A Common Binding Site on the Microsomal Triglyceride Transfer Protein for Apolipoprotein B and Protein Disulfide Isomerase*†

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The assembly of triglyceride-rich lipoproteins requires the formation in the endoplasmic reticulum of a complex between apolipoprotein B (apoB), a microsomal triglyceride transfer protein (MTP), and protein disulfide isomerase (PDI). In the MTP complex, the amino-terminal region of MTP (residues 22–303) interacts with the amino-terminal region of apoB (residues 1–264). Here, we report the identification and characterization of a site on apoB between residues 512 and 721, which interacts with residues 517–603 of MTP. PDI binds in close proximity to this apoB binding site on MTP. The proximity of these binding sites on MTP for PDI and amino acids 512–721 of apoB was evident from studies carried out in a yeast two-hybrid system and by co-immunoprecipitation. The expression of PDI with MTP and apoB16 (residues 1–721) in the baculovirus expression system reduced the amount of MTP co-immunoprecipitated with apoB by 73%. The interaction of residues 512–721 of apoB with MTP facilitates lipoprotein production. Mutations of apoB that markedly reduced this interaction also reduced the level of apoB-containing lipoprotein secretion.

Apolipoprotein B (apoB) is an obligatory component of chylomicrons, very low density lipoproteins and low density lipoproteins, which transport lipid to all body tissues. The assembly of apoB-containing lipoproteins has an absolute requirement for a microsomal triglyceride transfer protein (MTP) complexed to an endoplasmic reticulum-resident chaperone, protein disulfide isomerase (PDI) (1–3). The MTP complex facilitates the loading of apoB with lipid. ApoB that does not acquire sufficient lipid to form a lipoprotein is rapidly degraded by the proteosome (4–7).

ApoloB and MTP, along with the major lipid transport proteins of arthropods, share a common ancestry with the vitellogenins (VTGs) of nematodes (8). The VTGs transport lipid to the yolk sac (9). We have used the crystal structure of lamprey lipovitellin (LV) (10), the mature product of VTG, to derive molecular models of the amino termini of MTP and apoB (8). In the modeled structures, the amino-terminal ∼300 residues of each protein form a β-barrel. The next 300 residues form a double-layered α-helical structure containing 17 helices. The carboxyl-terminal portion of this structure is stabilized by a conserved buried salt bridge, which in lamprey LV underlies the homodimerization interface (10). In MTP, the surfaces of outer helices 15 and 17 form the major PDI binding site (8). This interaction is required for the production of soluble and active MTP (11, 12) and may, in addition, anchor MTP at the site of apoB translocation, as PDI alone contains the “KDEL” endoplasmic reticulum retention sequence (13, 14).

MTP and apoB interact during lipoprotein assembly (15, 16). We have studied this interaction with a series of carboxy-terminally truncated forms of apoB (8). The results established that residues 1–264 of apoB (apoB5,8) interact with the predicted β-barrel of MTP (8). In the present study, we have identified a second region of apoB that interacts with a binding site on MTP in close proximity to the major PDI binding site.

MATERIALS AND METHODS

Yeast Two-hybrid—Vectors pSB202 and pμG4–5, the reporter plasmid pSH18–34, and EGY48 were gifts from Professor Brent (Harvard Medical School, Boston, MA). MTP cDNAs were fused to the amino terminus of LexA. ApoB and PDI were fused to the B42 transcription activation domain. The PDI-aeb construct encodes amino acids 18–274 of PDI, representing the first three domains of PDI (designated “aeb” by Freedman et al. (17)), and was constructed from a cDNA clone provided by Professor Kivirikko (Collagen Research Unit, University of Oulu, Finland). Transformations were undertaken as described (18).

Sequence Alignment—A WU-BLASTP search of the Protein Data Bank, SwissProt, +SPupdate +PIR, and the nonredundant GenBank Coding Sequences translation data bases was performed with residues 1–1000 of lamprey LV, using blosum 62 as the scoring matrix. The most significant matches were tobacco hornworm apolipoporphin, human (Homo sapiens) apoB, Drosophila melanogaster retinoid/fatty acid binding protein, and H. sapiens MTP (p values of 3.4 × 10⁻¹⁶, 2.2 × 10⁻¹⁴, 1.6 × 10⁻¹², and 2.7 × 10⁻¹⁰, respectively). GenBank accession numbers are X75500 (H. sapiens MTP), Y00354 (Xenopus laevis VTG), M88749 (Ichthyomyzon unicuspus lamprey VTG), and X03044 (Caenorhabditis elegans VTG) 5. Sequences were aligned with the Clustal version W11.6 program (19) using default values and minimal manual adjustments, without reference to the position of intron/exon boundaries.

Expression Constructs—Details of oligonucleotides are available on request (from C. C. S.). Polymerase chain reaction and restriction enzymes were used to manipulate the MTP, apoB, and PDI sequences. Epitope tags were fused in-frame to the carboxyl termini of cDNAs and
juxtaposed to a terminator codon. The baculovirus vectors were pVL1392 and 1393 (Invitrogen BV, Leek, The Netherlands). All constructs were sequenced.

**Baculovirus Expression—** Cells were maintained in Grace's medium (Life Technologies, Inc.), supplemented with 10% insect-qualified fetal calf serum (Life Technologies, Inc.). Transfections were with liposomes, linearized BacPAK 6 viral DNA (CLONTECH), and the appropriate baculovirus transfer plasmid. The apoA-I virus was a gift from Dr. David Booth (Imperial College School of Medicine, London). Infections were at a multiplicity of 2.5. Cells were harvested 42–46 h postinfection. Cells for labeling were washed and resuspended in 7 ml of methionine-free Grace's medium and gently agitated at 27 °C for 45 min. Labeling was for 75 min with 0.43 mCi of L-[35S]methionine (Pro-Mix, Amersham International PLC). Analysis of expression was undertaken on microsomal fractions at 4 °C. Cells were washed in phosphate-buffered saline, pH 7.4, homogenized in 0.25M sucrose containing 20 mM imidazole, pH 7.4, and protease inhibitors, layered onto a gradient of 1.8 M sucrose in 20 m M imidazole and 0.5 M sucrose in 20 m M imidazole, and centrifuged at 100,000 \( \times g \) for 60 min. The pellet of microsomes at the 0.5–1.8 M sucrose interface was resuspended in buffer A (10 mM Tris, pH 7.4, containing 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, and protease inhibitors). ApoB, apoA-I, and MTP-FLAG were immunoprecipitated with saturating quantities of sheep anti-human apoB (Boehringer Mannheim from Roche Diagnostics Ltd.), anti-apoA-I (Genzyme Diagnostics), rabbit anti-human MTP antibodies, or anti-FLAG M2 affinity gel (Sigma), respectively. Immobilized proteins were washed with buffer A and recovered by boiling in SDS sample buffer.

**Expression of ApoB and MTP in COS-1 Cells—** The details of the coordinate expression of MTP and apoB have been described, as have the L-[35S]methionine labeling of cells and the immunoprecipitation of apoB (2). The chase was for 3 h.

**RESULTS** To screen for sites of interaction between apoB and MTP, distal to residues 1–264 of apoB, we used a yeast two-hybrid system. Short, overlapping cDNAs encoding residues 247–1147 (apoB5–25) (Fig. 1A) were assayed for interaction with the MTP amino-terminal (residues 22–304), predicted \( \alpha \)-helical (residues 298–603), and carboxyl-terminal domains (residues 604–894). ApoB residues 349–583 (apoB8–13) and 512–721 (apoB11–16) produced an interaction with the predicted \( \alpha \)-helical domain of MTP (Fig. 1B). The strongest interaction was with apoB residues 512–721 (apoB11–16). No other site of interaction was detected (data not shown).

To fine map the region on MTP that interacts with apoB, predicted helices 1–8 (residues 297–442), 9–13 (residues 447–529), and 13–17 (residues 517–603) of MTP were expressed with residues 349–583 (apoB8–13) and 512–721 (apoB11–16).
The interactions with predicted helices 13–17 of MTP were 10- and 5-fold higher than with predicted helices 1–8 and 9–13, respectively. These results indicate that residues 512–721 (apoB11–16) of apoB interact with helices 13–17 of MTP, which also form a major binding site for PDI (8). The PDI binding site on MTP was also mapped in a yeast two-hybrid system and corroborated by the characterization of mutant forms of full-length MTP expressed in COS-1 and Sf9 cells.

To fine map the region on apoB that interacts with MTP, we examined the interaction of subfragments of apoB11–16 (resi-
dues 512–721) with predicted helices 13–17 of MTP (Fig. 1).

The removal of residues 512–524 from the amino terminus of residues 512–721 of apoB (apoB11–16) abolished the interaction. The deletion of residues 701–721 and 664–721 from the carboxyl terminus of residues 512–721 of apoB (apoB11–16) reduced the interaction by 29 and 49%, respectively. These data indicate that helices 13–17 of MTP (residues 517–603) interact with a site encompassing predicted helices 13–17 (residues 512–592) of apoB and the carboxyl-terminal portion (residues 593–721) of apoB16. The homologous interacting regions of apoB and MTP (Fig. 2) are encoded by exons with conserved intron demarcations (Fig. 2, A and B).

In view of the co-localization of the binding sites for PDI and residues 512–721 of apoB (apoB11–16) to helices 13–17 of MTP, we examined whether mutations of MTP that disrupted the interaction of MTP with PDI (8) might also impair apoB bind-

**Fig. 3. Characterization of the interaction of apoB and PDI with mutant MTP proteins.** Mutations were introduced into helices 13–17 of MTP and expressed with residues 18–274 of PDI or residues 512–721 of apoB11–16 in a yeast two-hybrid system. Values are mean ± S.D. (n = 10). *, p < 0.005 and **, p < 0.001, compared with the apoB interaction.

**Fig. 4. Expression of MTP with apoB in the baculovirus expression system.** A, Western blot analysis of the interaction of apoB17 with MTP. Immunoprecipitation was done with rabbit anti-human MTP polyclonal antibodies and performed in 1% Triton X-100 to solubilize the lipoprotein assembly complex (15, 16). The immunoreactive products were detected with anti-apoB and anti-MTP antibodies. The first two lanes are controls. The last two lanes show that the amount of apoB associated with MTP was reduced by coordinate expression of PDI. Data are shown for one of two similar experiments. B, the co-immunoprecipitation of carboxyl-terminally truncated forms of apoB with FLAG-tagged MTP. Cells were labeled with [35S]methionine under steady state conditions. Immunoprecipitations were with anti-FLAG M2 affinity resin. MTP, apoB, and PDI were size-fractionated by SDS-polyacrylamide gel electrophoresis and visualized with a PhosphorImager. Note that lower amounts of MTP are solubilized in cells expressing apoB11 and apoB13 relative to cells expressing apoB16 and apoB17. On a mole to mole basis, PDI expression reduced the amount of apoB11, -13, -16, and -17 co-immunoprecipitated with MTP. PDI did not decrease apoB expression (data not shown). The results show one of three similar experiments. C, co-immunoprecipitation of MTP with carboxyl-terminally truncated forms of apoB. Immunoprecipitations were with anti-human polyclonal apoB antibody. The first lane is a control for the specificity of the antibody. The second lane shows that the association of MTP with apoB5.8 (residues 1–264) was unaltered by PDI expression. The final three lanes show that PDI expression successively decreased the amount of MTP associated with apoB11 (residues 1–499), apoB13 (residues 1–590), and apoB16 (residues 1–721). Quantification was by ImageQuant analysis with a PhosphorImager (Molecular Dynamics).
ing. MTP R540A and R540H, which disrupted the predicted buried salt bridge, and a series of mutant MTPs that had been used to map the PDI binding site (8) were expressed with either PDI (residues 18–274) or apoB (residues 512–721). The predicted positions of the salt bridge residue (Arg-540) and of mutated residues Val-520, Lys-521, Arg-526, Tyr-554, Met-555, Lys-558, Ile-592, Arg-594, and Arg-595 at the surface of helices 13–17 of MTP are shown in Fig. 2C. MTP R540H and double mutant R594A/R595A had a more marked effect on the interaction of MTP with apoB than on the interaction of MTP with PDI (Fig. 3). Mutants R540A, Y554A/M555A, Y554A/M555A/K558A, and Y554A/M555A/I592A were more deleterious for the interaction of MTP with PDI. The disruption for mutants V520A, K521A, R526A, and I592A was similar for both proteins (Fig. 3). These results suggest that the apoB and PDI binding sites on the a-helical region of MTP are in close proximity.

The proximity of binding sites on MTP for apoB and PDI was further evaluated by studies performed in a baculovirus expression system (Fig. 4). ApoB was co-immunoprecipitated with MTP using anti-MTP antibodies, whereas MTP was co-immunoprecipitated with anti-apoB antibodies. The coordinate expression of PDI reduced the amount of apoB17 (residues 1–781) co-immunoprecipitated with anti-apoB antibodies. The coordinate expression of PDI reduced the amount of apoB17 (residues 1–781) co-immunoprecipitated with MTP by around 25%. This increase corresponds to a 3-fold higher level of MTP in these cells, presumably because of increased solubilization of MTP by PDI (Fig. 4B). Thus, on a mole to mole basis, PDI substantially reduced the amount of apoB11 and apoB13 co-immunoprecipitated with MTP. Similar results were obtained in an independent experiment in which MTP was co-immunoprecipitated with anti-apoB antibodies. PDI reduced the amount of MTP co-immunoprecipitated with apoB16 (residues 1–721), apoB13 (residues 1–590), and apoB11 (residues 1–499) by 73, 52, and 29%, respectively (Fig. 4C). No reduction was observed with apoB12 (residues 1–264). These results support the close proximity of the binding sites on MTP for residues 512–721 of apoB and 18–274 of PDI and, in addition, indicate that the aminoterminal limit of this apoB binding site may extend further upstream than residue 499 of apoB.

Next, we evaluated whether mutations in predicted helices 13–17 (residues 512–592) of apoB (Fig. 2A) might disrupt the binding of amino acids 512–721 of apoB to MTP. The predicted buried salt bridge residues Arg-531 and Glu-557 in apoB (Fig. 2D) were individually replaced with alanine. R531H was also created, because the replacement of the homologous residue (Arg-540) in MTP with histidine causes abetalipoproteinemia (20). In the yeast two-hybrid system, R531A, R531H, and E557A reduced the interaction of helices 13–17 of MTP with amino acids 512–721 of apoB to 20.4 ± 6.9, 9.2 ± 3.2, and 56.1 ± 26% of wild type, respectively (Fig. 5A). To examine the consequence of this loss of binding between apoB and MTP for lipoprotein assembly, R531A, R531H, and E557A were introduced into apoB36, and their impact on the secretion of apoB-containing lipoproteins from COS-1 cells was examined. Mutants R531H and R531A, which had markedly reduced the interaction of amino acids 512–721 of apoB with MTP in the yeast two-hybrid system, reduced apoB secretion, whereas mutant E557A did not (Fig. 5B). Secretion levels were 20, 53, and 95% of wild type, respectively. Thus, the loss of the interaction between amino acids 512–721 (apoB11–16) of apoB and the carboxyl terminus of the predicted a-helical domain of MTP is deleterious for the production of apoB-containing lipoproteins.

**DISCUSSION**

In this study, we have identified a region on MTP that interacts with both apoB and PDI. The delineation of the apoB and MTP binding sites has been facilitated by the use of well characterized molecular models of residues 294–592 and 304–598 of apoB and MTP, respectively. The models were generated from the crystal structure of lamprey LV (10). The structural relationship between MTP, apoB, and VTG is supported by conservation of gene structure, primary sequence, predicted secondary structure, and site-directed mutagenesis (8).

We have previously shown that the predicted aminoterminal a-helices of apoB and MTP (first ~300 residues) interact (8). Here, we describe the interaction between residues 512–721 of apoB and helices 13–17 (residues 517–603) of MTP. This interaction was identified and characterized in a yeast two-hybrid system. The binding site on MTP for apoB is in close proximity to the major PDI binding site. Mutation of the buried salt bridge residue Arg-540 of MTP and of residues at the surface of predicted helices 15 (Tyr-554, Met-555, Lys-558) and 17 (Ile-592, Arg-594, Arg-595) impair the interaction of MTP with apoB and PDI, whereas the equivalent mutation of residues in helix 13 (Val-520, Lys-521, and Arg-526) of MTP do not. Accordingly, we propose that apoB and PDI have binding sites centered on predicted helices 15–17 (residues 556–598) of MTP. The fact that 4 of the 10 MTP mutations were more deleterious for the interaction of MTP with PDI than with residues 512–721 of apoB, whereas 2 were more deleterious for the interaction between MTP and apoB, suggests that apoB and PDI do not bind to the same site on MTP. However, the mutation data do not exclude the possibility that the critical residues on MTP for binding to apoB and PDI may differ and that these critical residues form part of a common binding site.

An interaction between MTP and residues 270–570 of apoB has recently been reported by Hussain et al. (21) in a third experimental system. The present study provides strong evidence that this apoB binding site encompasses helices 13–17 (residues 514–592) of the predicted a-helical domain of apoB, as well as structural motifs formed by residues 640–721 at the carboxyl-terminal end of apoB16. The site may also include residues 430–511 of apoB, which are predicted to form helices 9–12 of its a-helical structure (8). In the experimental system...
of Hussain et al. (21), an expression construct encoding residues 270–509 of apoB produced an interaction with MTP that was 58% of that observed with residues 270–570 of apoB, whereas a corresponding construct that comprised residues 270–430 of apoB produced virtually no interaction. These results, combined with our yeast two-hybrid data, indicate that the interaction of apoB with helices 13–17 of the predicted α-helical domain of MTP is centered on residues 512–592 (apoB11-apoB13) of apoB and includes flanking residues 430–511 (apoB9-apoB11) and 640–721 (apoB14-apoB16).

Our baculovirus expression studies also indicate that PDI and amino acids 512–721 of apoB bind to the same helical region of MTP. From our previous study (8) and the present yeast two-hybrid data we made two predictions. First, we predicted that the coordinate expression of PDI with MTP would not impair the interaction of residues 1–264 of apoB (apoB5.8) with the predicted β-barrel of MTP, which is confirmed in this study. Second, we predicted that PDI would reduce the interaction of MTP with apoB16 (residues 1–721), apoB13 (residues 1–590), and apoB11 (residues 1–499) and that the maximum reduction would be with apoB13 and apoB16. Thus, we show that PDI expression successively reduced the interaction of MTP with apoB11 (residues 1–499), apoB13 (residues 1–590), and apoB16 (residues 1–721).

The present study demonstrates that disruption of predicted helices 13–17 (residues 512–592) at the center of the distal binding site on apoB impairs the secretion of apoB-containing lipoproteins. Thus, we show that the mutation of the apoB buried salt bridge residue, Arg-531, which virtually abolished the interaction of MTP with apoB in the yeast two-hybrid system, had a marked impact on lipoprotein secretion. These results indicate that the assembly of apoB-containing lipoproteins is perturbed by the loss of interaction of the distal apoB binding site (residues 512–721) with MTP. An alternative explanation would be that the apoB Arg-531 mutation supports lipoprotein assembly but impairs the secretion of apoB-containing lipoproteins through aberrant misfolding. The present study does not directly address this issue. However, our data and those of Hussain et al. (21) are consistent with a loss of interaction between residues 512–721 of apoB and MTP as being the primary cause of reduced secretion. Incubation of cells with a pharmacological agent that specifically blocks this interaction represents an attractive strategy for determining its importance for lipoprotein production.

The location of the two binding sites on apoB and MTP would serve to align nascent apoB in a head to head orientation with MTP. This would position the predicted lipid binding domain of MTP with the lipid binding structures of apoB and presumably facilitate the transfer of lipid from MTP to apoB. The proximity of the distal apoB binding site and of the PDI binding site on MTP raises the question as to how this arrangement might facilitate lipoprotein assembly. It will be important to establish whether apoB displaces PDI from MTP only to be replaced again by PDI as lipidation progresses in the distal parts of the secretory pathway.

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