Subcellular localization of microsomal triglyceride transfer protein

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Abstract **Microsomal triglyceride transfer protein (MTP) is essential for the assembly of apolipoprotein B-containing lipoproteins. Within the endoplasmic reticulum, it transfers lipid from the membrane to the forming lipoprotein. Recent evidence suggests that it may also function within the Golgi apparatus. To address this hypothesis, we developed a polyclonal antibody to MTP and used it in a series of studies on mouse liver and McArdle-RH7777 (McA) cells. Western blot analysis demonstrated the presence of MTP within mouse hepatic-Golgi apparatus-rich fractions. In addition, in vitro lipid transfer assays demonstrated the presence of triglyceride transfer activity within the Golgi fractions. Immunohistochemical studies with mouse liver demonstrated the presence of MTP within all hepatocytes, but not in nonparenchymal cells. The subcellular location of MTP in McA cells was investigated using confocal microscopy. MTP colocalized with the *trans*-Golgi network (TGN) 38 and Golgi SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) of 28 kDa (GS28), markers for the *trans*- and *cis*-Golgi apparatus, respectively. Morphometric analyses indicated that ~17% of the MTP signal colocalized with the TGN38, while 33% of the *trans*-Golgi marker colocalized with the MTP. Approximately 17% of the MTP signal colocalized with the GS28, whereas 53% of the *cis*-Golgi marker colocalized with the MTP. The results provide unequivocal evidence for the location of MTP within the Golgi apparatus, and further highlight the importance of this organelle in the assembly of lipoproteins.**—Swift, L. L., M-Y. Zhu, B. Kakkad, A. Jovanovska, M. D. Neely, K. Valyi-Nagy, R. L. Roberts, D. E. Ong, and W. G. Jerome. **Subcellular localization of microsomal triglyceride transfer protein. *J. Lipid Res.* 2003.** 44: **1841–1849.**

Supplementary key words Golgi apparatus • very low density lipoprotein • intracellular transport

Microsomal triglyceride transfer protein (MTP) is essential for the assembly of triglyceride-rich lipoproteins by the liver and the small intestine [as reviewed in refs. (1, 2)]. It was first isolated from bovine liver microsomes by Wetterau and Zilversmit (3) and later from rat liver and intestine (4), and it was shown to transfer lipid between phosholipid membranes. Its role in the assembly of lipoproteins was predicted but not defined until the discovery that abetalipoproteinemia, a rare genetic disorder leading to markedly decreased plasma levels of apolipoprotein B (apoB) and triglycerides, was a result of mutations in the MTP gene (5). Development of pharmacologic inhibitors of the transfer activity of MTP further underscored the role of MTP in lipoprotein assembly. These inhibitors substantially decrease apoB and triglyceride secretion by cells in culture (6–8), block production of VLDL in rodent models, and normalize plasma lipoprotein levels in Watanabe heritable hyperlipidemic rabbits (9). Finally, studies have shown that expression of apoB truncations (>apoB-29) in nonlipoprotein-producing cells is accompanied by secretion of only trace amounts of the protein unless MTP is also expressed (10–13).

The assumption that MTP is localized within the endoplasmic reticulum (ER) of the cell (14) has been based on several observations: *1*) MTP was initially isolated from a microsomal fraction that consists primarily of the smooth and rough ER; *2*) MTP is essential for VLDL production, and the most widely accepted model for VLDL assembly, the two-step model, predicts that both steps occur within the ER (15); and *3*) MTP forms a heterodimer with protein disulfide isomerase (PDI) (14), and PDI contains a Lys-Asp-Glu-Leu (KDEL) sequence that retains it within the ER (16). It could therefore be assumed that the KDEL sequence would prevent the MTP/PDI complex from moving out of the ER to the Golgi apparatus.

Studies from our laboratory have suggested that MTP is not restricted to the ER but is present within the Golgi apparatus. By comparing the composition of nascent VLDL recovered from the rough ER with VLDL recovered from Golgi apparatus-rich fractions of rat liver, we estimated that

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Abbreviations: ER, endoplasmic reticulum; McA, McArdle-RH7777; MTP, microsomal triglyceride transfer protein; PDI, protein disulfide isomerase.

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up to 50% of the total VLDL triglyceride is added while the particle is en route to or within the Golgi apparatus (17). We recently identified a pool of newly synthesized triglyceride in the membranes of the Golgi complex from mouse liver (18). Furthermore, we have shown that the net turnover of this pool is rapid and is attenuated by the presence of the MTP inhibitor. These observations have led us to hypothesize that MTP is present within the Golgi apparatus and mediates the transfer of membrane triglyceride to nascent lipoproteins. In this paper, we report the presence of MTP in Golgi apparatus-rich fractions from mouse liver, and we demonstrate triglyceride transfer activity in these fractions using an in vitro lipid transfer assay. In addition, we have utilized confocal microscopy to probe the location of MTP within McArdle-RH7777 (McA) cells. Our studies clearly demonstrate the presence of MTP within the Golgi complex of the cell and further underline the importance of this organelle in the assembly of VLDL by the liver.

EXPERIMENTAL PROCEDURES

Materials

Rabbit anti-MTP was developed in our laboratory as described below. Goat anti-bovine MTP and MTP inhibitor (BMS 200150) were kindly provided by John Wetterau (Bristol Myers Squibb, Princeton, NJ). Mouse monoclonal anti-rat PDI was purchased from Affinity BioReagents (Golden, CO). Biotin-conjugated goat anti-rabbit IgG and anti-biotin antibody linked to alkaline phosphatase were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Alkaline phosphatase substrate was purchased from Dako (Carpinteria, CA). Anti-*trans*-Golgi network (TGN) 38 was purchased from Affinity BioReagents, and antibody to Golgi SNARE (soluble *N*-ethylamide-sensitive factor attachment protein receptor) of 28 kDa (GS28) was from Stressgen Biotechnologies Corp. (Victoria, BC, Canada). Donkey (Cy3)-conjugated AffiniPure Cy3 anti-rabbit IgG was purchased from Jackson Immuno-Research Laboratories, and fluorescein isothiocyanate (FITC)-coupled anti-mouse IgG was obtained from ICN Pharmaceuticals (Aurora, OH). Pansorbin was purchased from Calbiochem (La Jolla, CA). Unless otherwise specified, all cell culture reagents were provided by Invitrogen/Gibco (Carlsbad, CA). Egg phosphatidylcholine and cardiolipin were purchased from Sigma Aldrich (St. Louis, MO). Glycerol tri[1-^{14}C]oleate was purchased from Amersham Pharmacia Biotech (Piscataway, NJ).

Animals

Male ICR mice (~30 g) were purchased from Harlan Industries (Indianapolis, IN). The animals were maintained in the Animal Care Facility on food (Wayne Lab Blox, Allied Mills, Inc., Chicago, IL) and water ad libitum for at least 5 days but no longer than 10 days prior to the experiments.

Isolation of subcellular fractions

Golgi apparatus-rich fractions were isolated from mouse liver by the method of Swift et al. (19). Mouse hepatic microsomes were isolated as described by Valyi-Nagy et al. (18).

Cell culture

McA cells were cultured in DMEM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 50 mg/ml glutamine, 10% (v/v) FBS, and 10% (v/v) horse serum in 100 mm dishes at 37°C at 5% CO_2. For immunofluorescence studies, the cells (~1–2 × 10^4 cells in ~200 μl of medium) were plated in 8-well chamber slides (Nunc Lab-Tek Chamber Slide System, Fisher Scientific, Norcross, GA), and maintained in the incubator for 48–72 h before fixation and immunocytochemical labeling.

Antibody to MTP

Antibody to MTP was generated by immunizing rabbits with a 20-amino acid peptide (KYERLSTGRGYVSRRRKESC), representing residues 843 through 861 in the MTP protein plus an additional cysteine residue at the C-terminus. The peptide was synthesized by Peptidogenics (Livermore, CA) and coupled to the carrier protein keyhole limpet hemocyanin (KLH) via the cysteine residue. An emulsion of the coupled peptide with the adjuvant TiterMax Gold (Sigma Aldrich) was injected intradermally at multiple sites on the shaved backs of two rabbits. The rabbits were boosted with peptide coupled to BSA intradermally and intramuscularly after 5 weeks. Blood was collected 8 days after the boost. The IgG fraction was isolated from serum using a protein-A column, and antibodies specific for the peptide were purified by passing the IgG fraction over a SulfoLink (Pierce, Rockford, IL) column-containing peptide, coupled according to the supplier's protocol. The concentration of the antibody was 0.66 μg/μl.

SDS-PAGE and immunoblotting

Samples were solubilized in a NuPAGE lithium dodecyl sulfate (LDS) sample buffer and separated by SDS-PAGE using NuPAGE bis-tris gels (4–12% gradients) (Invitrogen/Gibco) with morpholinepropanesulfonic acid SDS-running buffer (20). The proteins were transferred to nitrocellulose membranes. The membranes were blocked in TBS with 5% nonfat milk, incubated with an antibody to MTP for 2 h at room temperature, washed extensively, and incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit IgG (Promega, Madison, WI) or rabbit anti-goat IgG (BioRad Laboratories, Richmond, CA). Bands were visualized using enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ).

Immunoprecipitation studies

Aliquots of mouse hepatic microsomes (15 μg) or Golgi apparatus (50 μg) were solubilized in 20 mM HEPES (pH 7.4), 1.0 mM EGTA, 1% Triton X-100, and 10% glycerol on ice for 20 min. The sample was then centrifuged at 4°C for 5 min at 14,000 *g* in an Eppendorf microfuge. The supernatant was transferred to a new tube, anti-MTP was added, and the sample was mixed and incubated on ice for 2 h. Pansorbin (10 μl of a 10% aqueous suspension) was added, and the mixture was incubated at 4°C for 2 h on a rotator. The antibody complex was pelleted by centrifugation, and the pellets were washed a total of three times with 1 ml of warmed (37°C) wash buffer [0.15 M NaCl, 10 mM Tris (pH 8.5), 1% Triton X-100, 1% sodium deoxycholate (NaDOC), 0.1% SDS, and 1 mM EDTA]. After the third wash, the pellets were resuspended in LDS sample buffer and heated at 95°C for 5 min. The samples were cleared by centrifugation for 5 min in the microfuge at maximum speed, and the proteins were separated by SDS-PAGE and blotted to nitrocellulose as described above.

Lipid transport assay

Mouse hepatic-Golgi and microsomal content fractions were prepared as described by Wetterau and Zilversmit (4). One-tenth volume of NaDOC solution (0.56%, pH 7.5) was added to the subcellular fraction with vortexing. The suspension was incubated on ice for 30 min and centrifuged in the Beckman Coulter TLA-120.2 rotor at 120,000 rpm for 30 min. The supernatants were recovered, dialyzed against 15 mM Tris-HCl (pH 7.5), 40 mM NaCl, 1.0 mM EDTA, and 0.02% sodium azide (15/40 buffer), and aliquots were assayed for MTP activity.

Lipid transfer from donor to acceptor membranes was measured in an assay similar to that described by Jamil et al. (21). Donor and acceptor small, unilamellar vesicles were prepared by bath sonication (Laboratory Supply, Hicksville, NY) in 15/40 buffer. The vesicles were kept at 4°C for 16 h after sonication prior to transfer experiments.

The transfer assay mixture contained donor membranes [40 nmol of egg phosphatidyl choline (PC), 3 nmol cardiolipin, and 0.08 nmol glycerol tri[1-^{14}C]oleate], acceptor membranes (240 nmol of egg PC), and 5.0 mg BSA in 15/40 buffer (total volume 700 μl). The reaction was initiated by adding the content fraction (8–48 μg protein) from mouse hepatic-Golgi fractions, and the samples were incubated at 37°C for 1 h. The reaction was stopped by adding 0.5 ml of DE52 cellulose resin (Whatman, Inc., and Fisher Scientific) preequilibrated in 15 mM Tris-HCl (pH 7.4), 1.0 mM EDTA, and 0.02% sodium azide (1:1; v/v). The samples were mixed for 5 min, and the cellulose resin was pelleted by centrifugation in a bench-top centrifuge. Aliquots of the supernatant and the pellet were counted in the scintillation counter after the addition of Bio-Safe II (Research Products International Corp., Mount Prospect, IL). MTP-mediated transfer was determined by subtracting background transfer in the absence of MTP from total transfer. Mouse liver microsomal contents were used as a source of MTP for control experiments.

Immunohistochemistry

Mouse liver was fixed in 10% neutral buffered formalin for ~24 h. Liver from a mouse in which hepatic MTP expression had been eliminated by Cre-mediated induction of a conditional (floxed) MTP allele was kindly provided by Dr. Stephen G. Young of the Gladstone Foundation. The tissues were embedded in paraffin and 5 μm sections cut. The paraffin was removed with xylene, and the tissues were equilibrated in TBS. Antigen retrieval was performed by heating the tissues in 10 mM citrate (pH 6.0) for 30 min at 100°C. To block nonspecific binding, the slides were incubated for 1 h at room temperature in TBS containing 3% (w/v) BSA. This blocking agent was also used to dilute the primary antibodies. Incubation with the affinity-purified MTP antibody [1:1,000 (v/v) dilution of the affinity-purified IgG fraction, optical density = 0.85] was carried out in a humidified chamber overnight at 4°C. The slides were then rinsed and incubated for 1 h at room temperature with biotin-conjugated goat anti-rabbit IgG. The slides were rinsed and incubated at room temperature for 1 h with the anti-biotin antibody linked to alkaline phosphatase, followed by incubation with alkaline phosphatase substrate for 20–30 min. Tissues were counterstained with hematoxylin and mounted with an aqueous mounting medium (Serotec, Raleigh, NC).

Immunocytochemistry

Cells were washed three times with PBS and fixed with 4% paraformaldehyde for 15 min at room temperature. The cells were washed three times with an intracellular buffer [75 mM potassium acetate, 2.5 mM magnesium acetate, 1.8 mM calcium chloride, and 25 mM HEPES buffer (pH 7.2)] at room temperature and permeabilized with 0.1% saponin in intracellular buffer with 0.4% BSA for 30 min at room temperature. All subsequent steps, including the washes, were performed in an intracellular buffer containing 0.1% saponin and 0.4% BSA. The cells were incubated overnight with primary antibodies at 4°C and then rinsed three times for 10 min. The appropriate secondary antibodies were applied for 2 h at room temperature. After three washes of 10 min each, the cells were mounted with ProLong Antifade (Molecular Probes, Eugene, OR). Primary antibodies were used at the following dilutions: anti-MTP, 1:1000 (v/v); anti-TGN38, 1:100 (v/v); and anti-GS28, 1:100 (v/v). Fluorescent secondary antibodies were used at the following dilutions: Cy3-conjugated anti-rabbit IgG, 1:1,000 (v/v) and FITC-coupled anti-mouse IgG, 1:100 (v/v). In double-labeling experiments, the two primary antibodies were applied simultaneously, followed by a mixture of the two secondary antibodies. In control experiments, fluorescence was shown to be negligible in the absence of a primary antibody. The anti-mouse IgG and the anti-rabbit IgG secondary antibodies did not bind to the polyclonal and monoclonal primary antibodies, respectively. There was no bleed-through of the Cy3 fluorophore at 488 nm or FITC at 568 nm. In one set of studies, McA cells were incubated for 2 h with cycloheximide (10 μg/ml) prior to fixation, permeabilization, and incubation with antibodies. Cycloheximide at 10 μM (2.8 μg/ml) has been shown to block apoB synthesis in McA cells (22).

Laser confocal microscopy

Images were collected and analyzed on a Zeiss LSM 510 confocal laser scanning inverted microscope using either a 40×/1.30 or a 63×/1.40 Plan-Apochromat objective. The 488 line of an argon laser or the 543 line of an HeNe laser were used for excitation of fluorescein and Cy3-labeled secondary antibodies, respectively, and Z-series scans were collected. Projections of the Z-series data were generated using Zeiss LSM 510 Image Browser software. Colocalization of the two fluorophores was computed as a percent of a one-color pixel that overlapped with a separate-color pixel. Analysis of colocalization was done using MetaMorph 5.0 (Universal Imaging Corp., Downingtown, PA), with the threshold set to display the brightest 95% of positive pixels. For display, images were converted into TIFF format and processed using Adobe Photoshop Software (version 5.5).

Protein determination

Protein was determined by the bicinchoninic acid method (Pierce), modified to eliminate interference by lipid (23) and using BSA as a standard.

RESULTS

An antibody to the large subunit of MTP was generated by immunizing rabbits with a 20-amino acid peptide representing residues 843 through 861 plus a cysteine residue for coupling to the peptide KLH. The antibody recog-

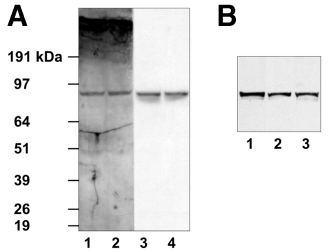

Fig. 1. A: Specificity of microsomal triglyceride transfer protein (MTP) antibody. Mouse liver microsomes (50 μg) were solubilized, separated by SDS-PAGE, and blotted to nitrocellulose. The blots were probed with antibodies to MTP as described in Experimental Procedures. Lanes 1, 2: antisera to MTP from Bristol-Myers Squibb diluted 1:1,500 (v/v); lanes 3, 4: MTP, our antibody diluted 1:20,000 (v/v) (33 ng/ml). B: Identification of MTP within mouse hepatic fractions. Golgi and microsomes were isolated from the mouse liver, and Golgi content fractions were prepared. Proteins were separated on 4–12% polyacrylamide gradient gels, blotted to nitrocellulose, and probed with an antibody to MTP [1:20,000 (v/v), 33 ng/ml]. Lane 1: microsomes, 30 μg protein; lane 2: intact Golgi, 50 μg; and lane 3: Golgi content fraction from 50 μg intact Golgi.

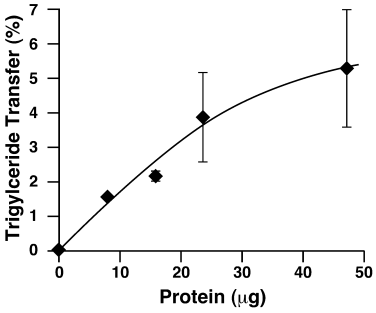

Fig. 2. Triglyceride transfer activity in mouse hepatic-Golgi content fractions. Hepatic-Golgi fractions were disrupted using sodium deoxycholate, and the membranes were pelleted by ultracentrifugation. The supernatant (Golgi content fraction) was used for the transfer assays. Results are expressed as a percentage of total [^{14}C]triglyceride transferred from donor to acceptor membranes as described in Experimental Procedures. Data represent the mean ± SD from three different Golgi preparations.

nized a 97 kDa protein in mouse hepatic microsomes that comigrated with a band recognized by an MTP antibody kindly provided by John Wetterau at Bristol Myers Squibb (**Fig. 1A**). The antibody did not recognize any other proteins even at dilutions as low as 1:3,000 (v/v).

Hepatic-Golgi apparatus-rich fractions from mice were probed for MTP after separation of the proteins by SDS-PAGE and transfer to a nitrocellulose membrane (Fig. 1B). MTP was identified in microsomes and intact Golgi apparatus fractions. Separation of the Golgi into membrane and content fractions demonstrated that the MTP was solely associated with the lumenal content fraction. Additional studies indicated that MTP was not associated with the VLDL ($d < 1.006$ g/ml) or d 1.006–1.210 g/ml fractions, but was present in the $d > 1.210$ g/ml fraction (data not shown). To probe for MTP activity within the Golgi apparatus, the lumenal contents from the Golgi fraction were recovered and the lipid transfer was measured. The results (**Fig. 2**) demonstrate the presence of triglyceride transfer activity within the Golgi fraction. Total transfer was ~6%, using 50 μg of Golgi content protein. The activity was heat sensitive and inhibited by an MTP inhibitor (BMS 200150). Using an equivalent amount of liver microsomal content protein, we found ~12–13% triglyceride transfer. NADPH cytochrome C reductase was not enriched in the Golgi fractions (19).

Sections of mouse liver were stained using the MTP antibody (**Fig. 3**). Immunoreactive product was prominent in all hepatocytes but was never observed in nonparenchymal cells (Fig. 3A). Interestingly, there seemed to be an accentuation of staining around the central veins within the tissue (Fig. 3B). No immunoreactivity was observed using the nonretained IgG fraction from the affinity column used to collect the specific IgG fraction. In sections from the liver-specific MTP knockout mouse, no immunoreactivity was observed in >95% of the hepatocytes (Fig. 3C, D); however, immunoreactivity was observed in the remaining cells. The positive cells were not randomly distributed throughout the section but were concentrated around the central veins. MTP-staining cells did not contain lipid droplets, as were observed in the MTP-negative cells (data not shown). Our observation that some hepatocytes showed positive staining for MTP is consistent with the fact that the gene is not inactivated in a small number of cells (24) and underscores the quality of our antibody.

To identify the intracellular locations of MTP, McA cells were probed with the antibody to MTP (**Fig. 4A**) and viewed with the confocal microscope. Fluorescence signals were seen throughout the cell. Although the pattern was not clearly reticulated, it was nevertheless consistent with a location within the ER. The signals obtained when probing the cells with antibody to TGN38 resulted in a typical Golgi pattern (Fig. 4B). The signals were concentrated in the juxtanuclear regions, consistent with patterns seen with other Golgi marker proteins (25). When the signals were superimposed, there was significant overlap, suggesting colocalization of the MTP with the TGN38 marker (Fig. 4C). For colocalization studies, the pinhole for detecting red fluorescence was set at one Airy unit that corresponded to an imaged depth of ~0.8 μM. The pinhole for detecting green fluorescence was set to image a similar depth. Thus, overlap of the fluorescence constituted colocalization in closely apposed if not identical regions. Not all of the MTP signals in the juxtanuclear regions overlapped with the Golgi marker, and it was clear that even within the Golgi regions, the MTP was not uniformly present. Morphometric analyses of several images revealed that 17.2% of the MTP (red) pixels colocalized with the TGN38 (green), and 33.0% of the TGN38 (green) pixels colocalized with the MTP (red) pixels (Fig. 4D). This suggests that 17% of the MTP was present in the *trans*-Golgi network and that 33% of the *trans*-Golgi network contained MTP. Three-dimensional reconstruction of the image sequences suggested patches of MTP fluorescence within regions of the Golgi rather than uniform distribution (data not shown).

Studies were also carried out using the antibody to MTP and the antibody to GS28, a marker for the *cis*-Golgi (**Fig. 5**). The signal for MTP represented primarily a pattern of localization within the ER (Fig. 5A), while the signals from the GS28 antibody were similar in appearance to those from the TGN38 antibody (Fig. 5B). When the images were superimposed, there was clear colocalization of the two signals (Fig. 5C). Analysis of the colocalization indicated 16.8% of the MTP (red) pixels colocalized with the GS28 (green), whereas 53.3% of the GS28 (green) pixels colocalized with the MTP (red) pixels (Fig. 5D). This suggests that 16.8% of the MTP was in the *cis*-Golgi and that 53.3% of the *cis*-Golgi contained MTP.

To determine whether MTP is carried to the Golgi apparatus with lipoprotein particles, we studied the localization of MTP in the *cis*-Golgi after blocking protein synthesis with cycloheximide. There was no difference in the degree of colocalization of MTP with GS28 in the cycloheximide-treated cells versus control cells. Approximately 19.0% of the MTP (red) pixels colocalized with the GS28 (green), whereas 51.0% of the GS28 (green) pixels colocalized with the MTP (red) pixels (data not shown). This indicates that in the absence of apoprotein synthesis, 19.0% of the MTP was in the *cis*-Golgi and 51.0% of the *cis*-Golgi contained MTP.

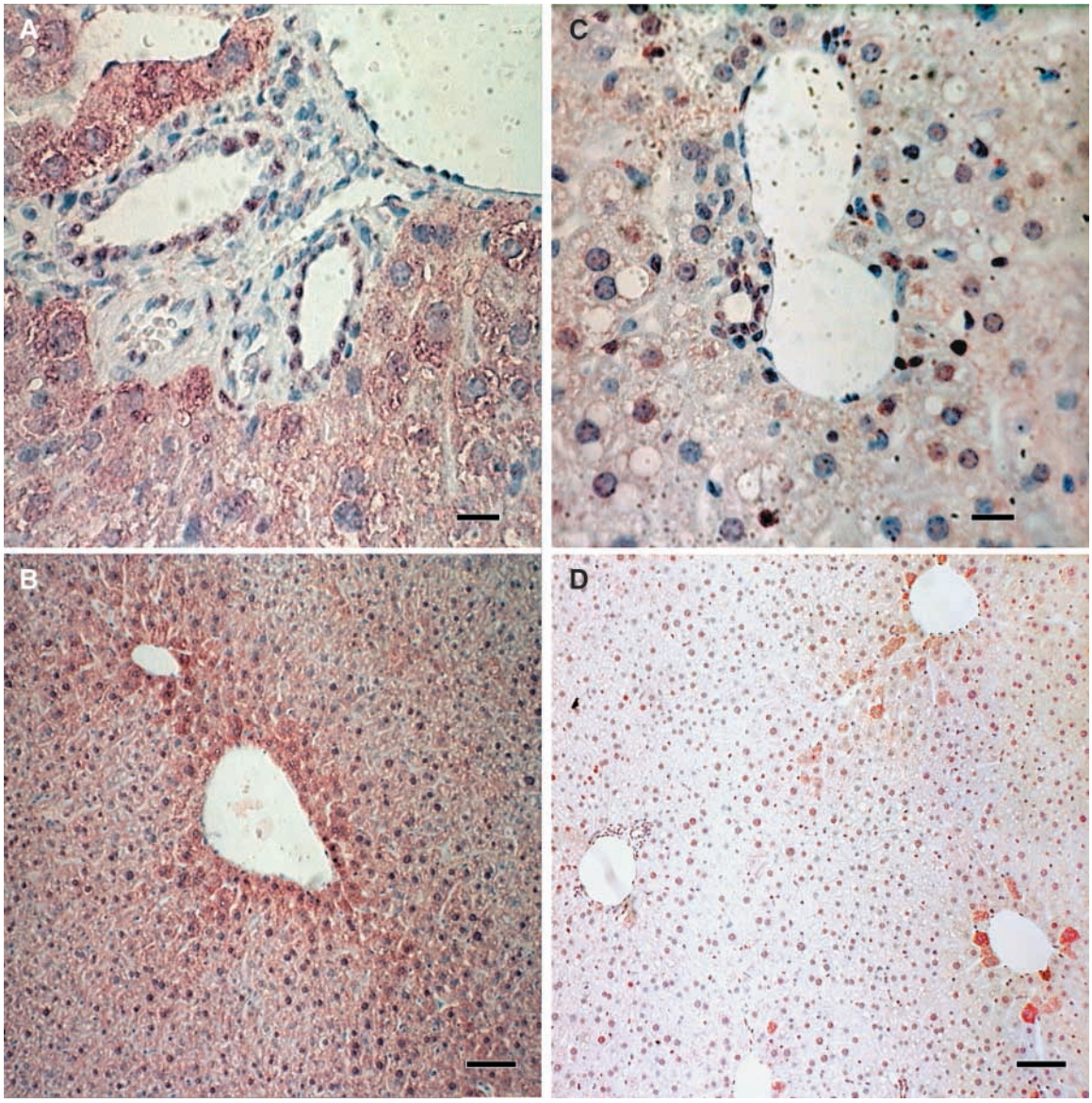

Fig. 3. Immunohistochemistry of MTP in wild-type and liver-specific MTP knockout mice. Mouse liver was fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained as described in Experimental Procedures. The section was lightly counterstained with hematoxylin to reveal cellular detail. The brown stain indicates the presence of immunoreactivity. A, B: wild-type liver. Note the absence of stain in nonparenchymal cells (A) and the prominence of staining around the central vein (B). C, D: liver from a liver-specific MTP knockout mouse. Some hepatocytes, located preferentially around the central vein, stained positively for MTP. The bars in A and C are 10 μm, while the bars in B and D are 40 μm.

Immunoprecipitation studies were carried out to determine whether MTP was associated with PDI in the Golgi apparatus. When Golgi and microsomal fractions were solubilized under nondenaturing conditions and immunoprecipitated with antibody to MTP, PDI was recovered with the immunoprecipitate (**Fig. 6**).

DISCUSSION

On the basis of previous studies in our laboratory (18, 19), we have hypothesized that lipid is added to nascent VLDL particles within the hepatic-Golgi apparatus in a MTP-dependent pathway. The present studies support this hypothesis by providing direct evidence for the presence of MTP within the Golgi. Using Western blots, we have identified MTP within Golgi apparatus-rich fractions of mouse liver (Fig. 1B) and demonstrated triglyceride transfer activity within the lumen of the Golgi (Fig. 2). Whereas our Golgi fractions are highly enriched in galactosyl transferase and exhibit little NADPH cytochrome C reductase activity, it is impossible to eliminate the possibility that slight contamination with ER could be the source of the MTP and the triglyceride transfer activity; however, using laser confocal microscopy we have shown colocalization of MTP with TGN38 and GS28 (Figs. 4, 5), which are accepted markers for the *trans*- and *cis*-Golgi, respectively (26, 27). Based on morphometric analyses, we estimate

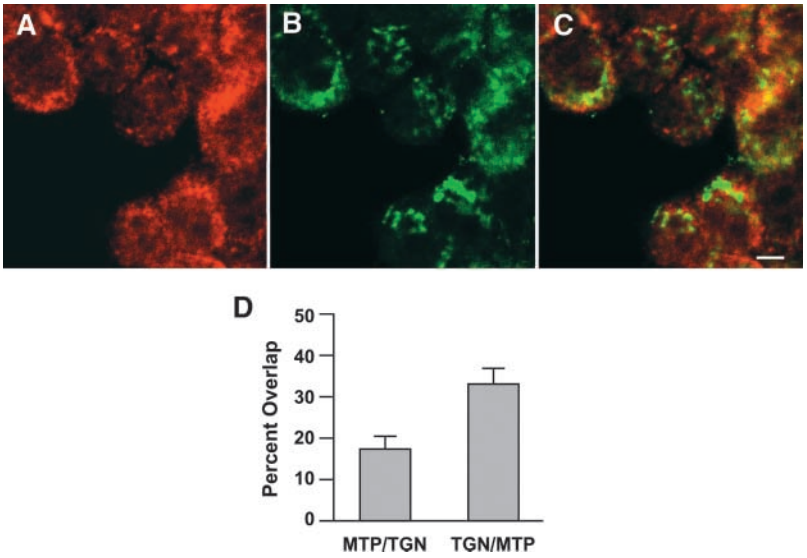

Fig. 4. Localization of MTP within the *trans*-Golgi apparatus in McArdle-RH7777 (McA) cells. Cells were probed simultaneously with rabbit anti-MTP (A) and mouse anti-*trans*-Golgi network (TGN) 38 (B), followed by anti-rabbit IgG conjugated with donkey (Cy3) and anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC). Colocalization is represented by yellow when images A and B are superimposed (C) (bar = 5 μm). D: Percent colocalization of MTP and TGN38 signals. MTP/TGN represents the percent of MTP signals that colocalize with TGN38 signals; TGN/MTP represents the percent of TGN38 signals that colocalize with MTP signals. Data represent mean ± SEM from analysis of five different images.

that ~17% of the MTP signal is colocalized with the *trans*-Golgi marker and 17% with the *cis*-Golgi marker. It is important to note that within a given cell, not all Golgi contain MTP. Only 33% of the *trans*-Golgi marker colocalized with MTP, whereas 50% of the *cis*-Golgi marker colocalized with MTP. This suggests that there may be functional differences between regions of the Golgi complex with regard to lipoprotein assembly. We also investigated the colocalization of MTP with calnexin, a membrane protein that is associated solely with the ER of the cell (28). As would be predicted, there was considerable colocalization of the signals from these two probes (data not shown). In addition, as a negative control, we studied the localization of calnexin and TGN38 and showed complete separation of the signals for these markers (data not shown).

The MTP antibody used for these studies was developed in our laboratories from a 19-amino acid peptide representing residues 843 through 861 in the MTP protein. This peptide was chosen because this region of the protein is highly conserved over several species and, based on the presence of charged amino acids, it is predicted to be strongly antigenic. The region was also selected because it is not involved in interactions with apoB (29) or PDI (30), both of which are known to associate with MTP and could potentially block recognition of the protein in tissues. The antibody was shown to be monospecific by Western blot analysis (Fig. 1A), and immunohistochemical studies in mouse liver revealed staining in hepatocytes but not in nonparenchymal cells (Fig. 3A, B). Interestingly, in the liver sections from the liver-specific knockout mouse, ~5% of the cells stained for MTP. The MTP-staining cells had little cytoplasmic lipid compared with the MTP-negative cells. Based on hepatic mRNA levels in these mice, Raabe et al. (24) reported that the Mx1-Cre transgene approach inactivates gene expression in >95% but not 100% of the hepatocytes. They also reported "occasional hepatocytes with few cytosolic fat droplets" (p. 1294 of Ref. 24). Our immunohistochemical studies using the MTP antibody are consistent with the careful observations of Raabe et al. (24), and provide additional evidence for the specificity and sensitivity of our antibody.

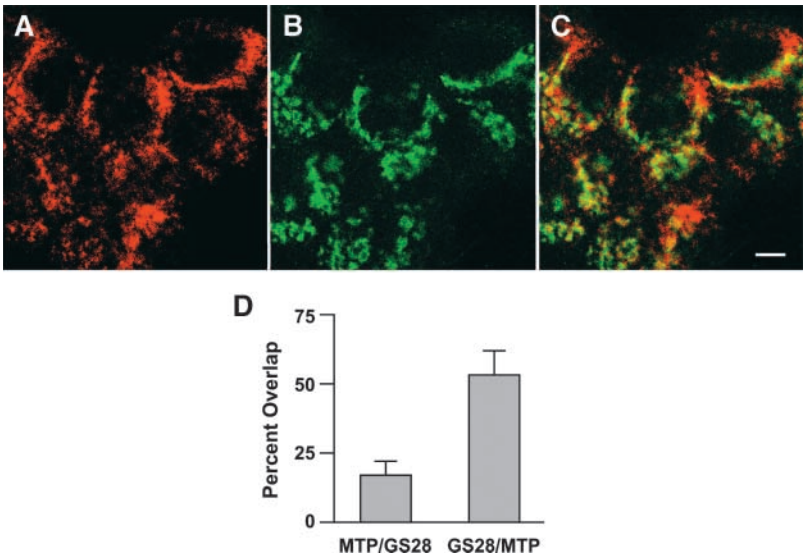

Fig. 5. Localization of MTP within the *cis*-Golgi apparatus in McA cells. Cells were probed with rabbit anti-MTP (A) and mouse anti-Golgi SNARE (soluble *N*-ethylmalemide-sensitive factor attachment protein receptor) of 28 kDa (GS28) (B), followed by anti-rabbit IgG conjugated with Cy3 and anti-mouse IgG conjugated with FITC. Colocalization is represented by yellow when images A and B are superimposed (C) (bar = 5 μm). D: Percent colocalization of MTP and GS28 signals. MTP/GS28 represents the percent of MTP signals that colocalize with GS28 signals; GS28/MTP represents the percent of GS28 signals that colocalize with MTP signals. Data represent mean ± SEM from analysis of six different images.

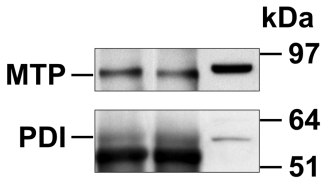

Fig. 6. Coimmunoprecipitation of protein disulfide isomerase (PDI) with MTP. Microsomal (15 μg) and Golgi (50 μg) fractions were solubilized in nondenaturing buffer and immunoprecipitated with MTP (lanes 1, 2). The immunoprecipitates and intact microsomes (3 μg, lane 3) were solubilized, separated by SDS-PAGE, blotted to nitrocellulose, and probed for MTP and PDI. The dark band at the bottom of the PDI blot is rabbit IgG.

These studies raise a number of questions. First, how does MTP get into the Golgi apparatus? Our studies suggest that it does not travel to the Golgi apparatus with lipoprotein particles, as the colocalization of MTP with Golgi markers in cycloheximide-treated cells was the same as in control cells. Treatment with cycloheximide at 10 μg/ml leads to a rapid cessation of apoB synthesis and lipoprotein assembly (22), and within 2 h the cell would be depleted of nascent lipoproteins. Immunoprecipitation studies suggest that MTP in the Golgi apparatus is associated with PDI, as PDI coimmunoprecipitates with MTP (Fig. 6). The ER-retention sequence (KDEL) on PDI should prevent the MTP/PDI complex from exiting the ER to the Golgi apparatus (31); however, other proteins with the ER retention signal have been shown to move to both *cis*- and *trans*-Golgi before retrieval to the ER, and under certain circumstances, proteins with ER-retention signals escape the ER completely (32). It is also possible that the KDEL sequence on PDI could be masked or that the sequence could be removed posttranslationally (33), allowing delivery of the MTP complex to another functional site (e.g., the Golgi apparatus). Dashti et al. (34) reported that McA cells transfected with truncated apoB-cDNA constructs secreted MTP with HDLs. They speculated that the binding of apoB to MTP displaces PDI, thus allowing MTP to be secreted. Additional evidence that MTP can escape the ER was provided by the immunogold electron microscopic studies of Levy and coworkers (35), who reported the presence of MTP/PDI complexes in the Golgi apparatus of enterocytes. These studies also indicate that the presence and role of MTP within the Golgi apparatus extends to both major sites of lipoprotein assembly.

A second question raised by these studies is why would triglyceride be added in the Golgi apparatus, or why doesn't MTP complete the addition of triglyceride in the ER? Certainly there is no clear answer to this question; however, some intriguing possibilities can be proposed. One possibility is that the Golgi apparatus ultimately controls the amount of triglyceride associated with a lipoprotein particle. To understand this, it is necessary to examine intracellular transport. The transport of secretory proteins from their site of synthesis in the rough ER to the Golgi apparatus occurs via 60–70 nm vesicles (36). If lipoproteins were transported in the same manner, then the size of the vesicle would limit the size of the particle that can be transported. For example, in a 60 nm vesicle, assuming the membrane is 9–10 nm, the inside diameter of the vesicle would be only 40 nm. This would barely be sufficient space for a nascent lipoprotein. A 40 nm lipoprotein might fit in an 80 nm vesicle, but it would require nearly all the space. It is not unusual to find 50 nm, 60 nm, and even 70 nm lipoproteins within the Golgi apparatus. Are these lipoproteins transported to the Golgi from the ER in some alternate transport pathway, or are they transported to the Golgi apparatus as smaller lipoproteins via the normal vesicular pathway, and additional triglyceride added in the Golgi? We believe the latter occurs. Thus, the size of the transport vesicles limits the size of the VLDL transported, and as apoB production is constitutive, it would necessarily limit the amount of triglyceride secreted with VLDL. If the Golgi apparatus has the ability to add triglyceride, then extra lipid could be added to a VLDL particle, increasing the mass of triglyceride exported from the cell.

The final question is: what does this finding mean with regard to the assembly of VLDL? The prevailing model for the assembly of VLDL by the liver consists of two steps (15). In the first step, small amounts of lipid (both neutral lipid and phospholipid) are added to apoB as the protein is translated and translocated across the rough ER membrane. This step produces a small HDL-like particle that is rich in protein and phospholipid and is relatively deficient in neutral core lipids. The major mass of core lipid is added in a second step that is visualized as a fusion between a protein-poor lipid droplet and the HDL-like particle. This model implies that VLDL assembly is completed within the ER, a conclusion supported by some studies (37).

A number of studies have suggested that the Golgi apparatus plays an active role in the assembly process (38–41).

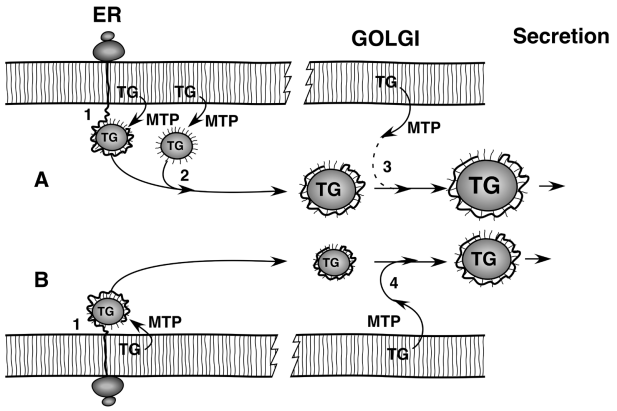

Fig. 7. Model of assembly of VLDL in the liver. A: Two-step assembly occurs in the endoplasmic reticulum (ER) as has been described (15), with further addition of triglyceride within the Golgi apparatus. This proposed third step utilizes Golgi membrane triglyceride in an MTP-dependent step, and may be of greater significance quantitatively when cellular triglyceride synthesis is stimulated. The third step is not absolutely necessary for the cell to secrete a VLDL particle. B: In this case, the second step does not occur in the ER, and the small, dense HDL particle is transported to the Golgi apparatus, where it can be converted to a VLDL particle via the MTP-dependent addition of triglyceride.

On the basis of studies of nascent hepatic lipoproteins, we estimated that as much as 50% of the triglyceride and perhaps 30% of the phospholipid are added to nascent VLDL in a post-ER compartment (17). We also demonstrated the presence of a large population of apoB-containing HDL particles in the Golgi apparatus of mouse liver (19); however, the major product secreted is VLDL, suggesting that there is a conversion of HDL-like particles to VLDL within the Golgi. Stillemark et al. (42) demonstrated that apoB-48 VLDL did not accumulate in the rough ER of McA cells, and suggested that completed apoB-48 VLDL was either rapidly transferred out of the ER or assembly steps occurred post-ER. Recently, Tran et al. (43) reported that assembly of apoB-100-containing VLDL in McA cells takes place in compartments at the distal end of the secretory pathway. They reported that the appearance of apoB-100 VLDL within the distal Golgi lumen occurred almost concurrently with the secretion of apoB-100 VLDL. In addition, they found an increase in the size of lipoprotein particles across the Golgi stack, suggesting lipid recruitment as the particles transit the Golgi apparatus. Certainly there is growing evidence that the Golgi plays an important role in the assembly of apoB-containing lipoproteins.

On the basis of our work and the studies of others, we propose a new model for the assembly of VLDL (**Fig. 7**). In our model, the first two steps in the assembly process are the same as has been proposed (15). We hypothesize that the product of the first two steps is a triglyceride-rich particle, 35–40 nm in diameter. These particles are transported from the ER to the Golgi apparatus by normal vesicle trafficking. Within the Golgi apparatus, additional triglyceride can be added to this particle in an MTP-dependent process. The source of this triglyceride is the Golgi membrane. This process, which we have termed the "third step," may be of greater significance quantitatively under conditions in which cellular triglyceride synthesis has been stimulated. In addition, HDL-like particles that do not undergo the second step addition of triglyceride within the ER may be converted to VLDL within the Golgi as suggested by our previous work (19). This new model suggests stepwise assembly of apoB-containing lipoproteins, and it emphasizes the critical role of the Golgi apparatus in this process.

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