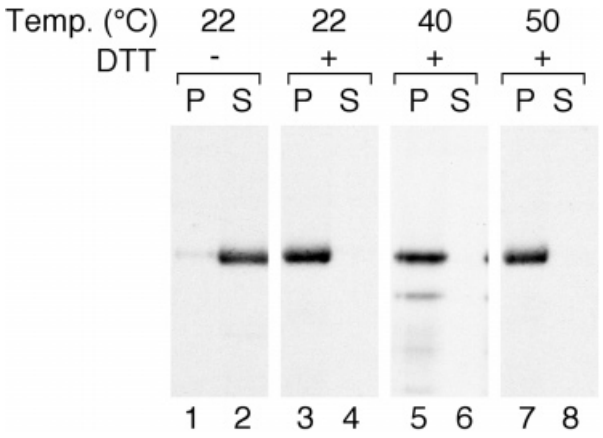

Fig. 3. Chemical reduction results in precipitation of apoB-17F in vitro. McA-RH7777 cells stably transfected with apoB-17F were radiolabeled for 24 h with [^{35}S]Met/Cys. Affinity-purified apoB-17F was incubated in the presence (+) or absence (−) of 50 mM DTT for 10 min at 22, 40, or 50°C as indicated. Samples were centrifuged at 16,000 *g* for 15 min. Pellet fractions (P) were solubilized directly in SDS-PAGE sample buffer. Protein in supernatants (S) was recovered by precipitation with trichloroacetic acid and subsequent solubilization in SDS-PAGE sample buffer. Fractions were analyzed by reducing SDS-PAGE and fluorography.

cal results were obtained with radiolabeled apoB-17F immunoaffinity purified from stably transfected McArdle-RH7777 cell medium (Fig. 2B). Together, these results indicate that the disulfide bonds in apoB-17F are predominantly solvent inaccessible and require thermal denaturation for complete chemical reduction in vitro.

Chemically reduced apoB-17F is insoluble in aqueous buffers

ApoB-17F was exposed to reducing conditions to determine how its vesicle-binding properties would be affected. Treatment at 50°C had no effect on the ability of apoB-17F to bind DMPC vesicles; however, on treatment at 50°C in the presence of 50 mM DTT no apoB-17F was recovered from the vesicle-binding assay (data not shown). The possibility was explored that chemical reduction may have compromised the solubility of apoB-17F. Affinity-purified [^{35}S]apoB-17F was incubated in the presence of DTT at various temperatures and subjected to centrifugation at 16,000 *g*. Pellet and supernatant fractions were analyzed by SDS-PAGE. As shown in **Fig. 3**, the solubility of apoB-17F is highly sensitive to DTT. Even under conditions that failed to produce a detectable alteration in gel mobility during nonreducing SDS-PAGE (e.g., incubation with 50 mM DTT at 22°C; Fig. 2), apoB-17F was rendered completely insoluble and was recovered in the pellet fractions (Fig. 3, lane 3). This result suggests that selective reduction of one or more disulfide bond in apoB-17F does occur at ambient temperature but without an accompanying alteration in mobility during nonreducing SDS-PAGE. Resolubilization of fully or partially reduced apoB-17F was unsuccessful unless denaturants such as SDS (Fig. 2) or 6 M guanidine were used (data not shown). Hence, the DTT-induced aggregation of apoB-17 is irreversible, a property similar to the behavior of delipidated apoB-100.

Characterization and affinity purification of wild-type and cysteine mutant forms of apoB-17F

As chemical reduction of apoB-17F rendered it insoluble, the relationship between native disulfide bonding and vesicle binding was explored by in vitro mutagenesis. It was previously demonstrated that pairwise cysteine-to-serine substitutions that disrupted disulfide bond 2 (Cys51Ser/Cys70Ser) or bond 4 (Cys218Ser/Cys234Ser) perturbed the ability of apoB-28 and apoB-50 to recruit lipid in the ER. In contrast, single pairwise substitution of cysteine residues required for formation of the five remaining disulfide bonds (bonds 1, 3, 5, 6, and 7) had little or no effect on apoB lipid recruitment or secretion (9, 10, 31). Hence, only disulfide bonds 2 and 4 are individually required for the α_1 domain to initiate the process of lipoprotein assembly. To discover how these mutations might affect vesicle-binding activity of the α_1 domain, the same pairwise cysteine-to-serine substitutions were introduced into apoB-17F. During steady state labeling, both the wild-type and disulfide bond 1 mutation (Cys12Ser/Cys61Ser; apoB-17F-C1), were secreted from transiently transfected COS cells (**Fig. 4**, lanes 1–4). However, the secretion of apoB-17-C2 and apoB-17-C4 was severely compromised, as evidenced by the almost complete absence of these proteins recovered from transfected cell medium (Fig. 4, lanes 6 and 8). Hence, the same pattern of cysteine-to-serine mutations that affected the ability of apoB-28 and B-50 to recruit lipid in the ER also perturbed the folding of apoB-17F in such a way that its secretion was disrupted (11).

To circumvent the limited secretion of apoB-17F-C2

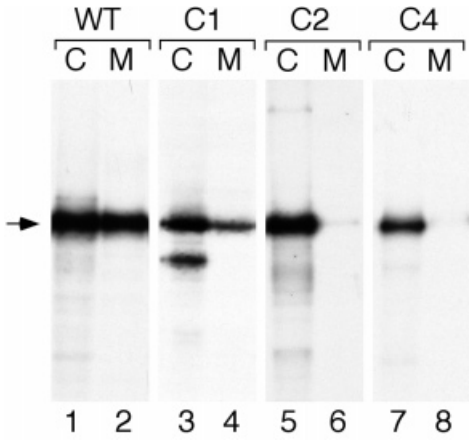

Fig. 4. Reduced secretion efficiency of apoB-17F-C2 and apoB-17F-C4. COS-1 cells transiently transfected with wild-type apoB-17F (WT) or apoB-17F containing mutations that disrupt disulfide bond 1 (C1), 2 (C2), or 4 (C4) were labeled for 3 h with [^{35}S]Met/Cys. Protein was recovered from cells (C) and media (M) by immunoprecipitation with anti-FLAG monoclonal antibody M2 and protein G-Sepharose, followed by SDS-PAGE and fluorography. The arrow indicates the position of apoB-17F proteins. The faster migrating band in lane 3 is a proteolytic breakdown product of apoB-17F-C1.

and apoB-17F-C4, intracellular forms were isolated from cellular detergent extracts by immunoaffinity chromatography. After binding of FLAG-tagged proteins, the anti-FLAG M2-agarose beads were washed extensively with PBS to remove detergent and protein was eluted with PBS containing FLAG peptide at 150 μg/ml. As observed in **Fig. 5**, both wild-type and mutant forms of intracellular apoB-17F were highly enriched in the eluted fractions and were recovered in soluble form. Less apoB-17F-C4 construct was recovered relative to the other forms, presumably because of reduced expression or intracellular stability.

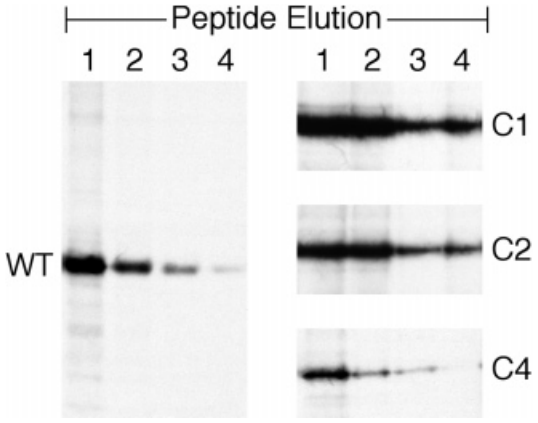

Fig. 5. Secretion-defective cysteine-to-serine mutant forms of apoB-17F are recovered from cell extracts in soluble form. COS-1 cells were transiently transfected with wild-type (WT), C1, C2, and C4 mutant forms of apoB-17F and then radiolabeled for 3 h with [^{35}S]Met/Cys. Cells were lysed and apoB was affinity purified by batch binding to anti-FLAG M2 agarose as described in Experimental Procedures. After extensive washing with PBS to remove detergent, bound proteins were displaced by four sequential rounds of incubation with PBS containing FLAG peptide at 1 to 50 μg/ml. After centrifugation at 16,000 *g* for 10 min, 10% of each peptide elution fraction (lanes 1–4) was analyzed by SDS-PAGE and fluorography.

Vesicle-binding properties of apoB-17F cysteine mutants

Affinity-purified apoB-17F, apoB-17F-C1, apoB-17F-C2, and apoB-17F-C4 were incubated with DMPC MLV, adjusted to 1.25 g/ml with solid KBr, and subjected to density gradient centrifugation. Fractions from the top and bottom were recovered and analyzed by SDS-PAGE. In the presence of DMPC, both wild-type apoB-17F and all the cysteine mutants were found predominantly in the top of the gradient (**Fig. 6A**). Hence, the mutations that were previously shown to disrupt the initiation of apoB-28- and apoB-50-containing lipoprotein assembly in vivo (i.e., C2 and C4) (9) had little apparent effect on apoB-17 DMPC vesicle binding in vitro. To determine whether these mutations affected the specificity of vesicle binding in terms of lipid species or vesicle topography, we incubated apoB-17F wild type and apoB-17F-C4 with either POPC MLV (Fig. 6B, lanes 1–4) or POPC SUV (Fig. 6B, lanes 5–8). Both apoB-17F and apoB-17F-C4 were capable of binding to POPC MLV and SUV (Fig. 6B). Although minor quantitative differences in vesicle binding were observed among the various forms of apoB-17F, repeat analyses suggested that these differences arose because of variability in the assay and not necessarily because of differences in the behavior of the mutants (data not shown). Further, it should be noted that both the pure DMPC and POPC vesicles used in these studies lack the heterogeneity of lipid composition characteristic of biological membranes.

Formation of recombinant lipoproteins

Herscovitz et al. (24) demonstrated that wild-type apoB-17 is capable of forming recombinant discoidal lipoproteins on incubation with DMPC, a capability also observed for apoB-17F (25). Although the studies performed above indicated that disulfide bond mutations do not affect vesicle binding, we explored the possibility that these mutations may affect the efficiency by which apoB-17F converts DMPC MLV into recombinant lipoproteins. As these studies were performed with trace quantities of apoB-17F protein recovered from cell extracts, it was not possible to perform biochemical or morphological analyses of the vesicle-binding products. We therefore analyzed the percentage of each protein present in the supernatant and pellet fractions after the vesicle-binding assays were subjected to centrifugation at 16,000 *g* (**Fig. 7**). When no apoB-17F was included in the vesicle-binding reaction, virtually all the DMPC (99.9%; data not shown) was recovered in the pellet fraction. Hence, the ability of apoB-17F to remain in the supernatant fractions can be attributable to conversion of the DMPC large MLV, which readily pellet, to recombinant lipoproteins or small vesicles that remain in the supernatant (24, 32).

COS cells transiently transfected with wild-type apoB-17F and apoB-17F-C1, -C2, and -C4 were radiolabeled with [^{35}S]Met/Cys and recombinant protein was affinity puri-

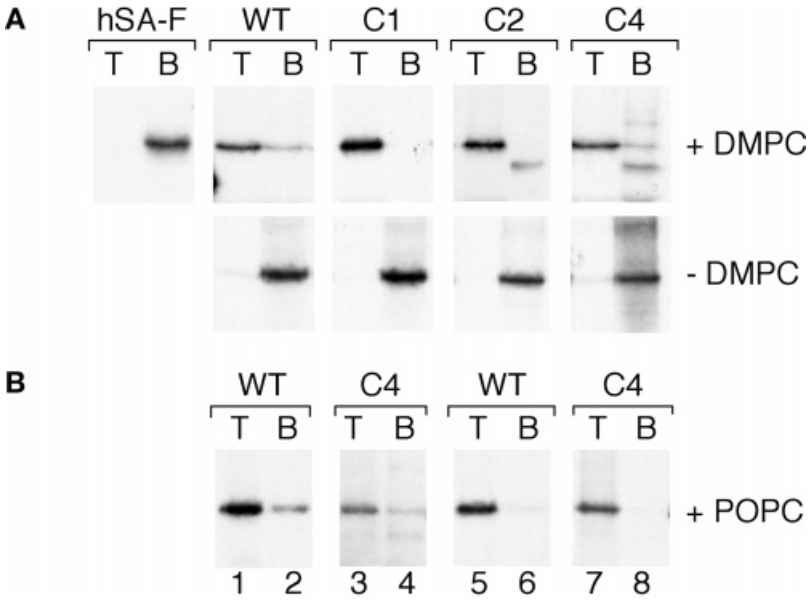

Fig. 6. Mutations that affect apoB-28- and apoB-50-containing lipoprotein assembly in vivo fail to block the ability of apoB-17F to bind to phospholipid vesicles in vitro. Affinity-purified [^{35}S]Met/Cys-radiolabeled protein (Fig. 5) was incubated with (+) or without (−) DMPC MLV as indicated (A) or with POPC MLV (B, lanes 1–4), and POPC SUV (B, lanes 5–8). Each sample was subjected to equilibrium density gradient centrifugation. Protein was recovered from the d < 1.25 g/ml top 1-ml (T) and d > 1.25 g/ml bottom 2-ml (B) fractions by trichloroacetic acid precipitation and analyzed by SDS-PAGE and fluorography. hSA-F = FLAG-tagged form of human serum albumin; WT = wild type.

fied from cell extracts. An equal number of radioactive counts of each protein was introduced into the vesicle-binding assay. After overnight incubation the reaction mixture was centrifuged at 16,000 *g*, the pellet and supernatant fractions were fractionated by SDS-PAGE, and band intensities were quantitated by phosphorimager analysis. The results of this analysis confirmed that both the wild-type and mutant forms of apoB-17F had similar efficiencies of vesicle binding and were also capable of converting the DMPC MLV into soluble lipoprotein particles (Fig. 7). Qualitative analysis of the particles generated was performed by density gradient centrifugation. Each form of apoB-17F gave rise to particles of densities in the range of 1.046–1.154 g/ml (**Fig. 8**). Repeat analyses revealed that the minor differences in densities observed among the various forms of apoB-17F were not significant.

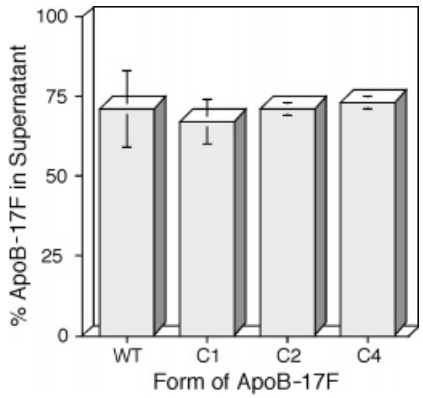

Fig. 7. Interaction of wild-type and cysteine mutant forms of apoB-17F with DMPC vesicles. Equal amounts of radiolabel of the indicated forms of apoB-17F were incubated with DMPC MLV for 20 h. Samples were centrifuged at 16,000 *g* for 15 min, supernatants were transferred to fresh tubes, and the centrifugation step was repeated. The two pellet fractions were resuspended in PBS, combined, and subjected to precipitation with 10% trichloroacetic acid (TCA). After centrifugation, to recover the precipitate, pellets were washed with ether–ethanol 1:1 (v/v) and dissolved by boiling in SDS-PAGE sample buffer. Supernatants were also precipitated with TCA, washed with ether-ethanol, and boiled in SDS-PAGE sample buffer. After SDS-PAGE and quantitation by phosphorimager analysis, the percentage of protein associated with the supernatant fraction was calculated. Each column and error bar represents the mean and range of duplicate assays.

DISCUSSION

The ability of apoB to interact with intracellular membranes is often cited as an important component of the lipoprotein assembly pathway. In the most common model, the nascent apoB polypeptide chain intercalates into the inner leaflet of the ER membrane as part of a process that nucleates intramembrane TG accumulation. On completion of translation, this TG droplet, surrounded by membrane-derived phospholipid, buds into the ER lumen as a soluble nascent lipoprotein particle (20–22). However, extensive analyses indicate that translation of the amino-terminal ~25% of apoB is sufficient for formation of a buoyant lipoprotein in the ER lumen (5, 22). This implies that the interaction of apoB with the ER membrane may occur only transiently during early stages of translation and is perhaps mediated by the α_1 domain. While the vesicle-binding property of the α_1 domain was previously demonstrated only for vesicles composed of DMPC, the current report demonstrating binding to both DMPC and POPC suggests that the α_1 domain can interact with bilayer structures composed of more physiological phospholipids. This finding, along with the demonstration of the rapid adsorption of apoB-17 at an oil-water interface and its dynamic interfacial properties (25), is consistent with critical protein-lipid interactions during an early initiating stage of cotranslational lipoprotein assembly. Whether this interaction mediated by the α_1 domain occurs at the ER membrane or by means of MTP-dependent transfer of lipid to lumenal apoB remains to be elucidated (16).

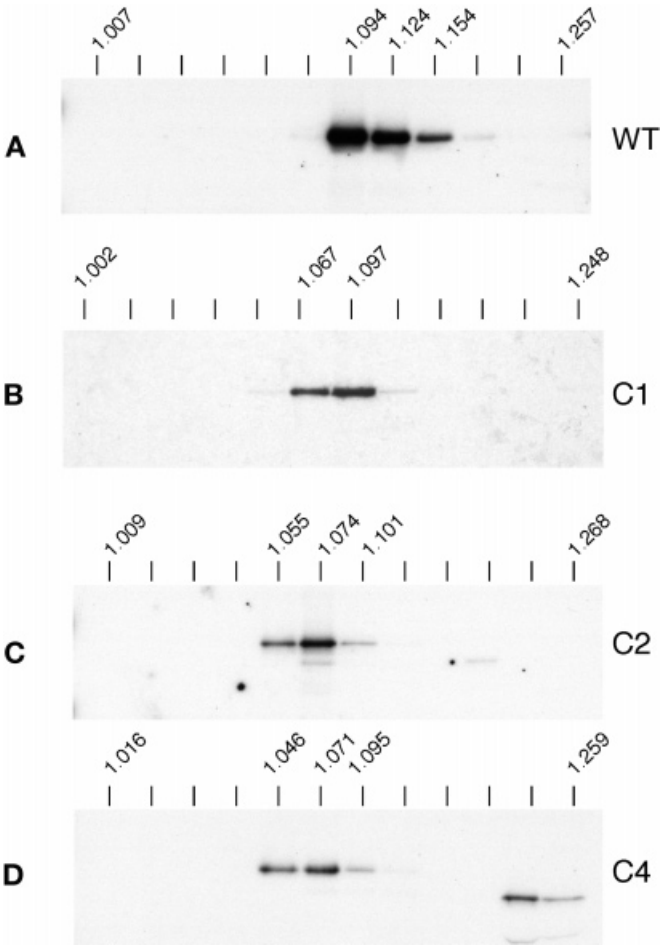

Fig. 8. Density distribution of apoB-17F wild-type (WT) and cysteine-to-serine mutants incubated with DMPC vesicles. Vesicle-binding assays were centrifuged at 16,000 *g* as described in Fig. 7. After the second spin, the supernatant was subjected to discontinuous density gradient centrifugation as described in Experimental Procedures. One-milliliter fractions, collected from the top of each tube, were subjected to trichloroacetic acid precipitation followed by SDS-PAGE and fluorography. Top and bottom densities, as well as densities of fractions where protein was found, are indicated.

To explore this issue, we tested the hypothesis that mutations with known capacities to disrupt lipoprotein assembly in transfected cells perhaps also affected the ability of apoB-17 to interact with lipid surfaces in vitro. Surprisingly, pairwise cysteine-to-serine substitutions in the α_1 domain that severely inhibited the ability of apoB-28 and apoB-50 to engage in lipoprotein assembly in vivo had no apparent affect on the capacity of apoB-17 to interact with phospholipid vesicles in vitro. This finding suggests that either (1) the vesicle/membrane-binding properties of apoB-17 are not important for the ability of apoB to participate in lipoprotein assembly in vivo or (2) this property is an important reflection of a role played by the α_1 domain, but is not dependent on native disulfide bonding. In the latter case, it follows that some other functional property of the α_1 domain of apoB essential for initiation of lipoprotein assembly in vivo is compromised by perturbed disulfide bonding.

A related but distinct property of apoB-17 that may be important for the initiation of particle assembly is its capacity to form phospholipid disks in vitro (24). While the hypothetical pathways outlined above relate to the accumulation of TG in the membrane followed by apoB-dependent budding into the ER lumen, it is also possible that apoB initiates particle assembly by selectively extracting only phospholipid from the ER membrane or by accumulating either existing or newly synthesized phospholipid via MTP-dependent lipid transfer. In addition to results reported here and elsewhere relating to the ability of apoB-17 to form recombinant lipoproteins, Rusiñol, Jamil, and Vance (33) demonstrated that when translated in vitro in the presence of hepatic microsomal membranes, apoB15 associated with newly synthesized phosphatidylcholine. Hence, the formation of a phospholipid-rich intermediate may precede the MTP-dependent formation of the TG core in a manner that may parallel lecithin:cholesterol acyltransferase-dependent maturation of discoidal HDL (32). However, it appears from the density gradient profiles of the vesicle-binding assays performed in this study that both wild-type and cysteine-to-serine mutations are effective at forming recombinant lipoprotein particles in vitro. One caveat associated with these studies is the possibility that the assays used are not sufficiently sensitive to reproducibly detect subtle differences in vesicle-binding behavior among the various forms of apoB-17F tested. Furthermore, the model DMPC and POPC vesicles used lack the heterogeneity of biological membranes.

A novel finding reported here is that even mild chemical reduction of apoB-17 results in its loss of solubility. This loss of solubility occurs even under conditions in which no mobility shift is observed on nonreducing SDS-PAGE. This is interesting in light of the characterization of apoB-17 as a globular and soluble domain (4, 34). One possible function of rapid disulfide bond formation during the assembly of secretory proteins in the ER is to drive hydrophobic domains, or adhesion zones, into the interior of the protein, a step that prevents exposure of hydrophobic surfaces that may cause intracellular aggregation (35, 36). Most certainly, one or more disulfide bonds in apoB-17 appear to be essential for the stabilization of these hydrophobic domains. The presence of these hydrophobic regions within the interior of the apoB-17 globular structure further supports its possible role in promoting a critical apoB-lipid interaction during an early stage of its assembly process.

An early lipid-binding step mediated by the α_1 domain of apoB was proposed by Segrest, Jones, and Dashti (16) as a means of initiating the cotranslational phase of apoB-containing lipoprotein assembly. This prediction is based on the partial lipovitellin homology in the α_1-β_1 boundary of apoB, which may form all or part of a lipid-binding pocket with the capacity to sequester several molecules of phospholipid and TG in the form of a "proteolipid" precursor particle. While it is clear that disulfide bond disruption has no effect on membrane binding and phospholipid sequestration activity of apoB-17 in vitro, it is possible that these same structural alterations may perturb independent steps required for formation of a putative phospholipid and TG-containing proteolipid intermediate.

Further structure-function profiling of the α_1 domain may reveal how its interactions with different types of lipids, MTP, and other factors in the ER may initiate the cotranslational phase of apoB-containing lipoprotein assembly.

We thank Li Hou and Abraham Gebre for technical assistance. This work was supported by National Institutes of Health Grant HL49373 (G.S.S. and J.S.P.).

Manuscript received 16 August 2000 and in revised form 7 November 2000.

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