A Mechanism of Membrane Neutral Lipid Acquisition by the Microsomal Triglyceride Transfer Protein*

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The microsomal triglyceride transfer protein (MTP) and apolipoprotein B (apoB) belong to the vitellogenin (VTG) family of lipid transfer proteins. MTP is essential for the intracellular assembly and secretion of apoB-containing lipoproteins, the key intravascular lipid transport proteins in vertebrates. We report the predicted three-dimensional structure of the C-terminal lipid binding cavity of MTP, modeled on the crystal structure of the lamprey VTG gene product, lipovitellin. The cavity in MTP resembles those found in the intracellular lipid-binding proteins and bactericidal/permeability-increasing protein. Two conserved helices, designated A and B, at the entrance to the MTP cavity mediate lipid acquisition and binding. Helix A (amino acids 725–736) interacts with membranes in a manner similar to viral fusion peptides. Mutation of helix A blocks the interaction of MTP with phospholipid vesicles containing triglyceride and impairs triglyceride binding. Mutations of helix B (amino acids 781–786) and of N780Y, which causes abetalipoproteinemia, have no impact on the interaction of MTP with phospholipid vesicles but impair triglyceride binding. We propose that insertion of helix A into lipid membranes is necessary for the acquisition of neutral lipids and that helix B is required for their transfer to the lipid binding cavity of MTP.

Chylomicrons (CM) and very low density lipoproteins (VLDL) are among the largest macromolecular complexes secreted from eukaryotic cells. The assembly of neutral lipids and phospholipids into CM and VLDL is nucleated around a single molecule of apoB and requires a microsomal triglyceride transfer protein (MTP) complexed to the endoplasmic reticulum-resident protein, protein disulfide isomerase (PDI). The function of the MTP-PDI complex is to supply apoB with sufficient lipid to form a soluble lipoprotein. Defects of apoB and MTP cause hypobetalipoproteinemia and abetalipoproteinemia, respectively (1, 2).

ApoB and MTP have structural homology with lamprey lipovitellin (LV) (3). LV contains an N-terminal β-barrel (amino acids 17–296), an α-helical structure (amino acids 297–614), and a C-terminal lipid binding cavity (4). The structural relationship between MTP, apoB, and LV is supported by conservation of the gene and protein structure (3, 5). Important features of the quaternary structure of the lamprey LV homodimer are adapted in MTP to form a heterodimer with PDI and to associate with apoB during lipoprotein production (3, 5). The defining difference between MTP, apoB, and LV is related to their C-terminal lipid binding structures, which associate with different amounts of lipid (3). LV binds principally phospholipid with a stoichiometry of ~35 molecules/subunit (6). MTP binds 1–5 molecules of lipid (7). ApoB has a long C-terminal extension (~3500 amino acids), which incorporates a large neutral lipid core (8).

Here, we have addressed the mechanism by which MTP-PDI acquires neutral lipid from phospholipid bilayers for the assembly of VLDL and CMs. In the absence of a crystal structure for MTP, we derived a homology model to guide mutagenesis and biophysical studies. The experimental data substantiate the overall predictions of the model and provide insights into the mechanism of lipid acquisition and binding.

EXPERIMENTAL PROCEDURES

Modeling—Models were developed on the alignment shown in Fig. 1 and the x-ray crystal structure of lamprey LV (Protein Data Bank accession number 1LLV), refined to an R-value of 0.19 at 2.8 Å resolution (4). The C-sheet was modeled using INSIGHT interactive graphics software and the Homology computer program (Biosym Technologies, San Diego) and the A-sheet with the general purpose modeling program O (9). Models were energy minimized and the quality of the coordinates assessed as described (3).

Mutagenesis and Expression Studies—Mutagenesis was performed by a polymerase chain reaction-based strategy (3). All constructs were sequenced before use. Transfections, preparation of cell extracts, and triglyceride transfer activities were performed as described (2, 3).

Triglyceride Binding and Fusogenic Activity—Wild-type (WT) and mutant MTP-PDI complexes were purified as described (10). Donor small unilamellar vesicles (SU vesicles) were prepared as described (2), purified to homogeneity (11), and incubated with MTP (w/w 70:1) for 2 h at 37 °C. Lipid-protein complexes were separated on a Sepharose CL-4B (26/100) column.

Peptide Assays and Conformational Studies—Peptides were synthesized and purified as described (11) and dissolved (1 mg/ml) in trifluoroethanol. Calcein leakage from SUVs was monitored as described (12). Lipid mixing between labeled and unlabeled vesicles was measured by a fluorescence probe dilution assay (12). The predicted conformation of MTP peptide A at the water-lipid interface was determined as described (13).

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**RESULTS AND DISCUSSION**

To build a model of the lipid binding cavity of MTP, we examined the crystal structure of lamprey LV (Fig. 1) and an alignment of the MTPs and VTGs (3) (Fig. 2). The walls of the lipid binding cavity of LV comprise two \( \beta \)-sheets, designated C and A, and a small section of the \( \alpha \)-helical domain. The C-sheet comprises seven strands and the A-sheet, 23 (4).

The alignment of the predicted C-sheet of human MTP (amino acids 613–722) with amino acids 624–768 of lamprey LV is an extension of the multiple sequence alignment of Mann \textit{et al.} (3). (Fig. 2). At the gene level, amino acids 613–684 of MTP and the homologous residues of the VTGs are encoded by conserved exons (5). The gaps between amino acids 681–682 and 688–689 of MTP span amino acids 694–702 and 710–734 of lamprey LV. These regions are either proteolytically removed from the LV homodimer or form part of the connecting structures (4). Thus, the alignment is consistent with the evolutionary preference for insertions/deletions to occur in regions connecting the secondary structure (14). The alignment also reveals homology between the first 126 amino acids of the A-sheet of lamprey LV and residues 723–825 of MTP. The alignment between amino acids 747–807 of MTP and residues 800–875 of lamprey LV was manually adjusted on the basis of the secondary structure of lamprey LV and the amphipathic sequences of MTP. In the alignment, a high proportion of the hydrophobic residues in this region of MTP are superimposed onto amino acids in lamprey LV that point inward toward the lipid binding cavity (Fig. 2). Hydrophobic amino acids are known to be favored within internal sequences of secondary structures and disfavored within loops or connecting structures (15).

The alignment suggests conservation of the lipid binding C-sheet of lamprey LV and of the lipid binding portions of \( \beta \)-strands 2–5 of the A-sheet of lamprey LV in MTP. Common \( \beta \)-helices in the MTP sequence also support the conservation of helix A and B of lamprey LV in MTP. Helix A contains an N-terminal sequence (PISVV) conforming to a proline-box motif (16). This motif includes an N-cap proline residue, has a preference for a valine residue at the third position (Val\textsuperscript{227}) of the helix, and apolar residues at the −1 and fourth positions. Helix B contains asparagine (Asn\textsuperscript{780}) as the N-cap residue, the most common residue at such a position (17).

**The Lipid Binding Cavity of MTP**—We used the realigned sequence of amino acids 613–826 of MTP and the atomic coordinates of lamprey LV to derive the model of the lipid binding structures of MTP. These structures in MTP may also include amino acids 827–894, but extensive sequence divergence from the LVs in this region precludes an evaluation. The models of the C- and A-sheets of MTP predict a hydrophobic cavity, consistent with biochemical studies (18). The MTP cavity is similar to, albeit smaller than, that found in lamprey LV (Fig. 3, a and b). Important evidence for the validity of the MTP structure derives from the finding that the predicted lipid binding surfaces contain a number of hydrophobic residues comparable to that found on the equivalent surface of lamprey LV (Fig. 3, a and b).

The predicted C-sheet domain (amino acids 603–712) of the lipid binding cavity of MTP contains six anti-parallel \( \beta \)-strands, and a short helix (Fig. 3b). The model of the A-sheet domain of MTP (amino acids 725–829) comprises four \( \beta \)-strands and two \( \alpha \)-helices, designated A (residues 725–736) and B (residues 781–786) (Fig. 3, a–c). Analogous to LV (Fig. 1), these helices reside in close proximity at the entrance to the lipid binding cavity of MTP (Fig. 3d). We envisage the back of the cavity of MTP closed off by dimerization with PDI, since the posterior openings to the lipid binding cavities of lamprey LV are closed off by homodimerization (4).

The predominantly \( \beta \)-sheet structure predicted for the lipid binding cavity of MTP is reminiscent of the lipid binding cavities of bactericidal/permeability-increasing protein (BPI) (19) and the fatty acid-binding proteins (FABPs) (20, 21). The BPI family of proteins includes phospholipid transfer protein and cholesteryl ester transfer protein (CETP) (19). The FABPs have a common topology comprising 10 antiparallel \( \beta \)-strands arranged in two \( \beta \)-sheets, typically having two short \( \alpha \)-helices that loop out to cover the mouth of the cavity. The helices are not required for the structural integrity of the lipid binding cavity (21). Rather, they regulate the kinetics of ligand transfer (21).

**The Entrance to the Cavity**—The functional importance of helices A (amino acids 725–736) and B (amino acids 781–786), located at the entrance of the lipid binding cavity of MTP, is suggested by the abetalipoproteinemia N780Y mutation (22). Expression of MTP N780Y in COS-1 cells produced near WT levels of soluble MTP (Fig. 4a) but no triglyceride transfer activity (Table I). The control mutant, N780A, produced near WT activity. Our model of MTP predicts that Asn\textsuperscript{780} forms the N-cap residue of helix B, and because a tyrosine is rarely found at this position (17), it is conceivable that the N780Y mutation
Fig. 2. Sequence alignment of the C- and A-sheet domains of the lamprey LV precursor, VTG, with representative VTGs and MTPs. Amino acids depicted in cyan have similar values (≥0.5) (31) in five or more sequences or in three MTPs and lamprey LV. Residues highlighted in yellow point toward the lipid binding cavity of lamprey LV or the predicted lipid binding cavity of human (H.s) MTP. Underlined residues (664 in Homo sapiens (H.s) MTP, 681 and 683 in Xenopus laevis (X.l) and chicken (G.g, Gallus gallus) VTG, respectively) show conserved intron/exon boundaries, the codon sequence of which is interrupted at the same position. Arrows indicate β-strands. Cylinders depict α-helices. Loops are shown in gray and residues with unassigned coordinates by a dashed line. The first strands of the C (red)– and A (blue)–sheets of lamprey LV, formed by amino acids 196–197 and 188–190, respectively, are not predicted for MTP (3).

To further evaluate the importance of helices A and B of MTP for the lipid transfer process, we introduced mutations predicted to destabilize their packing (Fig. 3c). The individual substitution of Ile725, Tyr771, Arg772, and Lys775 with alanine had a marked impact on lipid transfer activity (Table I), despite extensive network of interactions stabilizing the spatial arrangement between helices A and B. By contrast, alanine substitution of Ile725, Val728, Leu731, Ile735, Arg781, and Ile790, which are predicted to form few or no interactions, had no impact (Fig. 3c).

Helices A and B are located at the entrance to the lipid binding cavity of MTP. There, they form a hydrophilic ridge at the water-lipid interface (Fig. 3d), which we envisage as interacting with a lipid membrane during the lipid transfer process. The importance of amino acids (Val727, Leu731, Leu734, Arg781, Val782, Val785, Ile786) at the water-lipid interface was examined by alanine-scanning mutagenesis. Mutant proteins were individually expressed in COS-1 cells and WT levels of soluble MTP recovered (Fig. 4a). Triglyceride transfer activity was reduced in cells expressing L734A, R781A, and V782A, the activities being 33 ± 8, 59 ± 3, and 26 ± 4% of WT, respectively (Table I). The importance of Leu734 and Val782 for triglyceride transfer activity was confirmed by the markedly reduced activity of the double mutant L734A/V782A (Fig. 4e, Table I). In contrast, the individual combination of L731A, V785A, or I786A with L734A had no additional effect (Table I).

To further evaluate the functional importance of size, hydrophobicity, and geometry of residues at positions 734 and 782 of MTP, we mutated Val727 to Leu and Leu734 to Ser, Val, Ile, Phe, and Met (Fig. 4a). The activities of L734F, V782L, L734I, and L734M, V782L, L734I, 785A, and 786A with L734A had no additional effect (Table I).

From 13C NMR spectroscopic studies we envisage that the carbonyl groups of vesicle-solubilized triglyceride reside close to the vesicle surface. WT MTP-PDI increased the size of the phospholipid vesicles containing radiolabeled triglyceride and acquired radiolabeled triglyceride from vesicles (Fig. 4, b and c). The interaction of L734A with lipid was markedly impaired and the amount of radiolabeled

Table I. The importance of Leu734 and Val782 for triglyceride transfer activity was confirmed by the markedly reduced activity of the double mutant L734A/V782A (Fig. 4e, Table I). In contrast, the individual combination of L731A, V785A, or I786A with L734A had no additional effect (Table I).

Lipid Binding and Fusogenic Properties of MTP—WT and mutant MTP-PDI complexes were individually incubated with phospholipid vesicles containing radiolabeled triglyceride and evaluated for the dual MTP function of lipid interaction and triglyceride binding (Fig. 4, b and c). From 13C NMR spectroscopic studies we envisage that the carbonyl groups of vesicle-solubilized triglyceride reside close to the vesicle surface. WT MTP-PDI increased the size of the phospholipid vesicles (Fig. 4, b and c) and acquired radiolabeled triglyceride from vesicles (Fig. 4, b and c). The interaction of L734A with lipid was markedly impaired and the amount of radiolabeled
triglyceride acquired from the vesicles reduced. V782A and N780Y acquired very little radiolabeled triglyceride despite near normal ability to interact with lipid. The interaction of the double mutant L734A/V782A with lipid was similar to L734A but no radiolabeled triglyceride was acquired (data not shown). These results, and the reduced triglyceride transfer activities of L734A, V782A, and L734A/V782A (Table I), indicate that Leu$^{734}$ of helix A and Val$^{782}$ of helix B facilitate the triglyceride
acquisition and binding step of the MTP-mediated triglyceride transfer process. The results also suggest that helix A of MTP confers MTP with fusogenic activity. Fusion peptides were first identified in the envelope protein of viruses, where they facilitate the entry of the virus into a host cell (24). Typically, they comprise between 12 and 26 residues and are highly hydrophobic (24, 25). Predicted helix A contains 12 amino acids, 8 of which are hydrophobic. Its predicted helical structure is in accord with the helical conformation of viral fusion peptides in the active state (24, 25).

The proposition that helix A is fusogenic is also suggested by theoretical and experimental data on the isolated peptide PVKGLILLI. This peptide is predicted to adopt an oblique (-30° angle) orientation at a water-lipid interface (data not shown) and promote membrane destabilization. Accordingly, MTP helical peptide A causes the leakage of calcine from SUVs (Fig. 4d), intravesicular lipid mixing (Fig. 4e), and an increase in vesicle size (data not shown). Importantly, a peptide corresponding to the highly active mutant L734M increased intravesicular lipid mixing activity, suggesting a tight coupling between the lipid mixing activity of peptide A and MTP-mediated triglyceride transfer activity (Table I).

Concluding Comments—We have used molecular modeling to derive sufficient structural information to understand how the MTP-PDI complex might extract neutral lipid from its sites of synthesis, in the membranes of the endoplasmic reticulum (26) for transfer to apoB during lipoprotein assembly. Our data indicate that MTP has a fusion peptide at the entrance to a hydrophobic lipid binding cavity of MTP. We envisage that the negative charges on the phosphate groups of bilayer phospholipid molecules. Such interactions would be expected to increase the affinity of the cavity entrance of MTP with lipid.

**TABLE I.** Summary of mutants

| Mutation     | MTP position | Activity |
|--------------|--------------|----------|
| L725A        | hA, 1        | 101 ± 12 |
| V727A        | hA, 3        | 104 ± 27 |
| V728A        | hA, 4        | 116 ± 19 |
| L731A        | hA, 7        | 101 ± 17 |
| L731S        | hA, 7        | 103 ± 6  |
| L731A/L734A  | hA, 7; h1, 10| 46 ± 25  |
| L731S/L734S  | hA, 7; h1, 10| 3 ± 6    |
| L732A        | hA, 8        | 50 ± 5   |
| L733A        | hA, 9        | 105 ± 14 |
| L734A        | hA, 10       | 33 ± 8   |
| L734I        | hA, 10       | 31 ± 6   |
| L734V        | hA, 10       | 78 ± 17  |
| L734I        | hA, 10       | 102 ± 13 |
| L734F        | hA, 10       | 47 ± 9   |
| L734M        | hA, 10       | 132 ± 28 |
| L734A/V782A  | hA, 10; hB, 2| 7 ± 4    |
| L734A/V785A  | hA, 10; hB, 5| 39 ± 10  |
| L734A/V786A  | hA, 10; hB, 6| 39 ± 8   |
| L734A/I790A  | hA, 10; I    | 46 ± 9   |
| I735A        | hA, 11       | 102 ± 2  |
| V771A        | lp           | 39 ± 15  |
| R772A        | lp           | 56 ± 10  |
| K775A        | lp           | 72 ± 4   |
| R777A        | lp           | 94 ± 9   |
| K779A        | lp           | 81 ± 21  |
| N780A        | lp           | 87 ± 18  |
| N780Y        | lp           | 4 ± 4    |
| R781A        | hB, 1        | 59 ± 3   |
| V782A        | hB, 2        | 26 ± 4   |
| V782L        | hB, 2        | 120 ± 9  |
| V785A        | hB, 5        | 83 ± 17  |
| I786A        | hB, 6        | 104 ± 8  |
| I790A        | lp           | 98 ± 3   |

* For MTP position, hA, hB, and lp denote helix A, helix B, and loop between β-strand 3 and helix B of the A-sheet, respectively.

† L734A, V782A, L734A/V782A, and N780Y of MTP-PDI were purified from the baculovirus expression system. Each produced a comparable level of the MTP-PDI complex as wild type, indicating that mutation of Leu734, Asn 780, and Val 782 does not perturb the fold of the major binding site (amino acids 512–592) on MTP for PDI (3). The structural integrity of mutant proteins L734A, V782A, and L734A/V782A was also indicated by circular dichroism spectroscopy (10) (data not shown). Triglyceride transfer activities of purified L734A, V782A, L734A/V782A, and N780Y were 59.8 ± 16, 35 ± 8, 14 ± 2, and 0% of WT, respectively.
The discovery of lipid-interacting helical peptides at the entrance of the lipid binding cavities of the lipases (28), along with the predicted cavities of CETP (29) and lecithin cholesterol acyl transferase (30), suggests that the mechanism of lipid acquisition described here for MTP might apply to other lipid binding proteins.

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