Microsomal triglyceride transfer protein expression in adipocytes: A new component in fat metabolism

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Received 10 February 2005; revised 29 April 2005; accepted 9 May 2005

Available online 23 May 2005

Edited by Felix Weiland

Abstract Microsomal triglyceride transfer protein (MTP) is a carrier of triglyceride essential for the assembly of apolipoprotein (apo)B-containing lipoproteins by the liver and the small intestine. Its role in triglyceride transfer in tissues that do not secrete lipoproteins has not been explored. In particular, MTP would seem to be a candidate for a role in triglyceride metabolism within the adipocyte. To test this hypothesis, we probed adipocytes for the presence of MTP. Immunohistochemical and biochemical studies demonstrate MTP in adipocytes from brown and white fat depots of mice and human, as well as in 3T3-L1 cells. Confocal microscopy revealed MTP throughout 3T3 cells; however, MTP fluorescence was prominent in juxtanuclear areas. In differentiated 3T3 cells MTP fluorescence was very striking around lipid droplets. In vitro lipid transfer assays demonstrated the presence of triglyceride transfer activity within microsomal fractions isolated from rat adipose tissue. In addition, quantitative rtPCR studies showed that MTP expression in mouse white fat depots was approximately 1% of MTP expression in mouse liver. MTP mRNA in differentiated 3T3 cells was approximately 13% of liver expression. Our results provide unequivocal evidence for the presence of MTP in adipocytes and present new possibilities for defining the mechanisms by which triglyceride is stored and/or hydrolyzed and mobilized.

Keywords: Lipid transport; Immunohistochemistry; Differentiation; Golgi apparatus; Endoplasmic reticulum; Confocal microscopy; 3T3-L1 cells

1. Introduction

Fatty acids, stored as triglycerides within lipid droplets in adipocytes, constitute the primary energy reserve in mammals. These lipid droplets are composed of a core of triglyceride surrounded by a limiting osmiophilic boundary generally thought to be a phospholipid monolayer. A number of proteins have been found to be associated with this monolayer [1]. The accumulation of triglyceride within the droplet is determined by the balance between fat synthesis (lipogenesis) and breakdown (lipolysis). While both processes have been the subject of much investigation, neither process is completely understood.

Lipogenesis is under hormonal and nutritional control [2], and these effects are mediated primarily through sterol regulatory binding protein [3–5]. While studies have led to a better understanding of how this process is regulated, little, if anything, is known about how newly synthesized triglyceride is transported from its site of synthesis to the fat droplet. Lipolysis is mediated primarily through hormone sensitive lipase (HSL) and controlled by cAMP-dependent protein kinase/protein kinase A (PKA) [6]. Hormonal stimulation of the cell leads to the phosphorylation of HSL [7,8] and perilipin [9]. Phosphorylation of perilipin is believed to lead to rearrangement of this protein on the surface of the lipid droplet allowing HSL access to the triglyceride [10], whereas, phosphorylation of HSL leads to translocation of the enzyme from the cytosol to the lipid droplet [11,12]. Together, these processes lead to a 50–100 fold elevation in lipolytic activity and mobilization of fatty acid. However, while HSL and perilipin constitute the core of the lipolytic reaction, it is not clear that all the factors in the lipolytic response have been identified. Zimmerman et al. [13] recently reported a second triglyceride lipase (adipose triglyceride lipase, ATGL) in mammalian adipose tissue that catalyzes the initial step in triglyceride hydrolysis. In addition, perilipin is not the only protein surrounding the lipid droplet, and its role in facilitating lipolysis has not been clearly defined. Other, yet to be identified proteins, may play key roles not only in lipogenesis and lipid transport but also in lipolysis.

Our laboratory has been studying the role of microsomal triglyceride transfer protein (MTP) in the assembly of lipoproteins by the liver. MTP is a heterodimeric protein consisting of the active 97 kD monomer linked with protein disulfide isomerase [14]. It facilitates the transfer of triglyceride, cholesterol ester, and phospholipid between synthetic membranes and is essential for assembly of apolipoprotein B (apoB)-containing lipoproteins by the liver and the small intestine [15]. In addition to the hepatocyte and the enterocyte, MTP has been shown to be expressed in the yolk sac [16,17], cardiac myocytes [18–20], testis [21], ovary [21], and kidney [16,21].

The established role of MTP as a triglyceride transfer protein makes it a candidate for a role in triglyceride metabolism within the adipocyte. Thus, we probed fat depots for the presence of MTP. In this paper we demonstrate the presence of MTP in adipocytes in brown and white adipose tissue as well as in 3T3-L1 cells. Our studies also demonstrate that adipocyte

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Abbreviations: MTP, microsomal triglyceride transfer protein; HSL, hormone sensitive lipase; apoB, apolipoprotein B; VLDL, very low density lipoproteins; CM, chylomicrons
MTP is functional. In addition, confocal analysis reveals the presence of MTP with lipid droplets in differentiated 3T3 cells. Our studies clearly demonstrate the presence of MTP in adipocytes, providing new possibilities for understanding the mechanisms by which triglyceride is stored and/or hydrolyzed and mobilized.

2. Materials and methods

2.1. Antibodies and reagents

Rabbit anti-MTP was developed in our laboratory and has been described previously [22]. Horseradish peroxidase conjugated rat anti-rabbit IgG was purchased from Promega (Madison, WI). 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). Donkey (Cy3)-conjugated AffiniPure Cy3 anti-rabbit IgG was purchased from Jackson Immuno-Research Laboratories. Rabbit anti β-actin antibody was purchased from Abcam, Inc. (Cambridge, MA).

2.2. Animals

C57BL/6 mice (4–8 weeks old) were purchased from Jackson Laboratories (Bar Harbor, ME). The animals were maintained in the Animal Care facility with a 12-h light/12-h dark cycle and were fed a mouse-chow diet (R50515, PMI Feeds Inc., St. Louis, MO, USA). Food and water were available ad libitum. All animal procedures were carried out in accordance with institutional guidelines with approval from the Animal Care Committee of Vanderbilt University.

2.3. Preparation of fat tissue extracts

Mice were anesthetized with a ketamine/xylazine cocktail. A midline incision was made and the mouse was killed by exsanguination. Fat depots were removed and homogenized for 10–15 s in 5 volumes of hexane/isopropanol (3/2 v/v) using a Polytron S-10 (Brinkman Instruments, Westbury, NY) equipped with a PTA 7K1 generator. The homogenates were allowed to stand at 4°C for 30 min. Proteins were pelleted by centrifugation at 4000 × g for 10 min. The supernatant was recovered, and protein concentration was determined by the BCA method and the contents were equilibrated in tris-buffered saline (TBS). Antigen retrieval was performed by heating in 10 mM citrate (pH 6.0) for 10 min at 100°C. To block nonspecific binding, the slides were incubated for 1 h at room temperature in TBS containing 3% (w/v) bovine serum albumin (BSA) and 1% normal goat serum. This blocking agent was also used to dilute the primary antibodies. Incubation with the affinity-purified MTP antibody (1:1000 dilution of the affinity purified IgG fraction; O.D. = 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 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 aqueous mounting medium (Sereote, Raleigh, NC).

2.4. Cell culture

3T3-L1 cells were obtained from American Type Culture Collection (Rockville, MD) and were maintained in DMEM supplemented with 10% fetal bovine serum, 4 mM glutamine, 4500 mg/L glucose, 1 mM sodium pyruvate, and 1500 mg/L sodium bicarbonate. To maintain cells in a pre-differentiated state, they were not allowed to reach confluence. Differentiation of 3T3-L1 cells into adipocytes was initiated by the addition of 10 μM dexamethasone, 0.5 mM isobutyl methyloxanthine, and 10 μg/ml insulin to confluent cells for two days. The cells were then cultured in medium containing 10 μg/ml insulin for two days after which they were maintained in complete media. The media was changed every two days until differentiation was complete (usually by day 8).

3T3-L1 cells were solubilized in 20 mM HEPES (pH 7.4), 1 mM EGTA, 1% Triton X-100, and 10% glycerol on ice for 20 min. The extracts were then centrifuged at 4°C for 5 min at 14000 × g in an Eppendorf microfuge. The supernatant was recovered, and protein concentration was determined by the BCA procedure. Aliquots were taken for SDS-PAGE as described below.

2.5. Immunohistochemistry

Fat tissue recovered from mice as described above was fixed in 10% neutral buffered formalin for ~24 h. The tissues were embedded in paraffin and 5 μm sections cut. The paraffin was removed with xylene, and the tissues were equilibrated in tris-buffered saline (TBS). Antigen retrieval was performed by heating in 10 mM citrate (pH 6.0) for 10 min at 100°C. To block nonspecific binding, the slides were incubated for 1 h at room temperature in TBS containing 3% (w/v) bovine serum albumin (BSA) and 1% normal goat serum. This blocking agent was also used to dilute the primary antibodies. Incubation with the affinity-purified MTP antibody (1:1000 dilution of the affinity purified IgG fraction, O.D. = 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 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 aqueous mounting medium (Sereote, Raleigh, NC).

2.6. Immunocytochemistry

3T3-L1 cells were grown in 8-well chamber slides (Nunc Lab-Tek Chamber Slide System, Fisher Scientific, Norcross, GA) and induced to differentiate as described above. Cells were washed three times with phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde for 15 min at room temperature. The cells were washed three times with intracellular buffer (75 mM potassium acetate, 2.5 mM magnesium acetate, 1.8 mM calcium chloride, 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 antibody incubations and washes, were performed in intracellular buffer containing 0.1% saponin and 0.1% BSA. The cells were incubated overnight at 4°C with affinity-purified MTP (1:1000 dilution), washed three times for 10 min each, and incubated with Cy3-conjugated anti-rabbit IgG (1:1000 dilution) for 2 h at room temperature. After three washes of 10 min each, the cells were mounted with ProLong Antifade (Molecular Probes, Eugene, OR).

2.7. Confocal microscopy

Images were collected and analyzed on a Zeiss LSM 510 confocal laser scanning inverted microscope using a 40 × 1.4 Plan-achromatic objective. The 543 line of a HeNe laser was used for excitation of the Cy3 labeled secondary antibody. For display, images were converted into TIFF format and processed using Adobe Photoshop Software (version 7.0).

2.8. Isolation of fat tissue microsomes

Rats were anesthetized with ketamine/xylazine and killed by exsanguination. Fat depots (inguinal, perirenal, and visceral) were removed and placed in four volumes of homogenizing buffer (4.0 ml/g) consisting of 0.1 M phosphate buffer, pH 7.35, 0.25 M sucrose, 1% dextran, and 0.01 M MgCl2. The samples were homogenized using the Polytron homogenizer at the lowest speed for 30–40 s and spun at 2500 × g for 15 min. The floating fat cake was removed, and the infranatant was carefully removed from the pellet and centrifuged in an SW41 rotor at 100000 × g for 60 min. The pellets were recovered, resuspended in phosphate buffered saline, and pelleted in the SW41 rotor under the same conditions. The washed microsomal pellet was resuspended in 50 mM Tris, pH 7.4, containing 50 mM KC1 and 5 mM MgCl2 to a protein concentration of 3.5 mg/ml. Microsomal luminal content fractions were prepared as described by Wetterau and Zilversmit [25]. Briefly, deoxycholate (1% solution) was added to the sample to a final concentration of 0.054%, and the samples were incubated on ice for 30 min. The membranes were pelleted in a TLA-100.4 rotor at 100000 rpm for 30 min in a Beckman TLS centrifuge. The supernatant, containing the microsomal content fraction, was dialyzed against 15 mM Tris, pH 7.4, 40 mM NaCl, 1 mM EDTA, 0.02% NaN3 (15/40 buffer) overnight with 3–4 changes of the buffer. Protein content was determined by the BCA method, and the contents were concentrated using UltraFuge filters (MSI, Westboro, MA). Hepatic microsomal content fractions were prepared in a similar manner but were not concentrated.
2.9. Triglyceride transfer assay

Triglyceride transfer from donor to acceptor membranes was measured in an assay similar to that described by Jamil et al. [26]. Small, unilamellar vesicles (donors and acceptors) 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 vesicles (40 nmol egg phosphatidycholine (PC), 3 nmol cardiolipin, and 0.08 nmol glycerol tri-[1-3H]oleate (~7300 dpm)), acceptor vesicles (240 nmol egg PC), and 5.0 mg BSA in 15/40 buffer (total volume 500 μl). The reaction was initiated by adding aliquots of the microsomal content fractions, and the samples were incubated at 37°C for 1 h. Typically 50 μg of liver microsomal content protein was added to the reaction. For fat microsomal contents we added aliquots equivalent to 325–500 μg of the unconcentrated content fraction. The reaction was stopped by adding 0.5 ml of DE52 cellulose resin (Whatman, Inc., and Fisher Scientific) pre-equilibrated in 15/40 buffer (1:1 v/v). The samples were mixed for 5 min, and the cellulose resin was pelleted by centrifugation. 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 (~260 dpm) from the total transfer and was expressed as pmol triglyceride transferred/mg protein/h.

Mice liver microsomal contents were used as a source of MTP for control experiments.

2.10. Quantitative RT-PCR

Total RNA was isolated from brown and white fat tissue from C57BL/6 mice and differentiated 3T3 cells using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instruction. Relarative quantification of MTP mRNA was performed using a FAM-labeled Taqman probe (Mn 00438015_m1) with the TaqMan One-Step RT-PCR Master Mix reagent kit (Applied Biosystems, ABI, Foster City, CA) on an ABI Prism 7700 sequence detection system according to the method developed by Su et al. [27]. The relative quantities of MTP message were normalized with 18S ribosomal RNA as an internal control. MTP mRNA expression levels in different tissues were expressed relative to the expression in liver from a C57BL/6 mouse. The data were analyzed using the comparative CT method [28] and were confirmed by the standard curve method.

2.11. SDS–polyacrylamide gel electrophoresis and immunoblotting

Samples were solubilized in NuPAGE LDS sample buffer and separated by SDS–PAGE using NuPAGE bis-tris gels (4–12% gradients) (Invitrogen) with morpholinepropanesulfonic acid SDS running buffer [29]. The proteins were transferred to nitrocellulose membranes. The membranes were blocked in TBS with 5% non-fat milk, incubated overnight at 4°C with antibody to MTP or β-actin (as a load control), washed extensively, and incubated for 1 h at room temperature with the appropriate secondary antibody conjugated with horseradish peroxidase. Bands were visualized using enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ).

3. Results

3.1. Immunohistochemistry studies

Immunohistochemical staining for MTP was observed in adipocytes in both white and brown fat surrounding mouse heart (Fig. 1A–C). The staining in brown fat was concentrated around the fat droplet suggesting the presence of MTP on the surface of the droplet. Because fat droplets in white fat adipocytes occupy a large portion of the cell, reducing the relative amounts of cytoplasm, it was not possible to assess the subcellular location of MTP in these cells. MTP staining was also observed in adipocytes of brown and white fat surrounding rat adrenal (Fig. 1D), and the pattern of staining was similar to that observed with fat depots around mouse heart. Mouse perirenal fat and mammary tissue fat depots displayed MTP staining (Fig. 1E and F). Importantly, staining was also detected in adipocytes in a human breast section (Fig. 1G), demonstrating that the presence of MTP in adipocytes is not restricted to rodents.

3.2. Immunocytochemistry studies

MTP immunolocalization was assessed in 3T3-L1 cells by confocal microscopy (Fig. 2). In non-differentiated cells, MTP was observed throughout the cell, but MTP signals were especially prominent in juxtanuclear segments, typical of Golgi staining. At higher magnifications the signals appeared to be vesicular in nature and to parallel individual Golgi cisternae (Fig. 2A). In differentiated cells, fluorescence throughout the cell tended to be increased (Fig. 2B–E). Fluorescence was also prominent around lipid droplets and appeared as small vesicular staining on the surface of the droplet (Fig. 2B and E). Fluorescent signals were not as prominent on larger droplets, but were quite distinct and intense on the smaller droplets. Signals in the juxtanuclear regions remained prominent, and by changing the focal plane, MTP signals within the Golgi apparatus were apparent (Fig. 2C and D).

3.3. Biochemical studies

Protein extracts from various fat depots were probed for the presence of MTP by western blot analysis (Fig. 3). The expected 97 kD MTP band was clearly visible in white (abdominal, inguinal, mesenteric, perirenal) and brown (scapular) fat depots. The mass of protein applied to the gels was 20 μg for the fat tissue extracts and 2.4 μg for the liver homogenate.
Lysates from 3T3-L1 cells collected before and after differentiation were also probed for MTP (Fig. 3). MTP was present in small amounts in cells that had not been grown to confluence. In cells that had been allowed to grow to confluence but not induced to differentiate, the MTP band was more prominent. In cells induced to differentiate, the MTP band was even further enhanced.

3.4. Triglyceride transfer studies

A luminal content fraction was recovered from rat adipose tissue microsomes, concentrated, and triglyceride transfer measured. For comparison, triglyceride transfer was measured in liver microsomal content fractions. Triglyceride transfer activity in the fat microsomal content fraction was not detected unless the luminal content fraction was concentrated. After concentrating the contents 5–6 fold, transfer was measured and averaged 1.79 ± 0.70 pmol triglyceride transferred/mg protein/h compared with 295 ± 52 pmol transferred/mg protein/h for liver microsomal content fractions (Fig. 4). Heating microsomal content fractions from liver and adipose tissue completely abolished all transfer.

3.5. MTP mRNA expression in fat tissue and 3T3 cells

The expression of MTP mRNA in white and brown fat was less than 1% of that in liver (Fig. 5). Expression of MTP in differentiated 3T3 cells was nearly 15 times higher than in fat tissue and approximately 13% of that observed in liver.

4. Discussion

The studies presented in this paper demonstrate the presence of MTP within adipocytes in several fat depots. Immunohistochemical studies indicate that MTP is associated with lipid droplets in adipocytes from both white and brown fat. Western blot analysis of proteins from these tissue depots confirms the presence of the 97 kD protein. Confocal microscopy revealed the presence of MTP in 3T3-L1 cells, particularly in the perinuclear regions that correspond to areas typically containing the Golgi complex. The MTP signals appeared to be vesicular in nature and to parallel individual Golgi cisternae. Differentiation of 3T3 cells into adipocytes resulted in increased MTP fluorescence within the cell and the appearance of MTP fluorescence on the surface of lipid droplets. In vitro triglyceride transfer assays revealed the presence of MTP activity in a rat adipose tissue microsomal fraction. Finally, by quantitative rtPCR we demonstrated the presence of MTP mRNA in adipocytes from brown and white fat as well as in 3T3 cells. We conclude that functional MTP is present within the adipocyte,
The expression of MTP in adipocytes is important for triglyceride droplet formation. MTP was discovered as an intracellular lipid transfer protein [30]. Its function combined with its location in the lumen of microsomes from liver and small intestine led to speculation that MTP plays a critical role in the assembly of very low density lipoproteins (VLDL) by the liver and chylomicrons (CM) by the small intestine [14,31]. The essential nature of MTP in the assembly of VLDL and CM was clearly defined by the discovery that the genetic disease abetalipoproteinemia results from a mutation in the gene encoding MTP, culminating in the accumulation of lipid droplets.

MTP mRNA has been reported in the yolk sac [16], a tissue that also expresses apoB [35,36], and it is likely that these two proteins function in the movement of lipid in the forming embryo [37]. Shoulders et al. [21] reported the expression of MTP mRNA in human testis, ovary, and kidney, but protein expression was not confirmed. Given the fact that testis and ovary are lipid-rich tissues, one might predict a role for MTP in lipid transport and storage. The function of MTP in the kidney is difficult to predict as the kidney in mammals is not believed to produce lipoproteins, although the kidney of chicks secretes both apoB- and apoA-I-containing lipoproteins [38]. Ours is the first report of MTP expression in fat tissue, although given the function of MTP, it is not surprising to find it in adipocytes where substantial processing and movement of triglycerides occur.

What then is the role of MTP in adipocytes? In hepatic lipoprotein assembly, MTP is thought to transport triglyceride from the endoplasmic reticulum (ER) membrane to apoB, the major structural protein of VLDL, as the protein is translated and translocated across the membrane [14]. In addition, studies with liver specific MTP knockout mice have shown that MTP is essential for transferring membrane-synthesized triglyceride to the lumen of the ER, forming a droplet that is subsequently added to the apoB-containing particle [39]. The mechanism by which this lipid droplet is formed in the hepatocyte ER may be similar to droplet biogenesis in adipocytes. It has been suggested that lipid bodies arise from microdomains of the ER that contain lipid biosynthetic enzymes [40,41]. Furthermore, freeze-fracture electron microscopy studies have led to the suggestion that the fat droplet monolayer surface is an area of the ER membrane leaflet [42], supporting the concept that triglyceride accumulates and lipid droplets develop in association with the hydrophobic regions of ER membrane bilayers. Our confocal studies demonstrate that MTP is clustered around small lipid droplets in differentiated 3T3 cells but is not as prominent around the larger lipid droplets. This suggests to us that MTP is participating in the accretion of lipid in the biogenesis of fat droplets.

Our studies not only provide conclusive evidence for the presence of MTP in adipocytes, but they also demonstrate that adipocyte microsomes contain triglyceride transfer activity. Based on microsomal luminal content protein, the activity in adipose tissue is less than one percent (0.6%) of that found in a liver microsomal fraction (Fig. 4), which is nearly identical with the relative levels of MTP mRNA in white fat and liver (Fig. 5). However, one must keep in mind that the liver is constantly exporting triglycerides. For example, in C57BL/6 mice the hepatic triglyceride production rate is 150 μmol/kg/h [29]. Thus, in a 30 g mouse, the liver secretes 3.6 mg triglyceride as VLDL each hour. In contrast, adipocytes under normal conditions accumulate triglyceride at very slow rates, and less MTP would be required for the formation of lipid droplets. Our observation that MTP is more highly expressed in differentiated 3T3 cells than in adipocytes from white or brown fat depots is consistent with the fact that these cells rapidly accumulate triglyceride.

Whereas our observations would seem to suggest a role for MTP in lipid droplet formation, we cannot discount the possibility that MTP may also be important in the lipolysis of triglyceride. Hydrolysis of triglyceride in the adipocyte is activated through a cAMP-dependent PKA pathway [6]. Upon activation, both HSL and perilipin are phosphorylated [7-9], and HSL is translocated to the surface of the droplet [11,12], where, in coordination with ATGL, triglycerides are hydrolyzed. However, the mechanism by which HSL or ATGL interact with triglyceride in the fat droplet is not known. Based on our present understanding of lipolysis, it is assumed that triglyceride molecules interact with the lipases without the aid of a carrier protein. However, there are numerous examples of carrier proteins facilitating enzyme-substrate interactions [43]. Indeed, fatty acid binding protein (FABP) has been shown to interact with HSL and activate the enzyme [44]. In an analogous manner MTP may facilitate the interactions of triglyceride with HSL and ATGL.

In conclusion we have provided evidence that MTP is present in adipocytes. Its established function as a triglyceride transfer protein may prove important in unraveling many of the unanswered questions surrounding adipocyte lipogenesis.
and lipolysis. Its discovery in the adipocyte opens new avenues for investigation of triglyceride trafficking, and these studies are now in progress.

Acknowledgments: This work was supported by National Institutes of Health Grants HL57984 and DK32642. The MTP antibody was generated in the Protein/Immunology Core of the Clinical Nutrition Research Unit (DK26657). We acknowledge Cambria Blaize for excellent technical assistance.

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