Absence of Microsomal Triglyceride Transfer Protein in Individuals with Abetalipoproteinemia

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Abetalipoproteinemia is a human genetic disease that is characterized by a defect in the assembly or secretion of plasma very low density lipoproteins and chylomicrons. The microsomal triglyceride transfer protein (MTP), which is located in the lumen of microsomes isolated from the liver and intestine, has been proposed to function in lipoprotein assembly. MTP activity and the 88-kilodalton component of MTP were present in intestinal biopsy samples from eight control individuals but were absent in four abetalipoproteinemic subjects. This finding suggests that a defect in MTP is the basis for abetalipoproteinemia and that MTP is indeed required for lipoprotein assembly.

Abetalipoproteinemia is an autosomal-recessive disease that is characterized by a virtual absence of plasma lipoproteins that contain apolipoprotein B (apoB) and by low plasma concentrations of triglyceride (TG) and cholesterol (*1*). These abnormalities are the result of a genetic defect in the assembly or secretion of very low density lipoproteins (VLDLs) in the liver and of chylomicrons in the intestine, resulting in retinitis pigmentosa, spinocerebellar degeneration with ataxia, and a bleeding diathesis secondary to malabsorption of fat-soluble vitamins. The molecular basis for the primary defect in abetalipoproteinemia has not been determined. TG, phospholipid, and cholesterol synthesis are not impaired (*1*), and linkage between the apoB gene and abetalipoproteinemia has been excluded by restriction fragment length polymorphism (RFLP) analysis in several families (*2, 3*).

We investigated the possibility that a defect in the microsomal TG transfer protein (MTP) may be the proximal cause of abetalipoproteinemia. MTP is a soluble protein present in the lumen of microsomes isolated from liver and intestine (*4*). It mediates the transport of TG, cholesteryl ester, and phosphatidylcholine (PC) between membranes (*5*). The ability of MTP to transport TG between membranes, together with its tissue distribution and subcellular location, has led to the suggestion that MTP functions in the assembly of plasma lipoproteins (*4*).

MTP has been purified from bovine liver and characterized (*5*). It is a heterodimer of 58- and 88-kD peptides (*6*). Characterization of the 58-kD component indicated that it is the previously described multifunctional protein, protein disulfide isomerase (PDI) (*7*). The role of PDI in the transfer protein complex is not known. At a minimum, PDI appears to be necessary to maintain the structural integrity of the transfer protein (*8*), but a larger role cannot be excluded. Because PDI by itself does not have lipid transfer activity, the 88-kD subunit is either the active component or it confers transfer activity to the protein complex.

MTP activities in duodenal or duodenal-jejunal junction biopsy samples obtained from abetalipoproteinemic (*9*) and normal control subjects after an overnight fast were compared. Intestinal biopsy tissue was homogenized and treated with deoxycholate to release TG transfer activity from the microsomal fraction (*10*). The membrane fractions were removed by centrifugation, and TG transfer activity was measured in the supernatants. In biopsy samples from all five normal control subjects tested (Fig. 1A), TG transfer activity (*10*) was readily detectable. TG transfer activity was not detected (≤5% of the mean of the normal subjects) in the biopsy tissue from any of the four abetalipoproteinemic subjects (Fig. 1B and Table 1). To demonstrate that the lack of detectable TG transfer activity in individuals with abetalipoproteinemia was not related to an inability of deoxycholate to release MTP from the microsomes, we sonicated the microsomes from one abetalipoproteinemic subject for 5 min after detergent treatment. Sonication releases TG transfer activity with an efficiency comparable to that of detergent treatment. Even after sonication, no TG transfer activity was detected.

To demonstrate that the lack of detectable TG transfer activity in the abetalipoproteinemic individuals was not related to an inability to detect activity in cells that contain large intracellular fat droplets, as occur in abetalipoproteinemia, we measured TG transfer activity in biopsy samples from a subject with Anderson's disease (also referred to as chylomicron retention disease) (*11*) and a subject with homozygous hypobetalipoproteinemia (*12*). In these two genetic diseases, defects occur in the assembly or secretion of chylomicrons, and affected individuals have large fat droplets in their enterocytes, analogous to individuals with abetalipoproteinemia. In addition, TG transfer activity was measured in an intestinal biopsy sample taken from a normal subject who had not fasted before the biopsy. In all three individuals, TG transfer activity comparable to that of the control subjects was measured (Table 1), confirm-

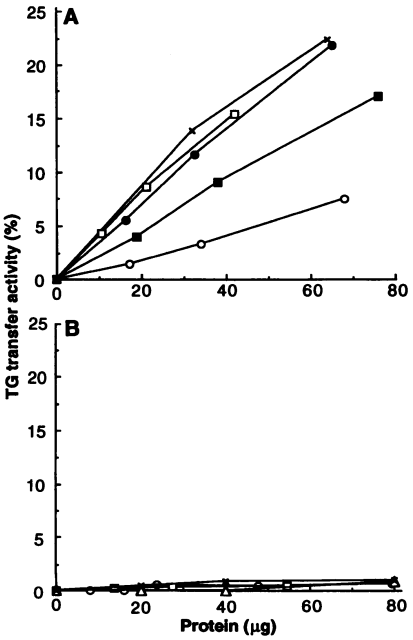

Fig. 1. TG transfer activity in (**A**) five normal and (**B**) four abetalipoproteinemic individuals. TG transfer activity was measured in homogenized intestinal biopsy samples. Results are expressed as the percentage of the total [^{14}C]TG transferred from donor to acceptor membranes in a 1-hour assay as a function of the amount of intestinal protein that was treated with deoxycholate to release TG transfer activity.

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ing that the presence of intracellular lipid droplets does not preclude the measurement of TG transfer activity.

Soluble proteins obtained after detergent treatment of the intestinal biopsy tissue homogenates were analyzed by protein immunoblotting (*13*) with antibodies to the 88-kD component of bovine MTP (*14*). The initial immunoblot analysis of two control subjects was performed with antibodies that had been affinity purified with bovine MTP. In both control subjects, a single band corresponding to the 88-kD component of bovine MTP was identified (Fig. 2A). To increase the probability of detecting small amounts of the 88-kD component of MTP (*15*), we used unfractionated antiserum in subsequent analyses, even though some cross-reactivity with other proteins was apparent. Bands comparable to that of the 88-kD component of bovine MTP were observed in all six control subjects examined (Fig. 2, B and C). In contrast, no protein corresponding to the 88-kD component was detected in the four abetalipoproteinemic subjects (Fig. 2D). A similar analysis was performed with the unfractionated intestinal biopsy tissue homogenates from two of the abetalipoproteinemic subjects. Again, no band corresponding to the 88-kD component of MTP was apparent. Two bands with mobilities intermediate between the 58- and 88-kD components of MTP were present in all six control and four abetalipoproteinemic subjects examined with unfractionated antiserum. Because these bands were not observed with affinity-purified antibodies (Fig. 2A), they have been attributed to contaminating antibodies that are specific for proteins other than MTP. As a control, immunoblot analysis with antibodies to PDI demonstrated the presence of the 58-kD component of MTP (PDI) in the two abetalipoproteinemic subjects tested (*16*).

Table 1. TG transfer activity in intestinal biopsy samples.

Subjects	Normalized TG transfer activity*
Normal controls (n = 5)	0.33 ± 0.16
Abetalipoproteinemia (n = 4)	0.011 ± 0.004
Anderson's disease (n = 1)	0.28
Homozygous hypobeta-lipoproteinemia (n = 1)	0.18
Nonfasted control (n = 1)	0.36

*The activity of a bovine MTP standard was measured each time an assay was performed. The TG transfer activity from each intestinal biopsy sample was divided by that of the standard MTP to normalize the activities between experiments. For the first two groups of subjects, TG transfer activity is the mean ± SD.

Our study suggests that MTP plays an obligatory role in the assembly of VLDL in the liver and chylomicrons in the intestine, probably by mediating the transport of lipid molecules from their site of synthesis in the endoplasmic reticulum (ER) membrane to nascent lipoprotein particles within the ER as they are assembled. This model for lipoprotein assembly is consistent with previous studies: Higgins and Hutson (*17*) showed that lipoproteins isolated from a rat liver Golgi fraction were consistently larger than those isolated from an ER fraction, which suggests the addition or transfer of lipid molecules to the nascent particles. The progressive addition of lipid to a developing lipoprotein particle was also demonstrated in the pulse-chase studies of Janero and Lane (*18*) and Boström *et al.* (*19*).

The absence of the 88-kD component of MTP in individuals with abetalipoproteinemia could be attributable either to its down-regulation to a nondetectable level or to a genetic defect in MTP or a factor that controls MTP concentration. Although MTP could be down-regulated in response to the cells not secreting lipoproteins, this explanation is unlikely because MTP concentrations were normal in the subjects with Anderson's disease or homozygous hypobetalipoproteinemia, diseases in which enterocytes do not secrete lipoproteins. Given that all other known aspects of lipoprotein synthesis and assembly—the expression of a normal apoB gene (*2, 3, 20*), as well as TG, phospholipid, and cholesterol synthesis—are not impaired in abetalipoproteinemic subjects, it is likely that the proximal cause of abetalipoproteinemia is a genetic defect in the 88-kD component of MTP or in the regulation of its synthesis or degradation.

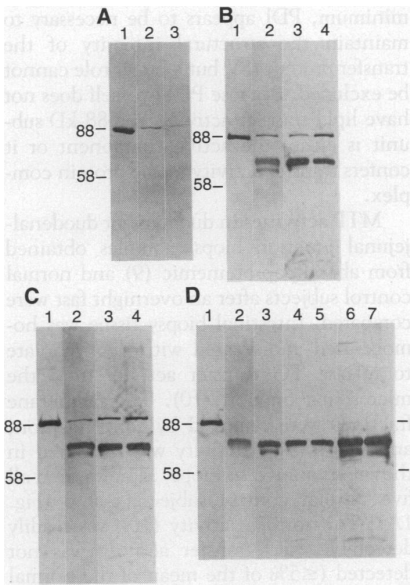

Fig. 2. Immunoblot analysis of MTP. Aliquots of purified bovine MTP (lane 1 of all four panels) or the 103,000*g* supernatant after treatment of homogenized intestinal biopsy samples with deoxycholate were subjected to SDS–polyacrylamide gel electrophoresis and immunoblotting with either antiserum or affinity-purified antibodies to the 88-kD subunit of MTP. (**A**) Lanes 2 and 3, soluble protein corresponding to 34 and 25 μg of homogenate protein, respectively, from two normal subjects, probed with affinity-purified antibodies. (**B**) Lanes 2 to 4, soluble protein corresponding to 23 μg of homogenate protein from three additional normal subjects, probed with unfractionated antiserum. (**C**) Lane 2, soluble protein corresponding to 15 μg of homogenate protein from a subject with Anderson's disease; lane 3, soluble protein corresponding to 25 μg of homogenate protein from an individual with homozygous hypobetalipoproteinemia; lane 4, soluble protein corresponding to 25 μg of homogenate protein from a nonfasted normal individual. Samples were probed with unfractionated antiserum. (**D**) Lanes 2 to 5, soluble protein corresponding to 18 μg (lane 2) and 23 μg (lanes 3 to 5) of homogenate protein from four abetalipoproteinemic subjects, probed with unfractionated antiserum. Lanes 6 and 7, 100 μg of unfractionated intestinal homogenate protein (from abetalipoproteinemic subjects corresponding to lanes 4 and 5) were subjected to electrophoresis and immunoblotting with unfractionated antiserum. The mobilities of the 58- and 88-kD components of bovine MTP are indicated. The figure represents a composite from several independent immunoblots.

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9. Of the four abetalipoproteinemic subjects, two were female (ages 14 and 39 years) and two were male (ages 3 and 49 years). All had healthy, normolipidemic parents. The subjects had plasma cholesterol concentrations of between 24 and 58 mg/dl and no detectable plasma apoB. Their current therapy includes low-fat diets and lipid-soluble vitamin supplements. The two adult patients were the subject of a previously described study (*20*).
10. TG transfer activity was measured by a modification of a previously described method (*4*). Before performing intestinal biopsies, we explained the nature of the study and its possible consequences to the subjects or their guardians and obtained informed consent. In some instances biopsy samples used in this study were part of material obtained for diagnostic purposes. Intestinal biopsy samples were frozen and stored at −70°C until analyzed. Biopsy tissue was homogenized in a polytron homogenizer (Polytron PT3000, Brinkmann) at half-maximal setting. Typically, one sample was homogenized in 0.25 ml of homogenization buffer [50 mM tris (pH 7.4), 50 mM KCl, 5mM

EDTA, leupeptin (5 μg/ml), and 2 mM phenylmethylsulfonyl fluoride]. A portion of the homogenate was diluted to 0.6 ml and adjusted to 1.4% SDS before measurement of the protein concentration [O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951)]. The homogenate was then diluted with homogenization buffer to a protein concentration of ~1.75 mg/ml. One part deoxycholate solution (0.56%, pH 7.5) was added to 10 parts diluted homogenate while mixing. Each sample was incubated at 4°C for 30 min and then centrifuged at 103,000*g* for 60 min. The supernatant was removed, diluted 1:1 with 15/40 buffer [15 mm tris (pH 7.4), 40 mM NaCl, 1 mM EDTA, and 0.02% NaN_3], and then dialyzed overnight against 15/40 buffer at 4°C. Portions of the dialyzed supernatant were assayed for TG transfer activity, and immunoblot analysis was performed to detect the 88-kD MTP subunit. TG transfer activity was measured as the protein-stimulated rate of TG transfer from donor small unilamellar vesicles (SUVs) to acceptor SUVs. Vesicles of the desired composition were prepared by bath sonication in 15/40 buffer as described previously (*5*). The donor and acceptor PC was labeled by adding traces of [^{3}H]dipalmitoyl PC {phosphatidylcholine L-alpha-dipalmitoyl-[2-palmitoyl-9,10,^{3}H(*N*)]; 33 Ci/mmol; DuPont Biotechnology Systems} to a specific activity of ~100 cpm/nmol. Donor vesicles containing 40 nmol of egg PC, 0.2 mole percent [^{14}C]triolein {triolein [carboxyl-^{14}C]; ~110 Ci/mol; DuPont Biotechnology Systems}, and 7.3 mole percent bovine heart cardiolipin (Sigma) were mixed with acceptor vesicles containing 240 nmol of egg PC and 0.2 mol percent unlabeled TG, 5 mg of fatty acid–free bovine serum albumin, and a portion of the MTP samples in 0.9 ml of 15/40 buffer, and the mixture was incubated for 1 hour at 37°C. The transfer reaction was terminated by the addition of 0.5 ml of a DEAE-cellulose suspension (*5*) and low-speed centrifugation to sediment selectively the donor vesicles containing the negatively charged cardiolipin. The measured amounts of [^{14}C]TG (transferred from donor to acceptor SUVs) and [^{3}H]PC (marker of acceptor SUV recovery) were used to calculate the percentage TG transfer from donor to acceptor SUVs. First-order kinetics were used to calculate the total TG transfer (*5*). To calculate the protein-stimulated rate of TG transfer, the rate of TG transfer in the absence of transfer protein was subtracted from that in the presence of MTP. To confirm that TG hydrolysis was not interfering with our ability to measure lipid transfer, after the assay of two subjects we extracted the acceptor vesicle lipid (which contained the transported lipid) and confirmed the identity of the TG by thin-layer chromatography. All the ^{14}C had a mobility identical to that of authentic TG, confirming that intact TG was transported in the assays. In addition, the human MTP was characterized for its heat stability. MTP was inactivated when heated to 60°C for 5 min. The loss of activity demonstrates that transfer activity attributable to an intracellular form of the cholesteryl ester transfer protein, which is stable at 60°C [J. Ihm, J. L. Ellsworth, B. Chataing, J. A. K. Harmony, *J. Biol. Chem.* **257**, 4818 (1982)], was not being measured.

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12. The clinical description and relevant data for patient C.D. with homozygous hypobetalipoproteinemia are presented elsewhere [G. Gay *et al.*, *Rev. Med. Interne* **11**, 273 (1990); J.-Y. Scoazec *et al.*, *Gut* **33**, 414 (1992)].
13. To identify the 88-kD component of MTP in tissue homogenates, we fractionated aliquots of proteins to be tested by SDS–polyacrylamide gel electrophoresis and then transferred the separated proteins to nitrocellulose with a Bio-Rad Trans-blot cell. After incubation with a nonfat milk solution, the nitrocellulose filter was incubated overnight at room temperature with an aliquot of antiserum to the 88-kD protein (1:300 dilution) or affinity-purified antibodies (1:25 dilution). Immunoreactive proteins were visualized with horseradish peroxidase–coupled goat antibodies to rabbit immunoglobulin G (Bio-Rad) and a standard developing solution.
14. The production and characterization of the antiserum to the 88-kD protein have been previously described (*7*). The antiserum immunoprecipitates MTP protein and activity, but direct inhibition of MTP activity has not been demonstrated. Affinity-purified antibodies were prepared as follows: Purified MTP (8 to 10 mg) was coupled to 4 ml of Bio-Rad Affigel 15. Antibodies were partially purified from the antiserum by $(NH_4)_2SO_4$ precipitation [226 mg of $(NH_4)_2SO_4$ per milliliter of serum]. After centrifugation, the pellet was suspended and applied to the MTP-affigel. The column was washed with 100 ml of 10 mM tris (pH 7.5), followed by 100 ml of 10 mM tris (pH 7.5) containing 500 mM NaCl. Antibodies were eluted with 50 ml of 100 mM glycine (pH 2.5) into 5 ml of 1 M tris (pH 8.0).
15. With unfractionated antiserum, the 88-kD band of MTP was detectable in the soluble proteins released from <3 μg of intestinal homogenate protein of three of four control subjects investigated. In the fourth subject, who had the lowest level of TG transfer activity of the controls, the 88-kD component of MTP was detectable in the soluble proteins from 10 μg of intestinal homogenate protein.
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21. We thank F. Maurer Chagrin (Hôpital Bichat, Paris, France) for providing biopsy samples from normal subjects, N. Verthier for technical assistance, and the University of Cincinnati for the antiserum to the 88-kD component of MTP. Supported by the Bristol-Myers Squibb Pharmaceutical Research Institute, the Centre National de la Recherche Scientifique, the Institut National de la Santé et Recherche Médicale (grant CRE 910301 and a France–U.S. cooperation grant), A.R.C.O.L. (the Committee for the Coordination of Research on Cholesterol in France), and NIH grant HL18577 (to L.P.A.).

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Tyrosine Phosphorylation of CD22 During B Cell Activation

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Ligation of the antigen receptor on B cells induces the rapid phosphorylation of tyrosine on a number of cellular proteins. A monoclonal antibody that recognized a tyrosine-phosphorylated cell surface protein that was present in activated B cells was generated. Amino acid sequence analysis showed that this 140-kilodalton protein was CD22, a B cell–specific cell surface glycoprotein and putative extracellular ligand of the protein tyrosine phosphatase CD45. Tyrosine phosphorylation of CD22 may be important in B cell signal transduction, possibly through regulation of the adhesiveness of activated B cells.

The B lymphocyte antigen receptor complex consists of membrane immunoglobulin (Ig), at least two accessory molecules (Ig-α and Ig-β) (*1*), several members of the Src family of protein tyrosine kinases (*2*, *3*), and a 72-kD protein tyrosine kinase that may be encoded by the *syk* gene (*4*, *5*). Cross-linking of surface Ig induces rapid increases in both tyrosine protein phosphorylation (6–8) and inositol phospholipid hydrolysis (9). Evidence suggests that the increased inositol phospholipid hydrolysis is induced, at least in part, by tyrosine phosphorylation. (i) Phospholipase C-γ, which is regulated by tyrosine phosphorylation in fibroblasts (*10*), is phosphorylated on tyrosine during B cell activation (*11*). (ii) The increase in free intracellular Ca^{2+} that results from inositol phospholipid hydrolysis is prevented by treatment of B cells with herbimycin, an inhibitor of tyrosine protein phosphorylation (*12*). (iii) Expression of the protein tyrosine phosphatase CD45 is required for the stimulation of phosphatidylinositol hydrolysis in a murine plasmacytoma (*13*).

Protein tyrosine phosphorylation may in fact represent the trigger or initial intracellular biochemical signaling event induced by the ligation of surface Ig. It is not clear how ligation of this receptor complex induces increased substrate phosphorylation, but it is likely that the Src-family kinases or the 72-kD kinase plays a role.

Phospholipase C-γ is not the only protein to undergo rapid tyrosine phosphorylation after cross-linking of surface Ig with antibody. Approximately ten newly phosphorylated proteins can be detected by immunoblotting of total cell lysates with antibodies to phosphotyrosine (6–8), including the *vav* proto-oncogene product (*14*), the 72-kD cytosolic protein tyrosine kinase (*4*, *6*), the 42-kD mitogen-activated (MAP)/

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