Expression of CETP and of splice variants induces the same level of ER stress despite secretion efficiency differences

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Abstract The cholesteryl ester transfer protein (*CETP*) gene has been associated with a variety of phenotypes, including HDL-cholesterol levels and, more sporadically, with cardiovascular disease, obesity, and extreme longevity. Alterations of CETP activity levels can be caused by single-base polymorphisms as well as by alternative splicing. In addition to the previously characterized alternative splicing that skips exon 9, we found additional minor variants and characterized the activity of the resultant proteins. The novel variants skipped exon 9 sequences and inserted one of two in-frame exons from Alu-derived intronic sequences. None of the alternatively spliced variants are efficiently secreted, and coexpression of them inhibits wild-type CETP secretion. Expression of the alternative spliced variants causes an induction of genes linked to the endoplasmic reticulum (ER) stress response, including the neighboring *HERPUD1* (homocysteine- and ER stress-inducible protein, ubiquitin-like domain-containing) gene. Unexpectedly, even though wild-type CETP is secreted much more efficiently than spliced variants, it induces the same degree of stress response as spliced variants, whereas a control secreted protein does not. CETP plays a complex role in modulating ER stress, with its expression inducing the response and its cholesteryl ester transfer activity and differential splicing modulating the response in other ways.—Lira, M. E., A. K. Loomis, S. A. Paciga, D. B. Lloyd, and J. F. Thompson. **Expression of CETP and of splice variants induces the same level of ER stress despite secretion efficiency differences. *J. Lipid Res.* 2008.** 49: **1955–1962.**

Supplementary key words HDL-cholesterol • atherosclerosis • Alu sequence • endoplasmic reticulum • cholesteryl ester transfer protein

Cholesteryl ester transfer protein (*CETP*) is an important regulator of HDL metabolism, catalyzing the transfer of cholesteryl esters (CEs) and triglycerides among plasma lipoproteins. Its role in modulating HDL-cholesterol (HDL-C) levels in humans is clear, but its importance in other processes has been debated (1). Its tissue expression profile, including liver, adipose, spleen, adrenal gland, and small intestine (2, 3), may provide hints at roles beyond HDL-C modulation. For example, in adipocytes, *CETP* has been shown to play a localized role by assisting in the process of CE uptake (4) and intracellular transport and storage (5). *CETP* mRNA and protein levels in adipocytes vary in relation to environmental changes (6, 7). This may be related to other observations in which elevated CETP levels were found in certain populations of obese individuals (8–11). Whether alterations in CETP levels help to promote obesity or are merely a consequence of it remains an open question.

A variety of polymorphisms in *CETP* have been shown to be reproducibly associated with HDL-C levels across numerous populations (as reviewed in Refs. 12, 13). Most studies of *CETP* genetics have not found associations with body mass index (BMI) or other measures of obesity, but there have been exceptions with particular single-nucleotide polymorphisms or in specific populations (14, 15). Complex interactions of *CETP* variation with obesity and other phenotypes within particular subpopulations have also been found (16, 17).

In addition to mutations that create defective splice sites and major defects in splicing (18, 19), alternative splicing of normal *CETP* mRNA may play a more subtle role in affecting CETP activity. The major alternatively spliced product lacks exon 9 and leads to the translation of a poorly secreted protein that is inactive in lipid transfer (20). The constitutively expressed, alternatively spliced form is retained mostly in the endoplasmic reticulum (ER) and has been shown to inhibit the activity and secretion of active protein (21). The rate of alternative splicing appears to be controlled, at least in part, by environmental factors, with oleic acid increasing the fraction of full-length transcripts in Caco-2 cells (22). In obese versus diabetic individuals, differential rates of alternative splicing in the liver

Manuscript received 12 February 2008 and in revised form 9 April 2008.

Published, JLR Papers in Press, May 28, 2008.
DOI 10.1194/jlr.M800078-JLR200

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have also been observed (11). Other splicing patterns in the *CETP* gene have been observed in individuals with normal genomic sequence (23).

Because the protein product of the major alternative splice variant of *CETP* is retained within the ER, there is a possibility that it could affect the ER stress or the unfolded protein response. Interestingly, the gene immediately upstream of *CETP*, *HERPUD1* (homocysteine- and ER stress-inducible protein, ubiquitin-like domain-containing), is known to be involved in the ER stress response (24). The potential role of *CETP* in modulating ER stress is particularly intriguing, given the impact that differences in lipid composition within the ER membrane have on ER stress as well as the role that ER stress appears to play in diabetes, obesity, and other metabolic disturbances (25).

EXPERIMENTAL PROCEDURES

Reverse transcription PCR

Human subcutaneous adipose tissue RNAs were obtained from Zen-Bio, Inc. cDNA was synthesized from 2 μg of total RNA using random hexamers from a first-strand cDNA kit obtained from Amersham BioSciences according to manufacturer's protocol. PCR primers spanning CETP exons 8 to 16 were used to amplify a fragment of CETP cDNA. PCR was performed in 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphate, 0.5 μM forward and reverse primers (forward primer: acctggagtcccatcacaag; reverse primer: atctgcagcagcaggaagc) and 2.5 U HotStar Taq polymerase (Qiagen, Inc.). Amplification was performed with the cycling conditions: 95°C for 10 min followed by 30 cycles at 95°C for 1 min, 60°C for 30 s, 72°C for 30 s, followed by 5 min incubation at 72°C. Of the first PCR amplification, 1/25th was used as template for a secondary PCR using the same conditions. PCR products were run on either a 2% metaphor agarose gel (FMC Bioproducts, Rockland, ME) or a 3:1 Nusieve agarose gel, gel-purified, and cloned into PCRII-Topo vector (Invitrogen). Sequence analysis was done on an Applied Biosystems 3730 DNA analyzer using M13 forward and reverse primers. For tissue distribution experiments, human total RNAs from liver, spleen, adrenal gland, small intestine, adipose, and heart were obtained from BD BioSciences Clontech. RT-PCR was done using the above conditions.

Cloning and expression of variants

The complete alternatively spliced *CETP* cDNAs were generated from human subcutaneous adipose tissue and cloned into a modified version of pSecTag2/Hygro (Invitrogen) carrying N-terminal His_6 and V5 tags. Transient transfections were done on a human liposarcoma cell line (SW872) and a human embryonic kidney cell line (HEK293S). SW872 cells were cultured in DMEM/F12, 10% FBS (certified, Invitrogen), with 50 μg/ml gentamycin at 37°C, 5% (v/v) carbon dioxide. Transient transfections on SW872 cells were carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Media was replaced with serum-free DMEM/F12 approximately 24 h post transfection. Transient transfections on HEK293S cells were carried out using FuGENE 6 (Roche Applied Science). HEK293S was cultured in DMEM (Invitrogen), 10% FBS (certified, Invitrogen) at 37°C, 5% (v/v) carbon dioxide. Media was replaced with 293 SFM II serum-free media approximately 24 h post transfection. For cell lines, media and whole-cell lysates were collected 48 h post transfection. Media was concentrated using Centricon Plus-20 filter units (Millipore). Whole-cell lysates were prepared in 50 μl of lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 24 mM $MgCl_2$, 0.5 mM Na-*o*-vanadate, 10% glycerol, 0.1% NP-40].

CETP activity assay

Lipid transfer activity was assessed using 6-({[4,4-difluoro-5-(2-thienyl)-4-bora-3a,4a-diaza-s-indacene-3-yl]styryloxy}acetyl) aminohexanoic acid (BODIPY)-CE assay as described (26). Ten microliters of cell lysates was combined with 80 μl of HDL (160-fold dilution in PBS; Biomedical Technologies, Inc.) in a black 96-well plate and incubated for 5 min at 37°C. Ten microliters of BODIPY-CE was added to the mixture, and fluorescence was determined in 10 min increments at 37°C in a Perkin Elmer Victor plate reader (excitation 485 nm, emission 520 nm). For each time point, values were normalized to mock-transfected cells.

Western analysis

Ten microliters of whole-cell lysate and concentrated serum-free media were electrophoresed on a 4–12% bis-acrylamide NuPage gel (Invitrogen). The proteins were transferred to nitrocellulose membrane using semi-dry transfer cell (BioRad). Mouse anti-V5 antibody (Invitrogen) and goat anti-mouse peroxidase-conjugated antibody were used, respectively, as probes. Super Signal West Pico chemiluminescent substrate (Pierce) was used to develop the blots. Protein detection was done using the GeneGnome chemiluminescence detection system (Syngene).

ER stress response gene expression

SW872 and HEK293A cells were plated in 12-well plates (3.5×10^5 cells/well) and allowed to adhere overnight. Cells were transfected with 3.2 μg DNA of the modified pSecTag2/Hygro (Invitrogen) plasmids described above. Transfections were carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. RNA was extracted from cells after 0, 6, 24, and 48 h (Qiagen RNeasy kit) and stored at −80°C until use. cDNA was synthesized from 1 μg RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) with a 10 min incubation at 25°C followed by 120 min at 37°C and 5 s at 85°C. mRNA expression for *GAPDH*, *DDIT3*, *HSPA5*, and *HERPUD1* was determined by quantitative PCR (TaqMan Fast Universal PCR Master Mix, Applied Biosystems) using one-fifth of the cDNA reaction on the ABI 7900HT with the following conditions: 95°C for 20 s followed by 40 cycles at 95°C for 1 s and 60°C for 20 s.

Protein expression and secretion of CETP(FL), CETP(−Ex9), and BPI (bactericidal permeability-increasing protein) were analyzed using whole-cell lysates and unconcentrated media from the transfections. Western analysis was done as described above using equal amounts of total protein for cell lysate samples or 10 μl of unconcentrated media.

RESULTS

Splice variation in *CETP* had been previously identified with exon 9, so this region was assessed in more detail in a variety of CETP-expressing tissues (**Fig. 1**). Primers were designed homologous to sequences in exons 8 and 16 so that all intervening splicing could be examined. As expected, the major bands corresponding to the full-length, wild-type transcript (697 bp) and the exon 9-deleted transcript (517 bp) were identified across the range of tissues tested. In heart and liver, the full-length transcript was the predominant species, whereas the exon 9-deleted species was more common in adipose, spleen, adrenal gland, and

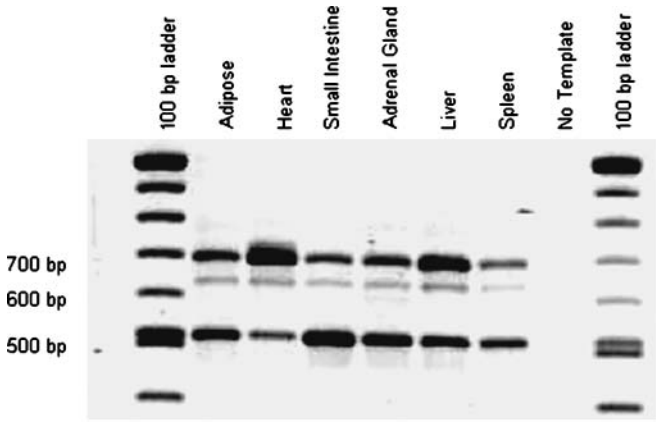

Fig. 1. Splice variants in different tissues. cDNA from various tissues was purchased from BD BioSciences and was amplified, electrophoresed on a Nusieve 3:1 agarose gel, and visualized. Fragments corresponding to full-length transcript (697 bp) and an alternatively spliced exon 9-deleted transcript (517 bp) were observed. An unidentified fragment migrating at approximately 600 bp was detected.

small intestine. In all tissues, a minor, previously uncharacterized band of intermediate size was also found.

To determine the identity of the minor variant, subcutaneous adipose tissue RNA from two female subjects was amplified and all three bands resequenced. The major bands corresponded to the expected full-length and exon 9-deleted sequences. The minor bands were also from the *CETP* gene and lacked exon 9, but both contained a novel but different exon. In one individual (BMI = 21), sequence analysis revealed that the inserted 108 bp was derived from an Alu-like sequence present in intron 10 of *CETP* (**Fig. 2A**). Flanking the 108 nucleotides are sequences with similarities to splice donor and acceptor recognition sites, suggesting the activation of cryptic splice sites (Fig. 2B). The sequence modifications yield a transcript coding for a protein missing the 60 amino acids from exon 9 and containing an additional 36 amino acids from the exonized sequence that we refer to as exon 10a. Despite both changes, the translational reading frame is maintained, resulting in a novel CETP protein that we refer to as CETP(−Ex9+Ex10a).

Sequence analysis of the ~600 bp fragment from the second subject (BMI = 37) revealed a transcript different from that isolated from the first subject. Like the other novel transcript, this lacked exon 9 sequences but instead of containing new sequences between exons 10 and 11, this transcript included an extra 102 nucleotides inserted between exons 11 and 12 (**Fig. 3A**). The insertion is also in-frame, adding an additional 34 amino acids to the protein. Sequence analysis showed that the 102 bp sequence originated from an Alu-like sequence found in intron 11 of *CETP*. Examination of the flanking genomic sequences revealed similarities to a consensus splice recognition site, indicating cryptic splice activation (Fig. 3B). The deletion of exon 9 (−Ex9) and incorporation of 102 nucleotides, termed exon 11a (+Ex11a), generates a newly identified variant of CETP that we designate CETP(−Ex9+Ex11a).

To evaluate the potential functional significance of the newly identified forms of CETP relative to full-length (FL) and exon 9-deleted proteins, cDNAs coding for CETP(FL), CETP(−Ex9), CETP(−Ex9+Ex10a), and CETP(−Ex9+Ex11a) were cloned into a modified pSecTag2/Hygro expression vector. The expression constructs were transiently transfected and expressed in a CETP-secreting human liposarcoma cell line, SW872, and a non-CETP-expressing cell, human embryonic kidney cells, HEK293S, previously used for generating specific *CETP* variants (26). Cell homogenates and media were collected from all clones 48 h post transfection and subjected to Western analysis. Using anti-V5 antibody to detect the recombinant CETP proteins, full-

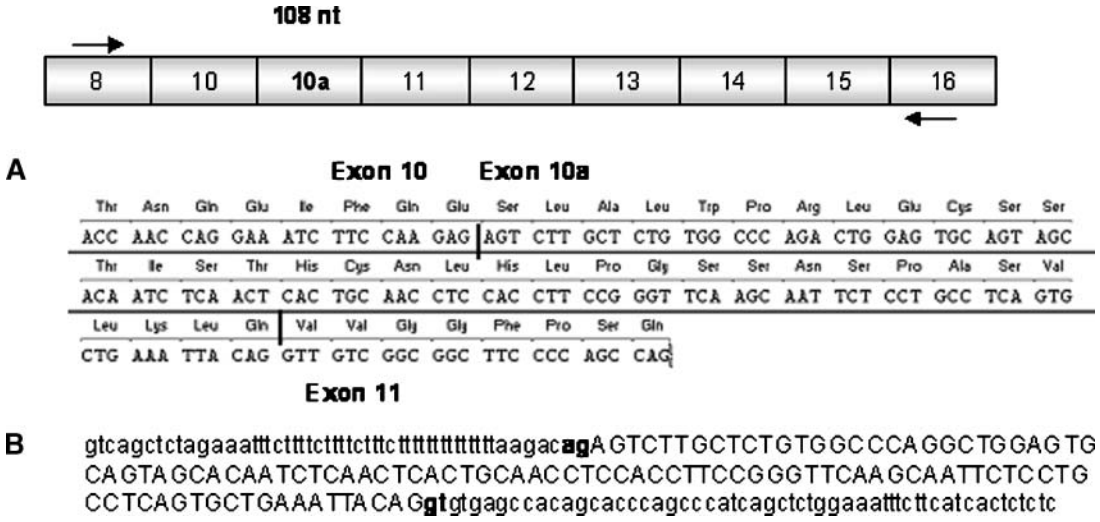

Fig. 2. Schematic representation of cholesteryl ester transfer protein (CETP) (−Ex9+Ex10a) showing deletion of exon 9 and incorporation of a 108 nt sequence between exons 10 and 11. A: Nucleotide and amino acid sequence of 108 bp insertion (exon 10a) with flanking exons. In-frame insertion results in addition of 36 amino acids. B: Region of CETP intron 10 showing exonized sequence (upper case) flanked by cryptic splice donor/acceptor sites.

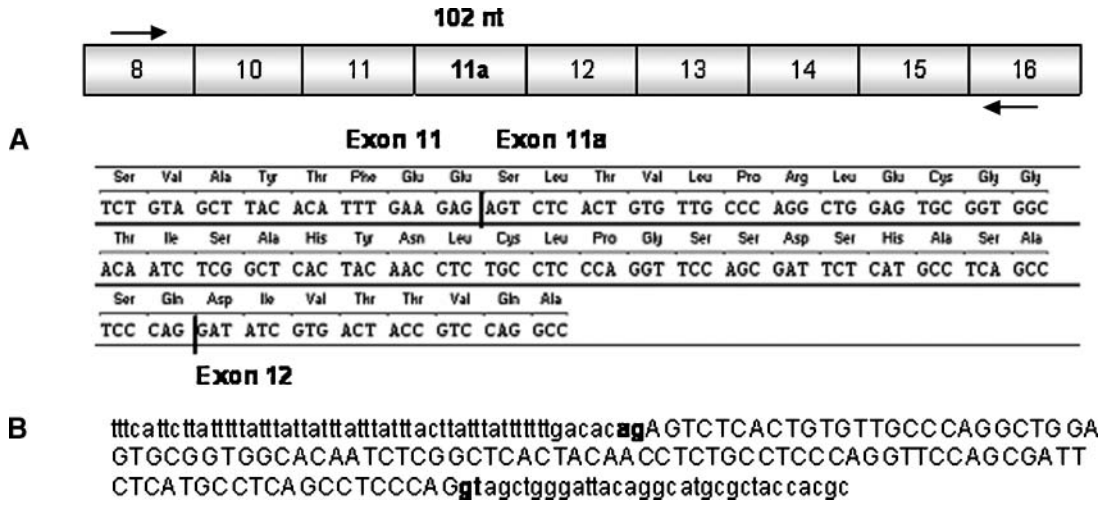

Fig. 3. Schematic representation of CETP (−Ex9+Ex11a) showing deletion of exon 9 and incorporation of a 102 nt sequence between exons 11 and 12. A: Nucleotide and amino acid sequence of 102 bp insertion (exon 11a) with flanking exons. In-frame deletion of exon 9 and insertion of exon 11a yields a protein lacking 60 amino acids and containing 34 amino acids. B: Region of CETP intron 11 showing exonized sequence (upper case) flanked by cryptic splice donor/acceptor sites.

length protein migrating at 60–65 kDa was detected in media and cell lysates for both SW872 and HEK293S cells (**Fig. 4**). The variant forms of CETP were expressed and visible as slightly lower molecular weight proteins in the cell lysates. The variants were visible but substantially reduced in the media from HEK293S cells and were undetectable in media from SW872 cells.

To assess the CE transfer activity of the newly identified variants, whole-cell lysates from transfected SW872 cells were analyzed in a BODIPY-CE transfer assay. The ability of CETP to transfer fluorescently labeled CEs from synthetic lipoproteins to HDL was measured. To normalize for endogenous CETP expressed by SW872 cells, relative fluorescence values from mock-transfected cells were subtracted from each time point. In contrast to full-length CETP protein, all alternatively spliced forms of CETP were found to be inactive in lipid transfer (**Fig. 5**).

The exon 9-deleted CETP protein has been characterized and shown to negatively affect the secretion of full-length CETP protein. The shortened CETP is retained mostly in the ER (21). To determine whether CETP(−Ex9+Ex10a) or CETP(−Ex9+Ex11a) proteins also have a negative effect when coexpressed with full-length protein, expression constructs bearing cDNAs for full-length and the alternatively spliced variants of CETP were cotransfected in SW872 cells. Western analysis confirmed coexpression of transfected proteins in cell lysates (**Fig. 6A**). However, secretion of full-length protein in the media was inhibited by the coexpression of the shortened CETP protein variants.

In addition to the effect on secretion, the impact of splice variation on activity was also examined by coexpressing CETP(−Ex9+Ex10a), CETP(−Ex9+Ex11a), and CETP(−Ex9) with CETP(FL) and assessing transfer activity with cell lysates from the cotransfected cells. A considerable reduction in full-length transfer activity was observed in the presence of the alternatively spliced variants of CETP (Fig. 6B), an effect consistent with previous results (21). To test whether the interaction between full-length and alternatively spliced isoforms occurs at the cellular level or during the in vitro assay, cell lysates from SW872 cells transfected singly with full-length *CETP* cDNA were combined with cell lysates from single transfections of alternatively spliced variants. The combined lysates were assayed for transfer activity. Full-length CETP transfer activity was unaffected, indicating that the inhibitory effect of the alternatively spliced isoforms occurs intracellularly and not during the assay itself.

We did not detect transcripts in which exons 10a or 11a were inserted when exon 9 was retained, but there is the possibility that such a transcript could occur in a particular tissue or metabolic condition not tested by us. To determine whether such a protein would be active, artificial cDNAs were constructed with full-length CETP sequence and insertions of the novel exons. These constructs were then transfected into HEK293S cells, and both the media and cell lysates were examined. Western analysis confirmed expression of the extended CETP variants with protein observable in the cell lysates. As in the other alternatively spliced products, there were very low levels of secreted protein in the media (data not shown). Both the media and cell lysates were assayed for CE transfer activity using the fluorescent BODIPY assay. No detectable activity was found with either protein containing the novel exons (data not shown).

The poor secretion of the spliced variants, despite good intracellular expression and their ability to inhibit secretion of wild-type CETP, suggested that they might cause disruptions in the ER, where CETP is processed for secretion. To investigate this, wild-type CETP and the exon 9-deleted CETP variant were transfected into both SW872 and HEK293A cells, and the expression of three known ER stress genes, *HERPUD1*, *HSPA5* (heat-shock 70 kd protein 5), and *DDIT3* (DNA damage-inducible transcript 3), was monitored as a function of time. Prior to transfection with *CETP*

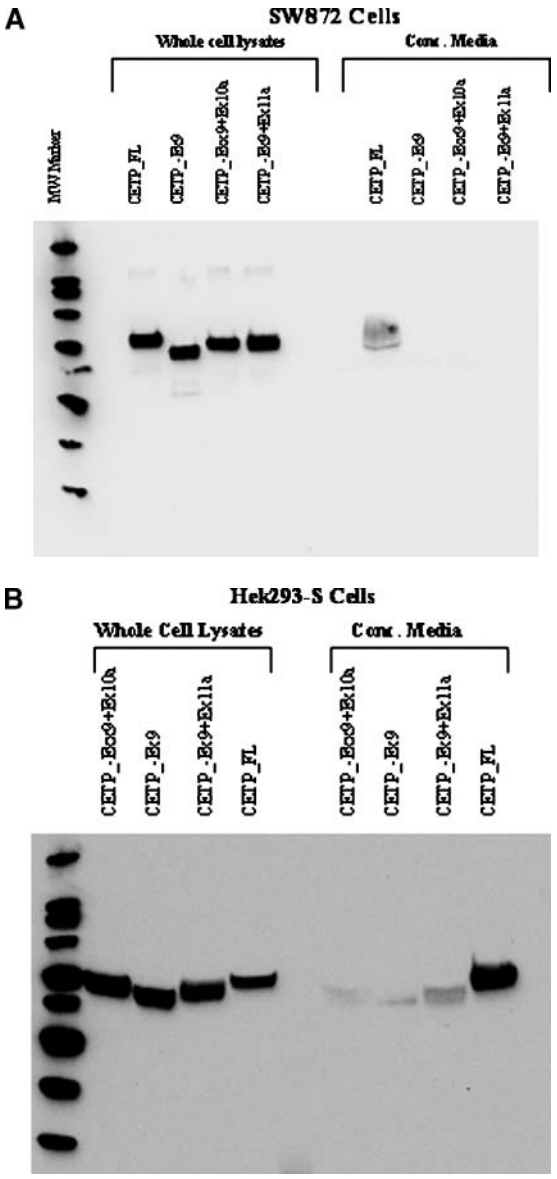

Fig. 4. Western analysis showing localization of various CETP proteins in cell lysates and media. Expression constructs containing different *CETP* cDNAs coding for full-length transcript CETP(FL), exon 9-deleted transcript CETP(−Ex9), and the newly identified transcripts CETP(−Ex9+Ex10a) and CETP(−Ex9+Ex11a) proteins were transiently transfected in human adipocytic cell line SW872 (A) and human embryonic kidney cell line HEK293S (B).

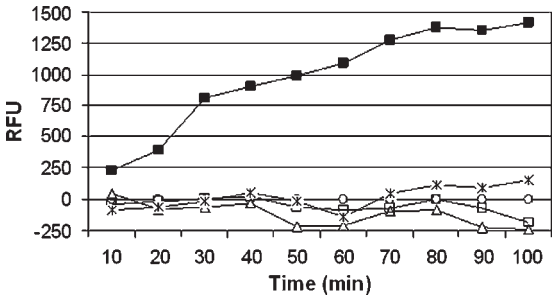

Fig. 5. 6-({[4,4-difluoro-5-(2-thienyl)-4-bora-3a,4a-diaza-s-indacene-3-yl]styryloxy}acetyl)aminohexanoic acid (BODIPY)-cholesteryl ester transfer activity of the CETP proteins derived from cell lysates from transiently transfected SW872 cells. Transfer activity indicated by relative fluorescence units (RFU) was monitored at 37°C. Values were normalized to time = 0 and mock-transfected cells at each time point. Full-length CETP (filled squares), CETP(−Ex9) (triangles), CETP(−Ex9 +Ex10a) (open squares), CETP(−Ex9+Ex11a) (asterisk), and the reference empty vector (circles) are shown.

and other vectors, a typical ER stress response with both HEK293A and SW872 cells was verified by treating with tunicamycin and monitoring expression of the chosen stress genes over time (data not shown).

In all transfections, empty expression vector and the same vector expressing the homologous protein BPI were included as controls. *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase), a control gene, was essentially unaffected by any of the expression vectors at any time. In contrast, *HERPUD1*, *HSPA5*, and *DDIT3*, although relatively unaffected at 6 h, showed increases in mRNA levels at 24 and 48 h. In HEK293A cells, empty vector and *BPI* caused changes in mRNA of less than 2-fold in *HSPA5* at 24 and 48 h, whereas transfection of the same amount of full-length and exon 9-deleted *CETP* caused increases of 8–12-fold. *HERPUD1* mRNA increased about 14-fold at 48 h for both full-length and exon 9-deleted *CETP*, whereas empty vector and *BPI* produced changes of less than 3-fold. For all transfected DNAs, the response of *DDIT3* was larger than those of *HERPUD1* and *HSPA5*. The control vector and *BPI* increased *DDIT3* by 6–9-fold at 48 h, whereas the two versions of *CETP* caused larger increases of 16–25-fold. The same rank order of effects was observed in SW872 cells, but the magnitude of the fold changes was smaller. The act of transfecting cells could, in theory, induce the ER stress response, as could the high level of expression caused by expression vectors. These factors alone, however, cannot explain the *CETP* effects, inasmuch as empty vector and the BPI expression vector do not generate the same level of response. The very high expression and secretion of BPI (**Fig. 7**), which has a similar sequence and structure relative to CETP, shows that it also is not simply an overloaded secretion machinery causing the ER stress response. The much higher levels of BPI secretion cause less of an effect than does secretion of a smaller amount of CETP.

To determine whether the expression of HERPUD1 might affect CETP expression, the expression vectors with each gene were cotransfected, CETP concentration was determined via Western analysis, and activity was determined using a fluorescent CE transfer assay as above. There may have been a modest decrease in CETP activity, but any differences were too small to be considered reliable (data not shown).

DISCUSSION

ER stress and the unfolded protein response have been linked to a variety of disease states, including many aspects of cardiovascular and metabolic disease (27). For example, signs of ER stress are readily detected in unstable plaques

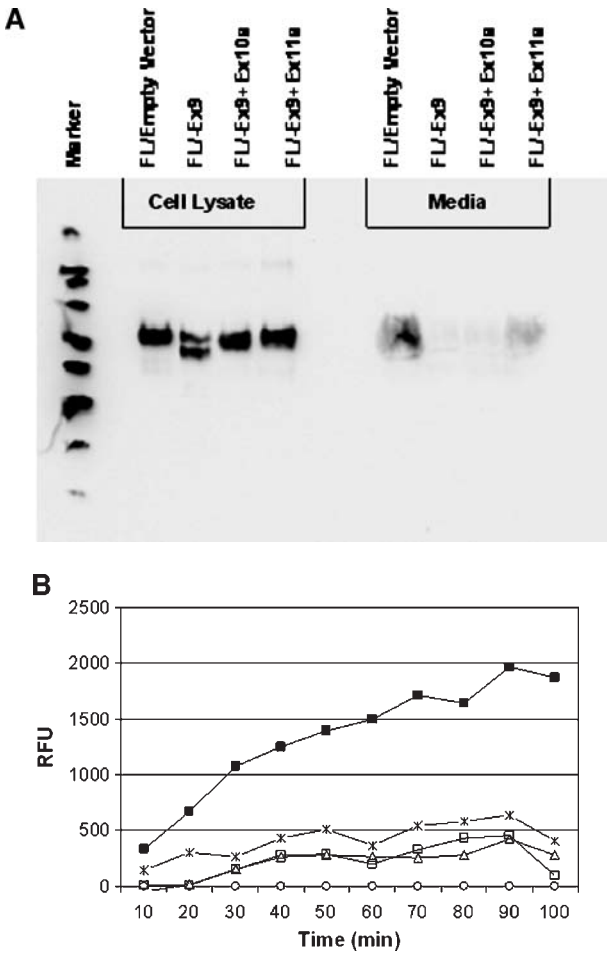

Fig. 6. Effect of coexpression of full-length *CETP* and alternatively spliced isoforms. Expression plasmids containing full-length *CETP* and alternatively spliced isoforms were cotransfected in SW872 cells. A: Western analysis of CETP proteins expressed in cell lysates and media. B: Effect on full-length CETP transfer activity by coexpression of the alternatively spliced variants. Full-length CETP was analyzed alone (filled squares) or cotransfected with CETP(−Ex9) (triangles), CETP(−Ex9+Ex10a) (open squares), or CETP(−Ex9+Ex11a) (asterisk). Empty vector alone (circles) is used as reference. RFU, relative fluorescence units.

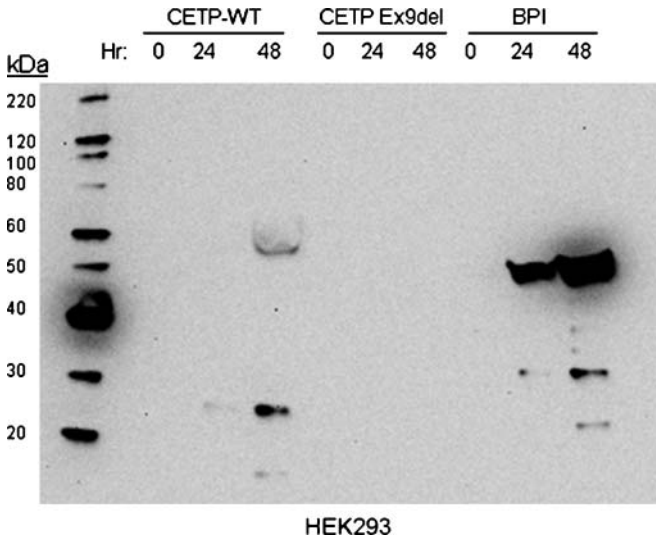

Fig. 7. Secretion of transfected proteins. The same amount of each expression vector was transfected into HEK293A cells and media collected at 24 and 48 h. Equivalent volumes of unconcentrated media were run on a polyacrylamide gel and Western blotted as above. Full-length CETP (lanes 2–4), exon 9-deleted CETP (lanes 5–7), and bactericidal permeability-increasing protein (BPI) (lanes 8–10) are shown.

in the vessel wall (28) as well as other tissues that mediate cardiovascular disease. Adipocyte stress has been shown to affect metabolic diseases (25), and adipocytes are particularly relevant because they play an important role in storage and transfer of multiple forms of lipids. Because a proper balance of lipids is necessary to maintain ER function, *CETP* could clearly play an important role, both directly, through its lipid transfer actions, as well as indirectly, by affecting ER stress if folded or secreted inefficiently or simply expressed at higher than normal levels. Lipid composition also affects the ratio of alternatively spliced transcript relative to normally spliced transcript (22), providing an additional mechanism for *CETP* to impact the process. The location of a known stress protein, *HERPUD1* (24), directly upstream of *CETP* and transcribed in the same direction, suggests that additional possible regulatory circuits may be present.

Alternative splicing has been shown to play an important role in the regulation of many genes, with this altered expression having an impact on many disease states (29). Many alternatively spliced genes have been found to include Alu sequences as "exonized" DNA (30). These Alu sequences must be present in the minus orientation so that the oligo T sequence can be used as the polypyrimidine splicing signal and the AG sequence starting eight base pairs downstream as the 3′ splice acceptor site. At the 5′ splice donor site, there is generally a C–T change at position 156 in the Alu consensus and +1 relative to the splice, creating a consensus GT splicing site. More often than the typical Alu sequence, the consensus G at position 153 in the Alu consensus is retained, +5 relative to the splice site (30). To a degree, exon 10a fits this pattern, starting and ending where predicted by other exonized Alu sequences, even though the resultant exon is several bases shorter because of a deletion 13 bp proximal to the splice site. Additionally, the Alu sequence that generates exon 10a is not full-length, diverging sharply from the consensus sequence just beyond where splicing occurs. Exon 11a is also not a standard exonized Alu sequence, in that there is no change at position 153 and the site of splicing does not match the position typically seen in Alu sequences. Instead, there are other changes just upstream of the normal splice site that generate an alternate AGGT sequence that is then used as a splice donor sequence rather than the one normally used in exonized Alu sequences.

With *CETP*, the major alternative splicing product can occur at high frequency, and it appears to be regulated in a physiologically meaningful way (22). The major alternatively spliced product, missing exon 9, is in-frame, but

the loss of 60 amino acids in the central region of the protein is expected to have major structural and functional consequences. Based on the CETP crystal structure (31), one of the two lipid binding sites and the neck region that allows transfer of lipids between binding sites will be eliminated. Indeed, this protein was previously shown to be inactive, and we have confirmed that finding. Our results for the tissue specificity of exon 9 deletion using PCR are also very consistent, compared with the earlier results using RNase protection (20). As also found previously, cotransfection of wild-type and exon 9-deleted constructs significantly reduced the wild-type activity. When either exon 10a or exon 11a is inserted in the exon 9-deleted sequence, the same result is obtained. Whether this would also occur in a physiological situation or whether this is unique to the cotransfection system is not clear.

Initially, we expected that the poorly secreted and probably unfolded exon 9-deleted CETP isoform would have a bigger impact on ER stress than the properly folded and secreted full-length CETP. However, as is clear from Fig. 7, even though full-length CETP is secreted much better than the exon 9-deleted version, it is secreted far less well than the similar protein, BPI, when expressed under the control of the same promoter. Thus, even native *CETP* expression is sufficient to induce ER stress, and mild changes in folding efficiency due to alterations in the ER stress response could have dramatic effects on the magnitude of CETP secretion.

Further complicating the potential role of *CETP* on ER stress is the *HERPUD1* gene, located immediately upstream of *CETP*. The degree of coregulation of these genes is uncertain, but their proximity introduces the possibility of a variety of feedback loops in which higher levels of *CETP* lead to increased ER stress and this feeds back on *CETP* activity. The roles that lipid composition in the ER, cholesterol synthesis, and other ER functions play in regulating secretion of CETP as well as CETP's impact on the ER are clearly complex and will require additional studies to dissect.

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