Several studies have demonstrated protein-protein interactions between microsomal triglyceride transfer protein (MTP) and apolipoprotein B (apoB). However, the binding sites involved in these interactions have not been elucidated. To identify an MTP binding site in apoB, we have expressed several apoB sequences as fusion proteins with the eight-amino acid FLAG peptide. The chimeras were transiently expressed in COS cells, and conditioned media were used to study the binding of these sequences to either immobilized or soluble MTP. A polypeptide containing amino acids 270–570 (B: 270–570), but not 1–300, bound to MTP. AGI-S17, an antagonist of apoB-MTP binding, inhibited the binding of B:270–570 to MTP but not to M2, a monoclonal antibody that recognizes the FLAG peptide. These data indicated that B:270–570 contains an MTP binding site. Next, sequences within 270–570 were subjected to C-terminal truncations at natural proline residues. B:270–509 bound less efficiently than B:270–570, whereas, B:270–430 and other shorter chimeras did not bind to MTP. Furthermore, truncations at amino acids 502 and 509 decreased MTP binding by 73 and 42%, respectively. These data indicate that B:430–570 in the α1-globular domain of apoB plays a crucial role in MTP binding and presumably in the initiation and maturation of apoB-containing lipoproteins.

Apolipoprotein biosynthesis is defective in abetalipoproteinemia due to mutations in the gene for microsomal triglyceride transfer protein (MTP) whereas, in hypobetalipoproteinemia, plasma lipoprotein levels are low due to mutations in the gene for microsomal triglyceride transfer protein. The requirement for MTP in apoB folding and assembly has been established by the use of inhibitors of MTP and the requirement for net transfer of lipids to apoB during its translation or may reflect a separate chaperone-like activity for MTP in apoB folding and assembly. Compounds that inhibited lipid transfer activity in apoB-containing lipoproteins was established by the use of inhibitors of apoB and MTP, which may be important for achieving net transfer of lipids to apoB during its translation or may reflect a separate chaperone-like activity for MTP in apoB folding and assembly. Expression of apoB alone in most studies resulted in the intracellular synthesis and degradation of apoB polypeptides, but no secretion (6–8). Expression of apoB alone in most studies resulted in the intracellular synthesis and degradation of apoB polypeptides, but no secretion (6–8). In contrast, co-expression of apoB with MTP resulted in the synthesis and secretion of apoB polypeptides as lipoprotein particles (6–8). The requirement for lipid transfer activity of MTP in the assembly of apoB-containing lipoproteins was established by the use of inhibitors of apoB and MTP, which may be important for achieving net transfer of lipids to apoB during its translation or may reflect a separate chaperone-like activity for MTP in apoB folding and assembly. Lysine and arginine residues in the N-terminal 18% of apoB are critical for these interactions (18). However, the binding sites involved in these protein-protein interactions are not known. In the present study, we have used fusion proteins containing defined sequences of apoB to map an MTP binding site.

**EXPERIMENTAL PROCEDURES**

**Materials**—MTP was purified to homogeneity and assayed as described earlier (17, 19–22). Antibodies used for enzyme-linked immunosorbent assay have been described previously (23, 24). Antibodies against purified MTP were kindly provided by Dr. Haris Jamil of Bristol-Myers Squibb, Princeton, NJ. An anti-FLAG monoclonal antibody, M2, and other reagents were purchased from Sigma. AGI-S17 was kindly provided by Drs. Russell Medford and Uday Saxena of Atherosciences Inc., Norcross, GA.

**Expression of FLAG/ApoB Chimeras in COS Cells**—To identify a putative MTP binding site in apoB, several FLAG/apoB chimeras were constructed. Various apoB DNA sequences were amplified by the polymerase chain reaction and cloned into the NcoI and Apal site of the pCMV/FLAG expression vector (25, 26). The expression plasmids contained the CMV immediate early promoter, a 30-amino acid signal sequence from bovine preprolactin, the FLAG octapeptide, and 33 amino acids of mature bovine prolactin, followed by the pertinent apoB sequences (25). The plasmids were transiently expressed in COS cells using the diethyl aminoethyl-dextran transfection method (25–27). Conditioned media were used to measure the secreted chimeras by virtue of their binding to M2 and were simultaneously used to study MTP binding.

**Binding of FLAG/ApoB Chimeras to Immobilized MTP and M2**—M2 (3 μg/well) or MTP (1 μg/well) were immobilized (2 h at 37 °C or 18 h at 4 °C) in microtiter plates, washed (three times), and incubated (30 min, 37 °C) with PBS containing 0.05% Tween 20 (PBS-Tween). After three
COS cells were transfected with truncated plasmids. Microtiter wells were coated with M2 (3 μg/well), a monoclonal antibody that recognizes FLAG, or purified, heterodimeric MTP (1 μg/well). Wells were incubated in triplicate with 100 μl of different conditioned media and washed, and then the bound peptides were quantitated using polyclonal sheep anti-human apoB antibodies and alkaline phosphatase-labeled anti-sheep IgG as described previously (17, 23, 24). The optical densities were determined at 405 nm. The data are representative of 10 independent experiments.

Table I: Binding of different apoB sequences to the immobilized MTP

| FLAG-chimeras expressing apoB sequences (amino acids) | Immobilized anti-FLAG M2 (optical densities) | Immobilized MTP (optical densities) | Ratio (MTP/M2) | Binding (% of 270–570) |
|-----------------------------------------------------|--------------------------------------------|-----------------------------------|----------------|------------------------|
| 270–570                                             | 0.76 ± 0.10                                | 0.71 ± 0.15                       | 0.93           | 100                    |
| 1–300                                               | 0.65 ± 0.02                                | 0.03 ± 0.02                       | 0.04           | 4                      |
| 1–502                                               | 0.73 ± 0.11                                | 0.18 ± 0.07                       | 0.25           | 27                     |
| No DNA                                              | 0                                          | 0                                 |                |                        |

Washes with PBS-Tween, the microtiter wells were incubated with conditioned medium from transfected cells for 2 h at 37 °C in triplicate. For controls, triplicate wells were incubated with nonconditioned medium or PBS-Tween. Wells were then washed with PBS-Tween, incubated with sheep anti-human apoB polyclonal antibodies (1:1000 dilutions) for 1 h at 37 °C, washed, incubated (1 h, 37 °C) with alkaline phosphatase-labeled anti-sheep IgG, washed, and incubated with p-nitrophenyl phosphate (1 mg/ml in 10 mM ethanolamine, 0.5 mM MgCl₂, pH 9.5) for various times (17, 23, 24). The absorbance at 405 nm was determined using a Microplate Reader (Dynatech Labs, Chantilly, VA). The highest optical densities from control wells were subtracted to determine the specific binding to M2 and MTP. The values in M2-coated wells provided a measure of the secretion of different chimeras, whereas the values in MTP-coated wells represented binding to MTP. We could not determine the kinetic parameters for the binding of individual peptides to MTP because it was difficult to measure their concentration due to differences in antibody epitopes in different regions of apoB.

Binding of FLAG/ApoB Chimeras to Soluble MTP and M2—Transfected COS cells were radiolabeled with [³⁵S]Met/Cys-labeling mixture (Expire³⁵S³⁸S, NEN Life Science Products) and conditioned medium (1 ml) were incubated overnight with purified heterodimeric MTP (1 μg/ml), followed by rabbit anti-bovine MTP (5 μl) and anti-rabbit IgG (5 μl). In parallel, conditioned medium (1 ml) were incubated with M2 (15 μg/ml) and anti-mouse IgG. Immune complexes were recovered using protein G-Sepharose (Amersham Pharmacia Biotech), washed, separated on polyacrylamide gels, and autoradiographed using PhosphorImager 445Si (Molecular Dynamics, Sunnyvale, CA).

RESULTS

We had previously demonstrated that optimum binding of MTP occurs with the N-terminal 18% (1–781 amino acids) of apoB (17, 18). Patel and Grundy (16) showed that apoB13 also interacts with MTP. Based on these studies, we thought that a high affinity MTP binding site would be present in the N-terminal region of apoB. To identify the binding site, we expressed different regions of apoB as FLAG/apoB chimeras (25, 26). FLAG (DYKDDDDK) is an octapeptide that is commonly used as an epitope tag. The cDNAs were transiently transfected into COS cells, and the conditioned media were used to examine the secretion of FLAG/apoB chimeras using an anti-FLAG monoclonal antibody, M2 (Table I). The FLAG/apoB chimeras containing B1–300, B270–570, and B1–502 were secreted to a similar extent. These data indicate that B:1–300 co-immunoprecipitated with M2 and MTP was 6-fold higher than that of B:270–570, indicating that the amount of B:1–300 secreted was that much higher. Nonetheless, only B:270–570 was co-immunoprecipitated with MTP. The amount of B:270–570 co-immunoprecipitated with M2 and MTP was similar. In control experiments, anti-mouse IgG or anti-MTP IgG did not precipitate B:1–300 or B:270–570 (data not shown). These data indicate that B:270–570 recognizes both soluble and immobilized MTP.

To confirm further that B:270–570 contains an MTP binding site, we studied the effect of AGI-S17 on the binding of B:270–570 to MTP (Fig. 2). AGI-S17 is an antagonist that inhibits LDL-MTP binding, has no effect on the lipid transfer activity of MTP and decreases apoB secretion in hepatoma cells.⁷ AGI-S17 (10 μM) inhibited 70% of the binding of B:270–570 to immobilized MTP, but did not inhibit binding to immobilized M2 (Fig. 2). These studies further support the evidence that B:270–570 contains a binding site for MTP.

Next, we hypothesized that an MTP binding site might be smaller than the 300 amino acid residues of B:270–570. To test this hypothesis, we deleted amino acids from the C terminus of B:270–570. Consideration was given to truncating polypeptides at naturally occurring proline residues to avoid disruption of helical structures predicted in this region (28, 29). Plasmids expressing various truncated forms of the B:270–570 were transiently transfected in COS cells, and the amounts of synthesized and secreted peptides were determined by radiolabeling, immunoprecipitation, and exposure to PhosphorImager screens (Fig. 3). The amounts of different FLAG/apoB chimeras synthesized by transfected cells were similar; however, the amounts of secreted chimeras were very different. B:270–570 was efficiently secreted by these cells, whereas B:270–394 was

Fig. 1. Coimmunoprecipitation of apoB polypeptides with MTP. COS cells were transiently transfected with B1 (amino acids 1–300) or B270 (amino acids 270–570) plasmids expressing FLAG-apoB fusion proteins. After 60 h, cells were radiolabeled with [³⁵S]Met/Cys. Conditioned media were incubated with either M2 (anti-FLAG antibody) or MTP and were then immunoprecipitated using either anti-mouse IgG or anti-MTP IgG, respectively, as described under “Experimental Procedures.” The immunoprecipitated proteins were separated on polyacrylamide gels and exposed to a PhosphorImager screen.

TABLE I

MTP Binding Site in ApoB

| Fusion proteins | Binding Site Ratio (optical densities) | Binding Site Ratio (optical densities) | Binding Site Ratio (optical densities) | Binding Site Ratio (optical densities) |
|-----------------|---------------------------------------|---------------------------------------|---------------------------------------|---------------------------------------|
| B:270–570       | 0.11                                   | 0.18                                   | 27.0                                  | 100                                   |
| B:1–300         | 0.02                                   | 0.04                                   | 4.0                                   | 27.0                                  |
| B:1–502         | 0.15                                   | 0.93                                   | 100                                   | 100                                   |

of the MTP binding. These studies indicated that optimum MTP binding occurs with B:270–570 of apoB, and truncations in this region at amino acid 502 results in significant loss (~70%) of this binding.

The experiments were then extended to study these interactions in solution (Fig. 1). For this purpose, MTP or M2 was incubated with radiolabeled B:1–300 and B:270–570, and complexes were recovered by immunoprecipitation with antibodies to MTP or M2 (Fig. 1). In this experiment, the amount of B:1–300 co-immunoprecipitated with M2 was 6-fold higher than that of B:270–570, indicating that the amount of B:1–300 secreted was that much higher. Nonetheless, only B:270–570 was co-immunoprecipitated with MTP. The amount of B:270–570 co-immunoprecipitated with M2 and MTP was similar. In control experiments, anti-mouse IgG or anti-MTP IgG did not precipitate B:1–300 or B:270–570 (data not shown). These data indicate that B:270–570 recognizes both soluble and immobilized MTP.

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A. Bakillah, unpublished observations.
Microtiter plates were coated with M2 or MTP. Conditioned medium under “Experimental Procedures.” Data plotted are means ± S.D. of disulfide bond formation in this region blocks the initiation of lipoprotein assembly (30, 31). Site-directed mutagenesis studies revealed that two disulfide bonds within the first 250 amino acids are crucial for the assembly and secretion of apoB-containing lipoproteins (26, 32). Our studies indicate that the MTP binding site in apoB is present downstream of these crucial cysteine residues. However, it is possible that native disulfide bond formation is required to expose the MTP binding site during lipoprotein assembly.

To obtain some information about the importance of the MTP binding site, we looked for conservation of the identified apoB sequence in other species. Complete apoB sequences from other species are not available in the data banks (see also Segrest et al. (29)). However, a partial pig apoB sequence (accession no. I46567.pir2) was found to be 74% identical to B:430–570. Within this site, the two identified α-helices were found to be 85% identical in human and pig. By comparison, the LDL receptor binding domains in human and pig have been shown to be 72% identical (33). The conservation of the two identified helices in these two species suggests that they may be functionally significant.

B:270–430 to M2 was similar to that observed for B:270–509 (Table II). These studies indicate that deletion of amino acids 430–570 in apoB disrupts its ability to interact with MTP.

**DISCUSSION**

Several lines of evidence indicated that amino acids 430–570 in apoB are critical for MTP binding. First, binding of FLAG/apoB chimeras to MTP was independent of FLAG and prolactin sequences but was dependent on the presence of apoB sequences corresponding to amino acids 270–570 (Table I). Second, specific apoB sequences were required for MTP binding. For example, B:270–570, but not B:1–300, interacted with MTP (Table I). Third, B:270–570 bound to immobilized as well as soluble MTP (Table I and Fig. 1). Fourth, binding of B:270–570 to MTP was inhibited by a compound that inhibits LDL-MTP binding (Fig. 2). Fifth, MTP binding was lost with successive C-terminal deletions in B:270–570 (Table II). Sixth, loss of MTP binding was independent of the length of the peptide. For example, B:1–502 and B:270–509 terminate within this sequence and bind poorly to MTP (Tables I and II).

Chou-Fasman analysis (data not shown) of amino acids 430–570 predicted the presence of two α-helices (B:496–508 and B:529–541) containing three positively charged amino acid residues each. We have previously shown that protein-protein interactions between apoB and MTP are ionic and that lysine and arginine residues in apoB are crucial for these interactions (17, 18). Thus, it is possible that these helices may in part be responsible for interactions between apoB and MTP.

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**TABLE II**

| Flag chimeras expressing apoB sequences (amino acids) | Immobilized anti-FLAG M2 (optical densities) | Immobilized MTP (optical densities) | Ratio (MTP/M2) | Binding (% of 270–570) |
|-------------------------------------------------------|---------------------------------------------|------------------------------------|---------------|------------------------|
| B:270–570                                             | 0.59 ± 0.02                                | 0.53 ± 0.05                        | 0.90          | 100                    |
| B:270–509                                             | 0.42 ± 0.03                                | 0.22 ± 0.02                        | 0.22          | 58                     |
| B:270–430                                             | 0.40 ± 0.02                                | 0.03 ± 0.02                        | 0.08          | 9                      |
| B:270–394                                             | 0.05 ± 0.02                                | 0.00 ± 0.01                        | 0.00          | 0                      |
| B:270–341                                             | 0.27 ± 0.03                                | 0.00 ± 0.01                        | 0.00          | 0                      |

**FIG. 2. Effect of AGI-S17 on the binding of B:270–570 to MTP.**

Microtiter plates were coated with M2 or MTP. Conditioned medium (100 μl) from transiently transfected COS cells was used in triplicate to study the binding of B:270–570 to immobilized M2 or MTP in the presence of different concentrations of AGI-S17 for 2 h as described under “Experimental Procedures.” Data plotted are means ± S.D.

**FIG. 3. Synthesis and secretion of different chimeras expressing C-terminally truncated forms of B:270–570.** Various C-terminal truncations were made in B:270–570 at naturally occurring proline residues. COS cells were transiently transfected with indicated plasmids and radiolabeled for 2.5 h with [35S]Met/Cys. Media and cell lysates were immunoprecipitated with M2, separated on polyacrylamide gels, and subjected to fluorography. pPLF is a C-terminal epitope-tagged form of bovine preprolactin and was used as a control. Migrations of different molecular mass markers (kDa) have been identified.

Secreted with the least efficiency. Next, we measured the secreted chimeras by enzyme-linked immunoassay using immobilized M2 (Table II). The secretion efficiency observed for these peptides was B:270–570 > B:270–509 and B:270–430 > B:270–341 > B:270–394, which is in agreement with the results obtained from radiolabeling and immunoprecipitation (Fig. 3). Next, we determined the binding of these peptides to MTP (Table II). The amounts of B:270–570 bound to M2 and MTP were similar since the ratio between their binding was close to one. This binding is consistent with the data in Table I. On the other hand, the ratio (MTP/M2) of B:270–509 binding was 0.52, indicating that loss of amino acids 510–570 decreased the binding to MTP by ~58% (Table II). Further truncation of B:270–570 to B:270–430 and B:270–341 resulted in complete loss of binding to MTP. This loss was not due to low levels of the chimeras being secreted because the binding ofapoB chimeras to MTP was independent of FLAG and prolactin sequences but was dependent on the presence of apoB sequences corresponding to amino acids 270–570 (Table I). Second, specific apoB sequences were required for MTP binding. For example, B:270–570, but not B:1–300, interacted with MTP (Table I). Third, B:270–570 bound to immobilized as well as soluble MTP (Table I and Fig. 1). Fourth, binding of B:270–570 to MTP was inhibited by a compound that inhibits LDL-MTP binding (Fig. 2). Fifth, MTP binding was lost with successive C-terminal deletions in B:270–570 (Table II). Sixth, loss of MTP binding was independent of the length of the peptide. For example, B:1–502 and B:270–509 terminate within this sequence and bind poorly to MTP (Tables I and II).

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The MTP binding site identified in the present study resides within N-terminal 13% of apoB. This region has been predicted to exist as an amphipathic globular domain (28, 29). Disruption of disulfide bond formation in this region blocks the initiation of lipoprotein assembly (30, 31). Site-directed mutagenesis studies revealed that two disulfide bonds within the first 250 amino acids are crucial for the assembly and secretion of apoB-containing lipoproteins (26, 32). Our studies indicate that the MTP binding site is present downstream of these crucial cysteine residues. However, it is possible that native disulfide bond formation is required to expose the MTP binding site during lipoprotein assembly.

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The involvement of B:430–570 in MTP binding is consistent with earlier studies that demonstrated that apoB-MTP binding occurs between C-terminally truncated apoB polypeptides and MTP (17, 18). It is also consistent with our earlier observations that heparin- and MTP-binding sites may be different (18). Heparin has been shown to bind residues 5–99, 205–279, 875–932, and 2016–2151 in apoB48 (34). Furthermore, the identified MTP binding site does not contain the heparin-binding consensus sequences (X-B-B-X-B-X and X-B-B-X-B-X) proposed by Cardin and Weintraub (35). Thus, the MTP binding site is located after the heparin binding site present at 205–279. In addition to heparin, apoB is also known to exhibit ionic interactions with the LDL receptor (36). The LDL receptor binding sites A and B are structurally distinct.

In conclusion, the data presented in this study suggest that B:430–570 plays an important role in MTP binding. Furthermore, this may be the site responsible for MTP binding within apoB that underlies the physical interactions observed between MTP and various truncated forms of apoB (15–18). The knowledge of the specific MTP binding site will facilitate efforts to elucidate the mechanism by which both the binding and lipid transfer activities of MTP, separately and/or in combination, contribute to the biogenesis of apoB-containing lipoproteins.

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