0021-972X/05/$15.00/0
Printed in U.S.A.

The Journal of Clinical Endocrinology & Metabolism 90(4):2250–2258
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doi: 10.1210/jc.2004-1325

Suppression of Hepatic Cholesteryl Ester Transfer Protein Expression in Obese Humans with the Development of Type 2 Diabetes Mellitus

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Cholesteryl ester transfer protein (CETP) is a plasma enzyme that can modulate the profile of lipoproteins and is thus considered: 1) a mediator of vascular disease; and 2) a therapeutic target for vascular disease. In the present study, we pursued a better understanding of the effect of type 2 diabetes on the expression of CETP in obese patients. Obesity was accompanied by a 20% elevation in plasma CETP that was eliminated with the development of diabetes. These differences were observed for both men and women and were due to variations in the amount of CETP protein in the plasma. The mRNA and protein of both the full-length (CETP^{FL}) and alternatively spliced (CETP$^{\Delta 9}$) forms of CETP were lower in the liver, but not in either sc or omental adipose tissue depots, of diabetic obese subjects. Sterol response element binding proteins 1 and 2 were also lower in liver homogenates, suggesting that these transcription factors may mediate the effects of type 2 diabetes on hepatic CETP expression. Thus, the suppressive effects of type 2 diabetes in obese subjects are observed in both men and women and may be due, at least in part, to a suppression of hepatic CETP expression. (*J Clin Endocrinol Metab* 90: 2250–2258, 2005)

CHOLESTERYL ESTER TRANSFER protein (CETP) is a glycoprotein that catalyzes the transfer of neutral lipids between the plasma lipoproteins. In this respect, this enzyme is involved in at least one arm of the reverse cholesterol transport process, an antiatherogenic process by which cholesterol is cleared from peripheral tissues (1). Even so, the atherogenic nature of CETP has been the subject of much debate, because both increased and decreased CETP expression have been linked to elevated risk and incidence of vascular disease via the effects of the enzyme on plasma lipoprotein concentration and composition (2). The expression of the CETP gene gives rise to two mRNA transcripts through alternative splicing, both of which give rise to protein products. The full-length product (CETP^{FL}) is a single peptide chain with a molecular mass of 67 kDa. The alternatively spliced transcript lacks exon 9, and its smaller protein product (CETP$^{\Delta 9}$) has been implicated in suppressing the expression and secretion of the full-length protein (3, 4).

Obesity is a metabolic condition afflicting over one third of the population of the United States (5) and is associated with the development of type 2 diabetes, cardiovascular disease, and some forms of cancer. Plasma CETP activity has been reported to be elevated in obese subjects (6–10). In women, this elevation is eliminated with the development of type 2 diabetes (6, 11, 12). It is unclear whether these differences between obese women with and without diabetes are beneficial or detrimental with respect to development of cardiovascular disease risk. In support of the beneficial role for CETP in this metabolic context, we reported that transgenic expression of CETP in db/db mice, a model of diabetic obesity that normally lacks the ability to express CETP, prevented the diet-induced development of atherosclerotic lesions (13). Given the controversy around the atherogenic/antiatherogenic nature of CETP that has received much attention in recent years (2), the contribution of suppressed CETP expression in the context of diabetic obesity continues to be debated. Because there is great interest in targeting CETP for the treatment of vascular disease (14), we have pursued a better understanding of the suppressive effects of type 2 diabetes on the obesity-induced elevations in plasma CETP activity.

In the present study, we measured CETP activity in the plasma of a large number of obese men and women with and without diabetes to determine whether the effect of diabetes on CETP activity is gender specific. We then examined whether the differential expression of CETP^{FL} and CETP$^{\Delta 9}$ in liver and two adipose tissue depots could be contributing to the differences in plasma CETP expression between obese subjects with and without diabetes. Our observations suggest that the suppression of plasma CETP activity with the

First Published Online January 11, 2005

Abbreviations: BMI, Body mass index; CETP, cholesteryl ester transfer protein; fg, fully glycosylated; CETP$^{\Delta 9}$, CETP lacking exon 9; CETP^{FL}, full-length CETP; FFA, free fatty acid(s); HDL, high-density lipoprotein; LDL-C, low-density lipoprotein cholesterol; LDL#, an indication of the number of LDL particles; LXR, liver X receptors; pg, partially glycosylated; SREBP, sterol response element binding protein; TC, total cholesterol; TG, triglycerides; VLDL, very low-density lipoprotein.

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development of type 2 diabetes in obese individuals occurs in both men and women and a suppressed level of $CETP^{FL}$ mRNA that is found in the liver, but not in adipose tissue, could be contributing to this differential level of expression. These observations provide insight into the regulation of CETP expression in the context of diabetic obesity in humans and have implications on therapeutic approaches that target CETP under these conditions in the treatment of vascular disease.

Subjects and Methods

Subjects

Body mass index (BMI) was determined as mass/height^2 (kilograms per square meter). A total of 129 nonobese (BMI < 30 kg/m^2), 95 obese (BMI > 30 kg/m^2), and 57 diabetic obese patients were studied in this investigation of CETP expression. Classification of type 2 diabetes was done with the criteria of the National Diabetes Data Group (15). Duration of diabetes was greater than 3 yr. Over 40% of those with type 2 diabetes were receiving some form of pharmaceutical therapy in addition to diet and physical activity recommendations, all of which was suspended 2 wk before sample collection. Patients were included in the study only after being informed of the potential risks and providing written consent to participate. All protocols used were approved by the Internal Review Board for Human Research before sample collection.

Plasma analyses

Blood samples were collected after an overnight fast in tubes containing EDTA (1 mg/ml), aprotinin (10 kallikrein inhibitor units), and sodium azide (0.1 mg/ml). Plasma was isolated with low-speed centrifugation (2500 $\times$ *g*) and stored at -80 C until the time of analysis. Samples were analyzed spectrophotometrically for glucose (Sigma 16-UV, Sigma Chemical, St. Louis, MO) and by microparticle enzyme immunoassay for insulin (IMx, Abbott Laboratories, Abbott Park, IL). Nonesterified fatty acids were determined colorimetrically (Wako Chemicals, Neuss, Germany). Lipid and lipoprotein profiles were obtained commercially (Lipomed Inc., Raleigh, NC) by nuclear magnetic resonance lipoprofiling as previously described (6, 16). Plasma leptin was determined by a RIA kit (Linco Research Inc., St. Louis, MO).

Tissue collection and treatment

A biopsy (100 mg) from the left lobe of the liver and a generous sample of sc and omental adipose tissue were collected from patients undergoing gastric bypass surgery for the treatment of severe obesity. Membrane extracts from hepatic and adipose tissues were prepared as previously described for the characterization of CETP protein (17). The samples were frozen in liquid nitrogen, and the frozen samples were stored at -80 C until analysis. Tissue homogenates were prepared (Polytron Kinematica, Lucerne, Switzerland) in an ice-cold solution containing 10 mM Tris buffer (pH 7.4), 0.25 M sucrose, 1 mM EDTA, 1 mM aprotinin, and 1 mM phenylmethylsulfonylfluoride (1:10 wt/vol). The homogenates were centrifuged at 150,000 $\times$ *g*, after which the supernatant was removed, and the pellet was resolubilized in the same buffer but with the addition of 1% Triton X-100. The resuspended pellet was centrifuged at 150,000 $\times$ *g*, and the supernatant was collected and stored at -80 C until analyzed for CETP activity and mass.

CETP activity and mass estimations

CETP activity in liver preparations and plasma was measured as the rate of [^{3}H]cholesteryl esters transferred from high-density lipoprotein (HDL) (donor) to apoprotein B containing lipoproteins (acceptor), as we have previously described (6, 18). The reaction was incubated at 37 C for 16 h in the presence and absence of TP2, a monoclonal antibody specific to CETP capable of binding and inhibiting activity. CETP-specific CET was calculated as the amount of CET that could be inhibited by TP2. The reaction was linear for CETP activity for label transfers of up to 45%. Samples run in separate assays were normalized to a standard of pooled plasma. Inter- and intraassay variations were both at least 4%. CETP mass was estimated by Western blot analysis, comparing samples to a known amount of partially purified CETP, as we have previously described (17). Mass estimates were normalized to protein concentration, determined by bicinchoninic acid Protein Assay (Pierce, Rockford, IL), using BSA as a standard. The primary antibody, TP2, was obtained from Dr. Ruth McPherson (Ottawa Heart Institute, Ottawa, Ontario). Deglycosylation experiments were done with a commercially available kit (Bio-Rad, Hercules, CA), using NANase II, O-glycosidase, and PNGase F.

RNA isolation and characterization

RNA was isolated from the liver and adipose tissue with the use of TRIzol Reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's specifications. Probes for use in the ribonuclease protection assay were prepared using a Maxiscript T7/T3 *in vivo* transcription kit (Ambion, Austin, TX). The plasmid used as the DNA template was acquired from Dr. Ruth McPherson. This Bluescript vector contains a 160-bp fragment of the human CETP gene spanning nucleotides 727 to 887. Antisense RNA probes were transcribed *in vitro* from the T3 promoter in the presence of [^{32}P]uridine 5′-triphosphate (DuPont NEN Life Science Products, Boston, MA). The reaction was carried out for 2 h at room temperature. The template was removed with deoxyribonuclease 1. CETP mRNA was determined with the use of the RPA II ribonuclease protection assay kit (Ambion). The probe was hybridized to RNA (30 μg of liver and 60 μg of adipose tissue) overnight at 45 C in a hybridization buffer containing 80% deionized formamide, 100 mM sodium citrate, 300 mM sodium acetate, and 1 mM EDTA (pH 6.4). Unhybridized RNA was then digested with a mixture of ribonuclease A and ribonuclease T1. The protected fragments (160 bp, $CETP^{FL}$ mRNA; 153 bp, $CETP^{\Delta 9}$ mRNA) were run on a 6% polyacrylamaide/7.5 M urea gel. The protected fragments in the gel were visualized using a phosphor imager and were normalized to total RNA.

Statistics

Data were analyzed by ANOVA or covariance (Systat/SPSS, SPSS Inc., Chicago, IL), as specified. When more than one group was involved, Bonferroni's *post hoc* test was used to determine differences between groups. Pearson correlation or Spearman's ρ coefficients were calculated to examine the relationships between specified parameters. Statistical significance was assumed when $P < 0.05$.

Results

Patient characteristics

The three groups did not differ in age, and no differences in weight or BMI were observed between obese and diabetic obese patients (Table 1). The obese group was characterized by hyperinsulinemia and euglycemia, whereas the diabetic group was characterized by both hyperinsulinemia and hyperglycemia. The lipid profiles of the three groups of patients were similar to what we and others have previously reported (6, 20–22). Total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), LDL particle concentration (LDL#, an indication of the number of LDL particles), total triglycerides (TG), very low-density lipoprotein (VLDL)-TG, and nonesterified free fatty acid (FFA) concentrations were elevated in obese subjects when compared with nonobese subjects and further elevated in diabetic obese subjects. In addition, the lipoprotein subpopulation distributions were also similar to what we have previously reported in nonobese, obese, and diabetic obese patients with nuclear magnetic resonance lipoprofiling (6). HDL and LDL size were smaller and VLDL size was larger in obese patients than in nonobese patients, perturbations that were magnified when obesity was accompanied by diabetes (data not shown).

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TABLE 1. Physical characteristics and plasma profiles of nonobese, obese, and diabetic obese patients

	Nonobese	Obese	Diabetic obese
n (f/m)	129 (93/36)	95 (80/15)	57 (49/8)
Age (yr)	39 ± 1	42 ± 1	42 ± 1
Weight (kg)	66 ± 1	143 ± 4[a]	147 ± 4[a]
BMI (kg/m^2)	24 ± 1	50 ± 1[a]	52 ± 1[a]
Glucose (mM)	4.88 ± 0.06	5.05 ± 0.06[a]	9.55 ± 0.50[a,b]
Insulin (pM)	29 ± 2	97 ± 7[a]	146 ± 13[a,b]
Leptin (ng/ml)	16 ± 2	87 ± 6[a]	57 ± 4[a,b]
TC (mM)	4.32 ± 0.10	4.68 ± 0.13[a]	5.07 ± 0.14[a,b]
LDL-C (mM)	2.92 ± 0.011	3.21 ± 0.12[a]	3.49 ± 0.11[a,b]
LDL# (nM)	1198 ± 44	1345 ± 47[a]	1519 ± 51[a,b]
HDL-C (mM)	1.09 ± 0.03	1.06 ± 0.03	1.03 ± 0.06
TG (mM)	1.14 ± 0.06	1.34 ± 0.08[a]	1.60 ± 0.12[a,b]
VLDL-TG (mM)	0.75 ± 0.06	0.93 ± 0.07[a]	1.16 ± 12[a,b]
NEFA (μM)	475 ± 31	635 ± 31[a]	775 ± 40[a,b]

Data from fasted subjects are expressed as means ± SEM and analyzed by ANOVA. TC, LDL-C, LDL#, HDL-C, TG, and VLDL-TG were determined by NMR Lipoprofiling (Lipomed). Other plasma characteristics were determined by colorimetric or immunoassays, as described in *Subjects and Methods*. F, Female; m, male; HDL-C, HDL cholesterol; NEFA, nonesterified fatty acids.

[a] Significantly different from nonobese group ($P < 0.05$).

[b] Significantly different from obese group ($P < 0.05$).

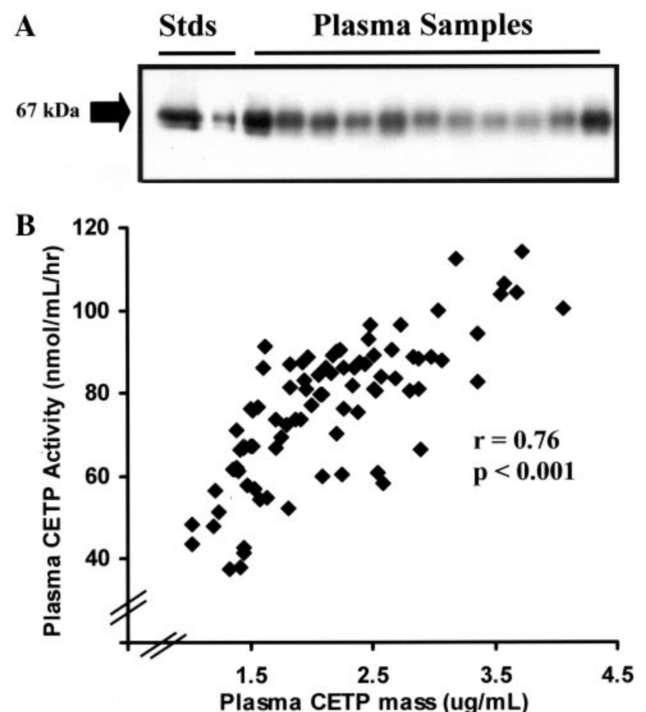

FIG. 1. Plasma CETP mass determination by Western blot. A, Plasma samples were diluted and run on a 12% acrylamide gel. CETP protein in each sample was quantified by running the samples with a standard (0.32 and 0.04 ng, shown) of semipurified plasma CETP protein. B, Plasma CETP mass determined by Western blotting was highly correlated to plasma CETP activity. CETP activity was determined as the amount of radiolabeled [^{3}H]cholesteryl esters transferred between exogenous donor and acceptor lipoprotein substrates that could be inhibited by the human CETP-specific monoclonal antibody, TP2, during a 16-h incubation at 37 C. Stds, Standards.

Plasma CETP expression

Plasma CETP activity was measured in two ways. We measured activity first by the more conventional radiolabel transfer assay and then by determining the amount of this radiolabeled transfer that could be inhibited by the monoclonal antibody, TP2. The results from the transfer and inhibition assays were highly correlated (r = 0.98; $P < 0.001$). The amount of inhibition by TP2 was approximately 91 ± 1% and was not significantly different between the groups. In a smaller number of these patients, a crude estimation of CETP mass was acquired by Western blot analysis and was observed as a single band (~67 kDa; Fig. 1A). CETP activity and mass were significantly related in the plasma of the subjects examined (r = 0.76; $P < 0.001$) (Fig. 1B). Thus, the conventional radiolabel transfer assay of CETP activity and that determined by the amount of radiolabel transfer that could be inhibited by TP2 appear to be good estimations of CETP mass.

TP2-inhibitable CETP activity and Western blot mass estimations of CETP protein that were observed in the plasma of the patients are shown in Table 2. Although women had higher levels of CETP activity and mass, both men and women showed the same differences with respect to obesity and diabetes. Plasma CETP activity was higher in obese than nonobese patients and lower in diabetic obese than obese patients. Thus, plasma CETP activity is elevated in obese patients without diabetes, but not in obese patients with diabetes, and these differences are due to alterations in the concentration of CETP protein.

In an effort to understand what factors may be influencing or affected by the levels of CETP in the plasma, the relationship of plasma CETP activity to several patient characteristics is shown in Table 3. Plasma insulin concentrations were not related to plasma CETP activity. Because this activity assay employed exogenous substrates, it is not surprising that TG, VLDL-TG, HDL, and FFA were not related to plasma CETP activity (data not shown). However, plasma glucose was negatively related, whereas BMI, plasma leptin, TC, LDL-C, and LDL# were positively related to plasma CETP activity. Interestingly, plasma leptin concentrations showed the strongest relationship to plasma CETP activity. The nonobese and obese subjects were examined separately; plasma insulin concentrations did not correlate to plasma CETP activity in any group. In nonobese subjects, plasma CETP activity was related to TC, LDL-C, and LDL#, whereas relationships with the other variables were nonexistent. In contrast, the analysis in obese subjects showed that both glucose and leptin concentrations were significantly related to plasma CETP activity, whereas the parameters of plasma cholesterol were not.

CETP expression in the tissues of obese and diabetic obese patients

Because a suppressive effect of diabetes was observed on plasma CETP in obese patients, we examined the expression of CETP in the liver, sc, and omental adipose tissue of a number of obese (n = 13) and diabetic obese (n = 7) individuals. The characteristics of these patients were similar to the larger sample population in which plasma CETP activity and mass were determined (Table 1). Furthermore, the differences between obese and diabetic obese patients in plasma

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TABLE 2. Plasma CETP activity and mass

	Activity (nmol/ml·h)[a,b]			Mass (μg/ml)[a,b]		
	Nonobese	Obese	Diabetic obese	Nonobese	Obese	Diabetic obese
Combined	68.9 ± 1.8 (129)	78.9 ± 2.3 (95)[c]	68.7 ± 3.3 (57)[d]	2.05 ± 0.13 (25)	2.33 ± 0.11 (42)[c]	1.86 ± 0.13 (20)[d]
Women	72.4 ± 1.5 (93)	80.2 ± 1.8 (80)[c]	71.0 ± 2.4 (49)[d]	2.10 ± 0.17 (17)	2.42 ± 0.13 (32)[c]	2.00 ± 0.18 (13)[d]
Men	65.5 ± 2.7 (36)	77.5 ± 3.9 (15)[c]	66.5 ± 4.7 (8)[d]	1.94 ± 0.18 (8)	2.03 ± 0.14 (10)	1.60 ± 0.09 (7)[d]

Plasma CETP activity was determined as the amount of cholesteryl ester transfer that could be inhibited by the human CETP-specific monoclonal antibody, TP2, during a 16-h incubation at 37 C. In a limited number of randomly chosen patients, plasma CETP mass was estimated by Western blot analysis. Plasma samples were diluted 1:150, and 10 μl of each sample was run on a 12% acrylamide gel and blotted to nitrocellulose. CETP protein was visualized by incubating the blot with the monoclonal antibody TP2, followed by an antimouse secondary antibody conjugated to horseradish peroxidase, and development in Supersignal. Data are shown as means ± SEM (n) and were analyzed by ANOVA for an effect of sex,[a] group,[b] and the interaction between the two factors. Bonferroni's *post hoc* test was used to test differences between individual groups.

[c] Significantly different from nonobese group ($P < 0.05$).

[d] Significantly different from obese group ($P < 0.05$).

CETP activity were reflective of that which had been observed in the larger sample population (85.3 ± 4.7 *vs.* 66.8 ± 6.5 nmol/ml·h; $P < 0.05$).

Hepatic $CETP^{FL}$ and $CETP^{\Delta 9}$ mRNA were significantly lower in diabetic obese patients than in obese patients without diabetes (Fig. 2, A and B). The $CETP^{FL}$ to $CETP^{\Delta 9}$ mRNA ratio was found to be similar to that previously reported for human liver (23, 24) and was lower in diabetic obese patients (2.7 ± 0.3 *vs.* 1.5 ± 0.3; $P < 0.05$). $CETP^{FL}$ mRNA was related to $CETP^{\Delta 9}$ mRNA ($r = 0.90$; $P < 0.001$). No differences were observed in sc or omental adipose tissue mRNA (Fig. 2, C and D).

Western blot analysis of liver homogenates revealed two bands (~62 to ~67 kDa) (Fig. 3A, lane 1). The upper band has a similar mobility to the protein found in the plasma (Fig. 3A, lane 2) and is consistent with the reported $CETP^{FL}$ protein that is secreted into the blood (3, 4, 23). Deglycosylation of liver samples resulted in the disappearance of the doublet between 62 and 67 kDa and the appearance of a single band at ~59 kDa (Fig. 3B, lane 2). Thus, the doublets observed in liver samples at 62 and 67 kDa are partially glycosylated (pg) and fully glycosylated (fg) forms of $CETP^{FL}$. Extended exposure of the blots revealed another band at ~56 kDa found in the liver (Fig. 3A, lane 3) but absent from the plasma (Fig. 3A, lane 4). This protein has a similar mobility to what has been reported for the poorly secreted, protein product of the alternatively spliced mRNA, $CETP^{\Delta 9}$ (3, 4, 23). This band sometimes appeared as a doublet also (Fig. 3B, lane 1). The differential expression between $CETP^{FL}$ and $CETP^{\Delta 9}$ did not allow the resolution of the two $CETP^{\Delta 9}$ bands after deglycosylation, but these are likely various forms of glycosylated $CETP^{\Delta 9}$ protein that have been previously reported in transgenic mice and transfected cell culture models (3, 4, 23).

TABLE 3. Relationship between several patient characteristics and plasma CETP activity

	Combined	Nonobese	Obese (with and without diabetes)
Insulin	0.08	−0.09	−0.02
Glucose	−0.13[a]	−0.09	−0.26[a]
BMI	0.16[b]	−0.07	0.07
Leptin	0.30[c]	0.17	0.31[c]
TC	0.16[a]	0.35[a]	−0.05
LDL-C	0.16[a]	0.32[a]	−0.02
LDL#	0.16[a]	0.33[a]	−0.04

Pearson correlation coefficients were calculated to examine the relationships between plasma CETP activity and several patient characteristics. Data were analyzed when all patients were grouped together and when separated into nonobese and obese groups.

[a] $P < 0.05$; [b] $P < 0.01$; [c] $P < 0.001$.

CETP protein mass of liver homogenates was estimated by comparing samples to semipurified standards on a Western blot (Fig. 3C), and average values are shown in Fig. 3D. The $CETP^{FL\text{-}fg}$ (upper band only), total $CETP^{FL}$, and $CETP^{\Delta 9}$ protein were lower in diabetic obese patients than in obese patients without diabetes. In the adipose depots, there were no significant differences in $CETP^{FL}$ or $CETP^{\Delta 9}$ protein between those with and without diabetes (data not shown).

Hepatic $CETP^{FL}$ mRNA was significantly related to the amount of $CETP^{FL}$ protein in liver homogenates (Fig. 4A), whereas $CETP^{\Delta 9}$ mRNA was more closely related to $CETP^{\Delta 9}$ protein (data not shown). The amount of $CETP^{FL\text{-}fg}$ protein was clearly a reflection of the total amount of hepatic $CETP^{FL}$ protein, as indicated by strong relationships with both total $CETP^{FL}$ protein (Fig. 4B) and $CETP^{FL\text{-}pg}$ protein ($r = 0.69$; $P < 0.001$). Furthermore, the relationship that plasma CETP activity has with $CETP^{FL\text{-}fg}$ protein (Fig. 4C) and the other parameters of hepatic $CETP^{FL}$ expression (data not shown) support the hypothesis that the differential expression of CETP in the liver in these subjects is contributing to the differences in plasma levels. In contrast, no such relationships to plasma CETP activity were observed with any aspect of hepatic $CETP^{\Delta 9}$ expression nor to the parameters measured in the adipose tissues. Taken together, these data suggest a differential expression of $CETP^{FL}$ between obese and diabetic obese patients at the level of mRNA that is reflected in hepatic and plasma CETP protein levels.

The transcription of CETP has been shown to be induced by the sterol response element binding protein (SREBP) family of transcription factors (25, 26). To investigate whether these factors might be involved in the differential effects of diabetes in the presence of severe obesity on CETP mRNA levels, liver homogenates were examined by Western blot for SREBP1 and SREBP2. We detected the full-length, membrane-bound protein for both isoforms and found them to be lower in those subjects with diabetes (Fig. 5). However, only SREBP2 was significantly related to CETP mRNA levels ($r = 0.51$; $P < 0.05$).

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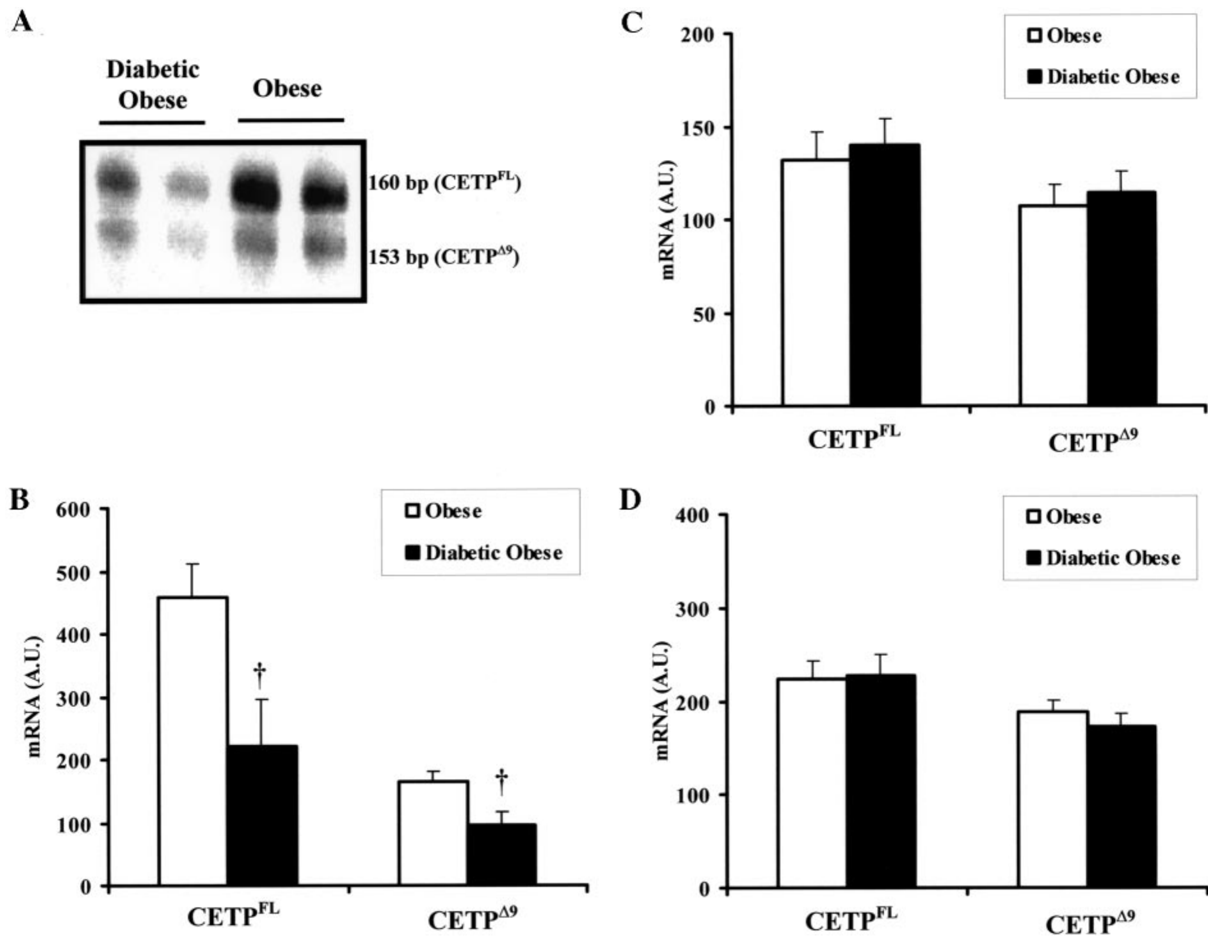

FIG. 2. Liver and adipose tissue CETP mRNA levels in obese patients with and without type 2 diabetes. A, A representative phosphor image of protected hepatic mRNA from two obese subjects and two obese noninsulin-dependent diabetes mellitus subjects is shown. A total of 30 μg of liver RNA were analyzed by a ribonuclease protection assay and normalized to total RNA. The radiolabeled riboprobe used protects both the $CETP^{FL}$ mRNA transcript (160-bp protected fragment) and the $CETP^{\Delta 9}$ mRNA transcript (153-bp protected fragment). The relative amounts of $CETP^{FL}$ and $CETP^{\Delta 9}$ mRNA in the liver (B), sc adipose depot (C), and omental adipose depot (D) are compared for obese (n = 13) and diabetic obese (n = 7) subjects. †, Significantly different from obese group ($P < 0.05$).

Discussion

This study is among the first to examine CETP expression in obese humans with and without type 2 diabetes in the plasma, liver, and adipose depots, reporting several novel findings. First, these results indicate that the obesity-induced elevation in plasma CETP activity previously reported in women (6–10, 27) is also found men. Second, the obesity-induced elevation in plasma CETP activity is a result of an elevation in the concentration of plasma CETP protein. Third, the suppressive effect of type 2 diabetes on the obesity-induced elevation in plasma CETP activity is a result of a lower concentration of CETP protein. Finally, the down-regulation of hepatic CETP mRNA levels and a consequential decrease in hepatic protein production and secretion may contribute to the suppressive effects of type 2 diabetes on plasma CETP in obese individuals. These results provide insight into the regulation of plasma CETP levels in the metabolic context that includes obesity and type 2 diabetes.

Effects of obesity and type 2 diabetes on plasma CETP levels

Although some studies have shown that plasma CETP activity is similar in diabetic individuals when compared with normal control patients (28–30), the subjects in these studies were primarily lean and mildly overweight individuals. When this issue is examined in obese subjects, diabetes has been reported to have a suppressive effect on plasma CETP activity (6, 11, 30). The findings in the present study show that the suppression of plasma CETP activity in obese subjects with type 2 diabetes is found in both men and women and is a result of alterations in CETP protein concentrations. Taken together, these observations indicate that obesity is accompanied by an elevation in plasma CETP levels that is eliminated or attenuated with the development of type 2 diabetes.

Several factors that have known connections to the regulation of CETP expression were observed to be related to CETP expression in this study. For example, it has been shown that cholesterol stimulates the transcription of the CETP gene (31–

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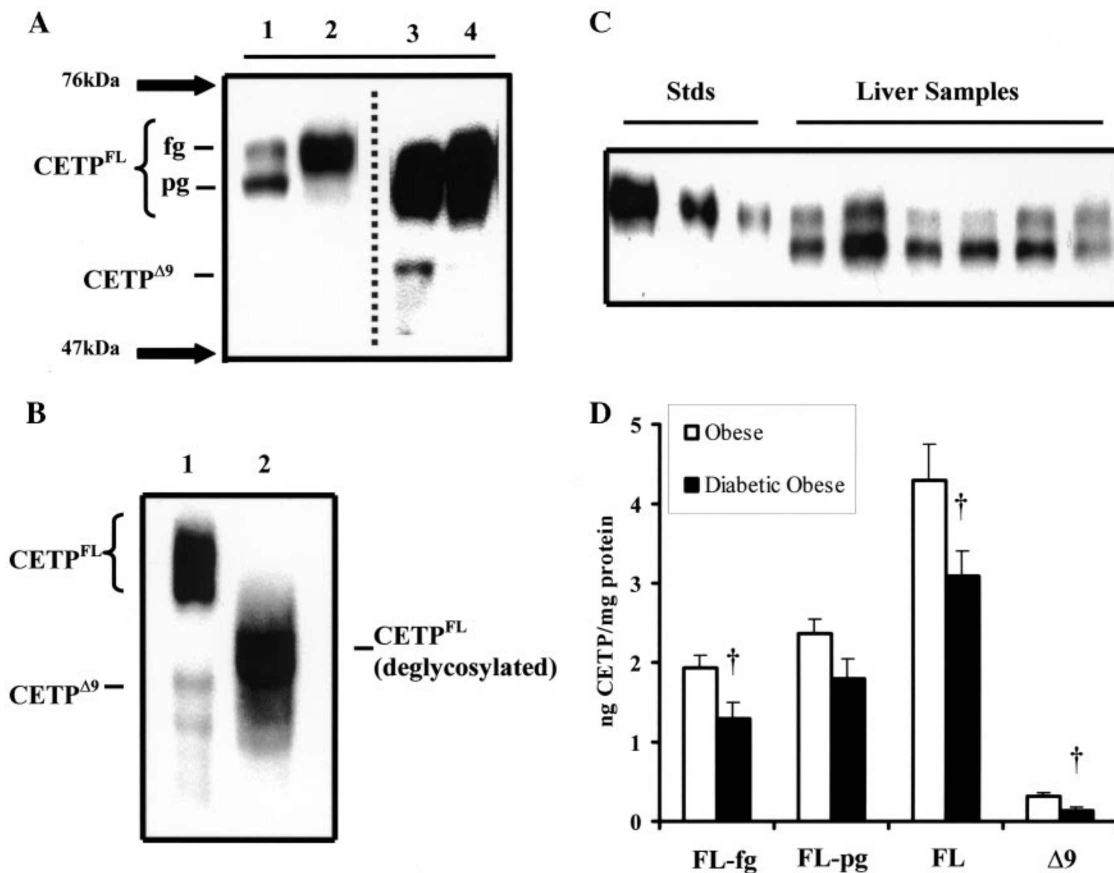

FIG. 3. CETP protein in plasma and liver homogenates. A total of 30 μg of protein were run on a 12% acrylamide gel and blotted to a nitrocellulose membrane. CETP protein was visualized by the sequential incubation in the monoclonal antibody, TP2, an antimouse secondary antibody conjugated to horseradish peroxidase, and Supersignal, followed by multiple film exposures. A, A single band was observed in the plasma (lane 2), whereas a doublet was observed in the solubilized membrane fraction (lane 1). A longer film exposure yielded a third band in this fraction (lane 3), assumed to be the protein product of the Δ9 transcript ($CETP^{\Delta 9}$ protein; ~57 kDa). This band was not observed in the plasma (lane 4). B, A liver particulate sample was run before (lane 1) and after (lane 2) N-linked and O-linked deglycosylation. The doublet observed was reduced to a single band, indicating the presence of $CETP^{FL\text{-}fg}$ and $CETP^{FL\text{-}pg}$. The blot shown is overexposed to show the relative mobility of deglycosylated $CETP^{FL}$ (lane 2) to that of the $CETP^{\Delta 9}$ protein (lane 1; ~57 kDa). C, An example blot showing how CETP protein in each sample was quantified by running the samples with a standard (0.16, 0.08, and 0.04 ng, shown) of semipurified plasma CETP protein. D, Average $CETP^{FL\text{-}fg}$, $CETP^{FL\text{-}pg}$, total $CETP^{FL}$, and $CETP^{\Delta 9}$ protein in obese and diabetic obese subjects after adjustment for β-actin protein levels (†, $P < 0.05$). Stds, Standards.

35). Radeau *et al.* (36) observed that membrane cholesterol content in human subjects was related to CETP mRNA levels in adipose tissue. The positive correlation between plasma cholesterol concentration and CETP expression that we observed in this study shows that this relationship was more pronounced in nonobese subjects but was not apparent in obese subjects with or without diabetes. Taken together, our data suggest that plasma cholesterol may be an important determinant of plasma CETP activity in nonobese subjects but may be less influential in obese individuals with or without diabetes.

It has been suggested by several researchers, including us, that insulin may be an important modulator of CETP expression in humans and that insulin resistance may lead to perturbations in plasma CETP levels (1, 11, 37, 38). This has been a controversial issue in the literature, because reports are not clear as to whether insulin might be a positive or negative regulator of CETP expression (17, 37–46), and no clear effect of insulin resistance has been observed (47, 48). In the present study, we observed that plasma insulin concentration was not related to any parameter of CETP expression, even when subjects with diabetes, in whom a β-cell defect may be profound, were eliminated from the analysis (data not shown). Alternatively, our analyses indicated that plasma leptin and glucose levels were more closely linked to plasma CETP levels, supporting the notion that neither insulin nor insulin resistance directly influences plasma CETP concentrations. However, insulin and/or insulin resistance may be indirectly affecting the total cholesteryl ester transfer rates *in vivo*. Cholesteryl ester transfer between lipoproteins is dependent on both CETP and the substrate lipoproteins, and the substrate lipoproteins are altered in insulin-resistant states (49, 50). Practically speaking, the higher VLDL-TG in subjects with diabetes may be accelerating the cholesteryl ester transfer rates *in vivo*, despite lower levels of plasma CETP protein. The methods used to determine CETP levels in this study are reflective of CETP protein concentrations and do not account for variations in the composition and concentration of endogenous lipoproteins. As such, we were not surprised that TG, VLDL-TG, HDL, and FFA were not related to our measurement of CETP activity.

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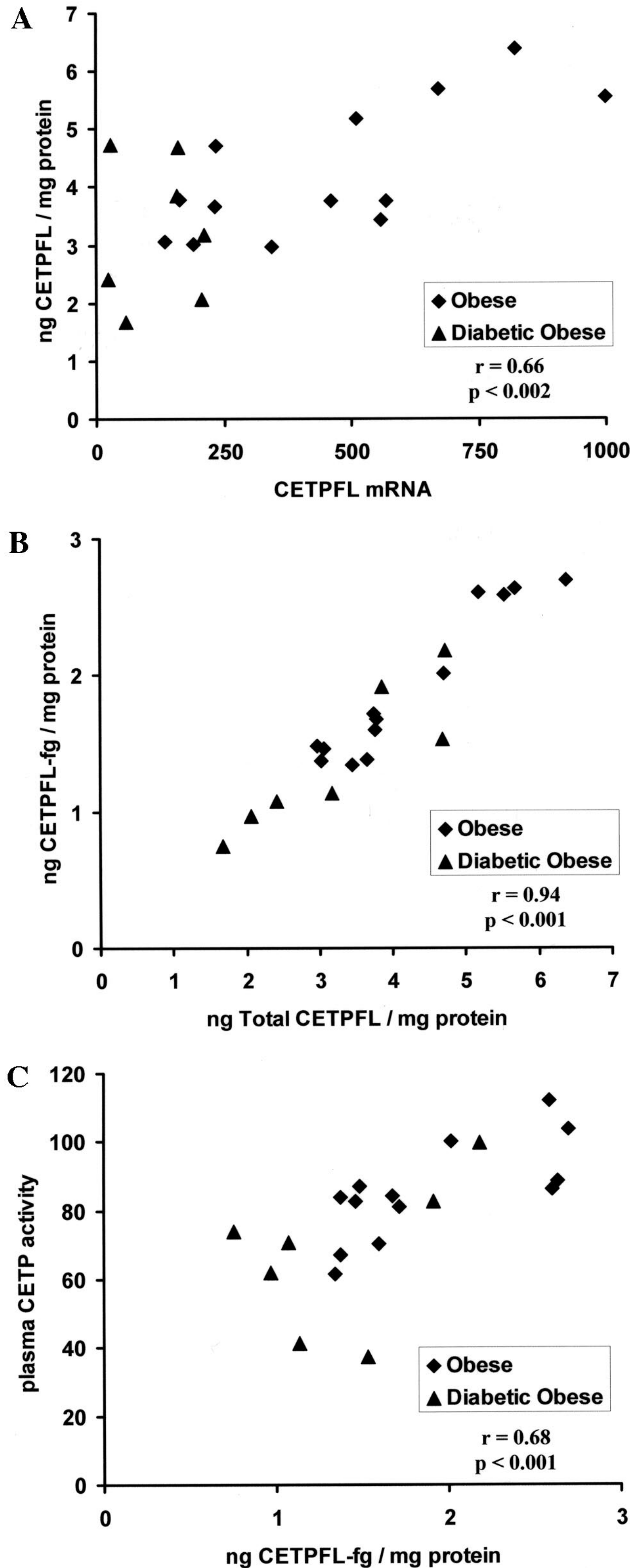

FIG. 4. Scatterplot representation of the relationships between hepatic mRNA, hepatic protein, and plasma CETP levels. A, Relationship between total $CETP^{FL}$ mRNA and total $CETP^{FL}$ protein. B, Relationship between total $CETP^{FL}$ protein and that amount that is fully glycosylated ($CETP^{FL\text{-}fg}$). C, Relationship between $CETP^{FL\text{-}fg}$ protein and plasma CETP activity.

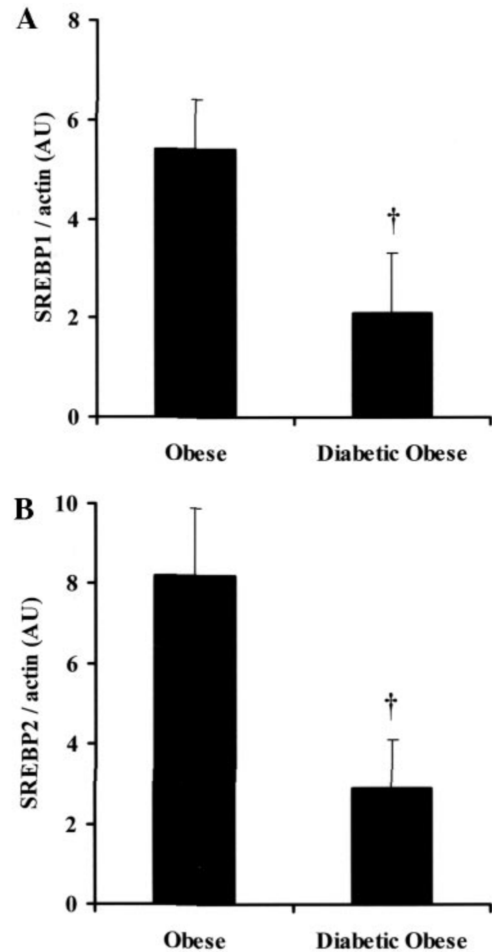

FIG. 5. Protein levels of SREBP1 and SREBP1 in liver homogenates. Average full-length SREBP1 (A) and SREBP2 (B) protein levels detected by Western blot in obese and diabetic obese subjects after adjustment for β-actin protein levels (†, $P < 0.05$).

Tissue-specific dysregulation of CETP expression in diabetic obesity

In humans, CETP is produced and secreted primarily from liver and adipose tissue (51), and our study is the first to examine the expression of CETP in these tissues from obese subjects with and without type 2 diabetes. Hepatic CETP expression is suppressed when obesity is accompanied by type 2 diabetes, an effect that was not observed for either adipose tissue depot that we measured. It should be noted that Radeau *et al.* (52) reported that plasma CETP activity was strongly related to CETP mRNA levels in sc adipose tissue of lean subjects. Although we confirmed this relationship in another group of lean patients (data not shown), we did not observe a relationship between plasma CETP activity and any parameter of CETP expression in sc or omental adipose tissue from these severely obese subjects (data not shown). These observations indicate that the contribution of each tissue to plasma CETP variations may be dependent on the metabolic context. Thus, the suppressive effect of diabetes on plasma CETP levels may be a result of altered regulation that is specific to the liver and, possibly, other CETP-expressing tissues that were not examined in this study.

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A novel finding of this study is the differential hepatic expression of both $CETP^{FL}$ and $CETP^{\Delta9}$ in a metabolic disorder like diabetes. In transgenic mice and in transfected cell lines, $CETP^{\Delta9}$ mRNA is translated into a truncated form of CETP that lacks activity and is poorly transcribed, translated, and secreted (3, 4, 23). Consistent with these reports, we observed that: 1) as total mRNA is increased in the liver, $CETP^{FL}$ mRNA may be increased preferentially over the $CETP^{\Delta9}$ transcript; 2) the amount of $CETP^{\Delta9}$ protein is relatively low when considering the $CETP^{FL}$ to $CETP^{\Delta9}$ mRNA ratio; and 3) no $CETP^{\Delta9}$ protein was observed in the plasma. Previous studies in cultured cells have also shown that $CETP^{\Delta9}$ protein binds $CETP^{FL}$ protein and prevents its secretion, possibly targeting it for degradation (3). Although our data are consistent with several expression and regulatory aspects of $CETP^{\Delta9}$ that have been observed in transgenic mice and cultured cells, $CETP^{\Delta9}$ does not appear to be involved in the dysregulation of CETP expression in diabetic obesity. The function of $CETP^{\Delta9}$ expression *in vivo*, either as a regulator of $CETP^{FL}$ expression or a diversionary pathway of transcription, remains unclear.

The suppressive effects of type 2 diabetes on the level of CETP mRNA implied an adjustment in: 1) transcriptional regulation; 2) transcript stability; or 3) both. In the present study, we pursued the first of these possibilities by examining the expression of some transcription factors known to regulate CETP transcription. The liver X receptors (LXRs) and the SREBP family of transcription factors have been shown to activate CETP transcription (25, 26, 53, 54). LXRs may have a dual mechanism of regulating CETP expression. LXRs can have direct effects on the CETP promoter, and they can have indirect effects by inducing the expression of SREBP proteins (55, 56). SREBP-1 expression has been shown to be impaired in muscle and adipose tissue from patients with type 2 diabetes (57), and we hypothesized that a similar effect in the liver could explain the effects of diabetes on CETP expression. The observation that the full-length, membrane-bound form of both proteins was suppressed in diabetic obese subjects is consistent with a role for SREBP proteins in mediating the effects of diabetes on CETP mRNA levels. However, the full-length SREBP proteins do not directly affect transcriptional rates. These proteins are processed through a regulated cleavage that releases the active, nuclear-targeted transcription factor (19). Furthermore, the parameter of SREBP1 and SREBP2 expression that we measured in both cases is influenced not only by the amount of SREBP mRNA being translated but also by the rate of cleavage that releases the N-terminal region for nuclear targeting. Unfortunately, we were unable to measure LXRs, SREBP mRNA, or the nuclear, cleaved form of the SREBP proteins in these subjects together, which would have provided a better picture of how SREBP proteins may or may not be involved.

Clinical relevance: CETP as a therapeutic target

In conclusion, diabetic obese patients have lower levels of plasma CETP activity and mass than obese patients without diabetes. This difference appears to be due, in part, to altered hepatic expression of CETP mRNA in these patients. Although plasma CETP levels may be lower in diabetic obesity, absolute rates of cholesteryl ester transfer may still be higher, because the *in vivo* transfer rate is also influenced by the lipoprotein substrates. Therefore, the suppressive effects of diabetes on the expression of CETP in obese individuals can be viewed as: 1) a detrimental impairment in the reverse cholesterol transport process that contributes to abnormal lipoprotein profiles and increased vascular disease; or 2) a beneficial response to an impaired process of cholesterol clearance that is depleting antiatherogenic HDL and accumulating atherogenic LDL. In either case, the fact that CETP is capable of modulating the composition and the concentrations of lipoproteins in the plasma makes it an attractive therapeutic and preventative target in the treatment of vascular disease (14). However, knowing which view correctly describes the perturbation in CETP expression in diabetic obesity is essential to appropriately target it in this metabolic context. Understanding the mechanisms behind the suppressive effects of diabetes on CETP expression in obesity will not only provide insight into this issue but also may yield other therapeutic targets for the treatment and prevention of vascular disease.

Acknowledgments

We thank Dr. Jim Otvos in the Department of Biochemistry at North Carolina State University for assistance in the processing and interpretation of nuclear magnetic resonance data and Drs. Lynis Dohm and Joseph Bower for their review and editing of the manuscript. Brian Roberts, Tom Green, and Rania Abdel-Rahman provided excellent technical assistance in the course of this study.

Received July 8, 2004. Accepted January 4, 2005.

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This work was supported by a grant from the National Institutes of Health (Department of Health and Human Services-Public Health Service DK 45029).

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