Association of cholesteryl ester transfer protein with HDL particles reduces its proteolytic inactivation by mast cell chymase

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Abstract **Human atherosclerotic intima contains mast cells that secrete the neutral protease chymase into the intimal fluid, which also contains HDL-modifying proteins, such as cholesteryl ester transfer protein (CETP), in addition to abundant amounts of nascent discoidal HDL particles. Here, we studied chymase-dependent degradation of *a*) CETP isolated from human plasma and *b*) CETP-HDL complexes as well as the functional consequences of such degradations. Incubation with chymase caused a rapid cleavage of CETP, yielding a specific proteolytic pattern with a concomitant reduction in its cholesteryl ester transfer activity. These chymase-dependent effects were attenuated after CETP was complexed with HDL. This attenuation was more effective when CETP was complexed with HDL_3 and HDL_2 than with discoidal reconstituted high density lipoprotein (rHDL). Conversely, rHDL, but not spherical HDLs, was protected in such CETP complexes against functional inactivation by chymase. Thus, in contrast to the complexes of CETP with spherical HDLs, the ability of the CETP-rHDL complexes to promote cholesterol efflux from macrophage foam cells remained unchanged, despite treatment with chymase. In summary, complexation of CETP and HDL modifies their resistance to proteolytic inactivation: spherical HDLs protect CETP, and CETP protects discoidal HDL. These results suggest that in inflamed atherosclerotic intima, CETP, via its complexation with HDL, has a novel protective role in early steps of reverse cholesterol transport.**—Lee-Rueckert, M., R. Vikstedt, J. Metso, M. Jauhiainen, and P. T. Kovanen. **Association of cholesteryl ester transfer protein with HDL particles reduces its proteolytic inactivation by mast cell chymase.** ***J. Lipid Res.*** **2008.** 49: **358–368.**

Supplementary key words proteolysis • cholesterol efflux • reverse cholesterol transport • high density lipoprotein

The various subclasses of HDL play a crucial role in the initiation of reverse cholesterol transport by mediating the transfer of excess cholesterol from the macrophages in atherosclerotic lesions to the liver for excretion (1). In plasma, several enzymes and lipid transfer proteins continuously remodel HDL, thus modulating HDL functions. Discoidal preβ-migrating HDL (preβ-HDL) particles are the preferred substrates for LCAT, which promotes their maturation to spherical α-HDL particles (2). The two plasma lipid transfer proteins, cholesteryl ester transfer protein (CETP) and phospholipid transfer protein (PLTP), in turn remodel HDL, with ensuing generation of preβ-HDL particles (3). Both preβ-HDL and α-HDL particles promote the efflux of cholesterol from macrophage foam cells via the ATP binding cassette transporters ABCA1 and ABCG1 (4).

CETP is a glycosylated protein of 476 amino acids that transfers cholesteryl esters, triglycerides, and phospholipids between circulating lipoproteins (5). In plasma, CETP is associated mainly with HDL particles, primarily the HDL_3 subclass (6). The recently defined crystal structure of CETP has suggested a novel mechanism involving the movement of lipids through a continuous tunnel, thus adding complexity to CETP structure-function relationships (7). Despite considerable research into the functions of CETP, its role in atherosclerosis still remains unknown (8, 9). In addition to the liver, CETP is synthesized by smooth muscle cells and monocyte-derived macrophages in the human aortic wall (10) as well as by adipocytes (11). However, the physiological function of CETP in these cells, or in the extracellular fluid surrounding them, has yet to be clarified. Interestingly, expression of CETP in macrophages is under regulation by liver X receptor and retinoid X receptor (12). Because cholesterol loading in macrophages via the formation of oxysterols upregulates these receptors, it also leads to increased secretion of CETP, a response that has been considered to prevent the accumulation of cellular cholesterol (13). The concomitant secretion of CETP and PLTP by macrophage foam cells (10, 14) raises the question of whether CETP may assist PLTP in facilitating cellular choles-

Manuscript received 31 August 2007 and in revised form 30 October 2007.
Published, JLR Papers in Press, November 8, 2007.
DOI 10.1194/jlr.M700392-JLR200

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terol efflux to HDL (15, 16). Because CETP mass was found to be abundant in the macrophage foam cells of human atherosclerotic lesions and CETP transfection in COS cells increased cholesterol efflux, a direct antiatherogenic role of CETP in the removal of cholesterol from foam cells has been suggested (17). It is intriguing to speculate that the role of CETP in atherosclerosis may differ in plasma and in atherosclerotic lesions, as has been suggested for PLTP (18–20).

Several types of inflammatory cells accumulate at the sites where atheromas develop, including mast cells (21). A fraction of the mast cells in the human arterial intima synthesize the neutral serine protease chymase and store it in their secretory granules (22). Upon stimulation, mast cells release chymase-containing granules into the intimal fluid, where chymase in its natural form (i.e., bound to the granule-derived heparin) remains partly resistant to protease inhibitors present in the intimal fluid (23) and, thus, may degrade a variety of extracellular and pericellular proteins (21). Importantly, mast cells, via the secretion of chymase and other active compounds, exert various effects in the cardiovascular system. We believe that by promoting cholesterol accumulation and plaque vulnerability, and by locally regulating hemostasis, mast cells in atherosclerotic lesions have the potential to contribute to the clinical outcomes of atherosclerosis, such as myocardial infarction and stroke (24).

Concerning the reverse cholesterol transport, we have found that chymase is able to degrade apolipoprotein A-I (apoA-I) in isolated HDL particles as well as in unfractionated plasma and intimal fluid (25). Furthermore, studies in vitro have demonstrated that chymase specifically depletes preβ-HDL particles (26), thus blocking cholesterol efflux mediated by the ABCA1 pathway (27). In contrast, limited proteolysis of HDL_3 particles by chymase does not impair their ability to activate LCAT (28) or to act as phospholipid acceptors in PLTP-mediated phospholipid transfer reactions (29). These observations reveal that the spherical α-HDL particles primarily remain functional even after being exposed to chymase. Chymase also degrades PLTP, thereby reducing its phospholipid transfer activity (29). However, it is not known whether chymase is able to degrade CETP, its structurally closely related lipid transfer protein. Here, we examined in vitro the effect of human chymase on the structural integrity and cholesteryl ester transfer function of human CETP and the influence of CETP binding on different types of HDL particles with respect to the susceptibility of CETP to chymase-mediated proteolysis. In addition, we investigated the effect of CETP on HDL-facilitated cholesterol efflux and whether chymase would modify this process. Our data provide an insight into the possible fate of CETP in atherosclerotic lesions, within which proinflammatory cells secrete proteolytic enzymes into the intimal fluid.

MATERIALS AND METHODS

Isolation and purification of CETP

CETP was isolated from human plasma by modifying an earlier procedure (30). Lipoprotein-deficient plasma was obtained by sequential ultracentrifugation of plasma at d = 1.21 g/ml. After adding 50 U/ml Trasylol and 5 mM β-mercaptoethanol, the lipoprotein-deficient plasma was applied to a Butyl-Toyopearl 650M column (column dimensions, 5 × 30 cm; TOSO Corp., Tokyo, Japan) and recycled overnight at 4°C at a flow rate of 10 ml/min. After washing of the column with 50 mM Tris-HCl and 1 mM EDTA, pH 7.4, the column was eluted with 3 mM Tris-HCl and 1 mM EDTA, pH 7.4, and 10 ml fractions were collected. Fractions containing CETP activity were pooled, 50 U/ml Trasylol and 5 mM β-mercaptoethanol were added, and the pool was applied to a heparin-Sepharose CL-4B affinity chromatography column (column volume, 50 ml) and recycled overnight at 4°C at a flow rate of 1 ml/min with 25 mM Tris-HCl and 1 mM EDTA, pH 7.4. Fractions containing CETP activity were pooled and applied to a HiTrap heparin column (column volume, 5 ml; GE Healthcare) with the above buffer at a flow rate of 2 ml/min, and the fractions containing CETP activity were pooled again.

Heparin affinity chromatography steps were used to separate CETP from the PLTP that specifically binds to heparin (31). The eluted pool containing CETP activity was next applied to a Mono Q HR 5/5 anion-exchange column (GE Healthcare) at a flow rate of 1 ml/min using 25 mM Tris-HCl and 1 mM EDTA, pH 7.4, buffer. After washing, the bound proteins were eluted with a linear NaCl gradient from 0 to 500 mM. Active fractions were applied to a hydroxyl apatite column (column volume, 2 ml; Bio-Rad, Richmond, CA) at a flow rate of 0.5 ml/min with 1 mM Na-phosphate buffer, pH 6.8, containing 150 mM NaCl. After washing of the column with the same buffer, the bound CETP was eluted with a linear gradient of Na-phosphate from 1 to 50 mM containing 150 mM NaCl for 60 min at a flow rate of 0.5 ml/min, and 1 ml fractions were collected. CETP activity eluted at the Na-phosphate concentration range of 15–25 mM. These fractions were devoid of LCAT and PLTP activity, and no apoA-I was detected in Western blot analysis. The active fractions were pooled and stored in 0.5 ml aliquots at −70°C. Five similarly purified CETP batches were used in this study, with individual batches showing a single broad band of CETP (apparent molecular mass of ~76 kDa) in Western blot analysis. Cholesteryl ester transfer activity ranged from 4.1 to 14 nmol/ml/h. No degradation of CETP protein was found after incubation of the preparations at 37°C for up to 24 h, revealing that the CETP preparations contained no detectable CETP-degrading protease activity.

Human chymase

Recombinant human chymase (specific activity, 80 N-benzoyl-L-tyrosine ethyl ester (BTEE) units/μg) was kindly provided by Teijin, Ltd. (Hino, Tokyo, Japan). This heparin-free chymase is fully inhibited by soybean trypsin inhibitor (SBTI; Sigma-Aldrich, St. Louis, MO) at a final inhibitor concentration of 100 μg/ml.

Isolation and radiolabeling of lipoproteins

LDL (d = 1.019–1.063 g/ml), HDL_2 (d = 1.063–1.125 g/ml), and HDL_3 (d = 1.125–1.210 g/ml) were prepared from freshly isolated normolipidemic human plasma by sequential ultracentrifugation using KBr for density adjustment. The concentrations of lipoproteins used in the experiments are given in terms of their protein content. The HDL_3 preparation used in this study contained 0–1.5% of preβ-migrating species. LDL was acetylated in the presence of acetic anhydride (32), and the acetyl-LDL was radiolabeled by treatment with [1,2-^{3}H]cholesteryl oleate (Amersham Biosciences, Piscataway, NJ) dissolved in dimethyl sulfoxide (33). The specific activity of the [^{3}H]CE-acetyl-LDL preparation was 46 dpm/ng protein. Isolated HDL_3 and HDL_2 contained traces of immunodetectable CETP, and their choles-

teryl ester transfer activity was very low (0.71 ± 0.12 and 0.53 ± 0.08 nmol/ml/h, respectively).

Preparation of discoidal reconstituted HDL

ApoA-I-containing reconstituted high density lipoprotein (rHDL) particles were prepared by the cholate dialysis method. In brief, egg yolk phosphatidyl choline [PC; 10 mg/ml stock in chloroform-methanol (9:1, v/v)] and cholesterol (1 mg/ml stock in benzene) were added to a glass vial, and the organic solvents were evaporated under nitrogen at room temperature. After this, the mixture was lyophilized for 30 min to remove the final traces of solvents. After lyophilization, 3 ml of TBS buffer (10 mM Tris-HCl, 1 mM EDTA, and 140 mM NaCl, pH 7.4) was added, the mixture was vigorously vortexed, and 1 mg of lipid-free apoA-I (kindly provided by Dr. Peter Lerch, Swiss Red Cross, Bern, Switzerland) was added. Sodium cholate (stock solution; 0.725 M in TBS) was then added (final concentration, 55 mM), and the mixture was gently vortexed (4 × 15 s) by strictly avoiding foaming and then incubated for 20 min at 24°C in a shaking water bath. Finally, the mixture was dialyzed against TBS for ~72 h at 4°C using 3,500 cutoff dialysis tubing, after which the final volume of the mixture was adjusted to 4 ml. The apoA-I/PC/cholesterol molar ratios of the discoidal rHDL particles ranged from 1:139:7 to 1:100:12.5, with the final preparation being composed of three subpopulations having diameters of 9.5 (major subpopulation), 12, and 15 nm. The size analysis was based on both the asymmetrical flow field-flow fractionation method (34) and native gradient gel electrophoresis (35).

CETP activity

CETP activity was analyzed by a radiometric method as a transfer/exchange of radiolabeled [^{14}C]cholesteryl oleate (Amersham Biosciences) between exogenously added human LDL and HDL, as described previously (36, 37). Radioactivity in HDL as a measure of transfer activity was determined by liquid scintillation counting. The activity of CETP was expressed as nanomoles of cholesteryl ester transferred per milliliter per hour.

Binding of CETP to apoA-I-containing particles

A brief incubation of lipid-free apoA-I or apoA-I-containing HDL with CETP generates CETP-containing complexes (38, 39). To promote CETP complexation, we incubated CETP (6.2–10.9 nmol/ml/h) for 1 h at room temperature with lipid-free apoA-I, or with mature α-HDL particles (HDL_2 or HDL_3), or with discoidal rHDL (50 μg each) in 120 μl of 5 mM Tris-HCl containing 150 mM NaCl and 1 mM EDTA, pH 7.4 (TNE buffer). As a control, CETP was incubated alone under identical conditions. Analysis of the mixtures was performed by gradient gel electrophoresis, as reported earlier (40), followed by immunoblotting using the CETP-specific monoclonal antibody (MAb) TP2 (41) (a kind gift from Dr. Yves Marcel, Ottawa, Canada). Consistent with previous reports (38, 39), incubation of CETP with either lipid-free apoA-I or the various HDL particles shifted CETP mobility to discrete bands of higher molecular weights reflecting the formation of apoA-I-containing complexes of CETP (data not shown).

Proteolysis of CETP by chymase

Chymase (18 BTEE units) was added to incubation mixtures containing CETP that had been incubated alone for 1 h at room temperature under the conditions described, or CETP complexed with various apoA-I-containing particles, and the incubations were continued at 37°C for up to 6 h. Aliquots of the mixtures were taken at different time intervals, and SBTI was added immediately to fully inhibit chymase activity. The aliquots were applied to 12.5% SDS-PAGE, and proteins were transferred onto nitrocellulose membranes, immunoblotted using the CETP MAb TP2, and finally visualized by chemiluminescence (ECL Plus reagent; GE Biosciences, Buckingshamshire, England) to detect intact CETP and its proteolytic fragments. Semiquantitative analysis of the band denoting intact CETP was performed by scanning (Bio-Rad Gel Doc 2000 densitometer) the X-ray film. To render comparable results from individual experiments replicated two to five times using different batches of CETP, the densitometric readings were expressed as percentages of the control band at time 0. Variation in CETP activity during proteolysis was determined in aliquots of the incubation mixtures at the given time points. To compare the results of experiments in which different batches of CETP were treated with chymase for various time intervals, CETP activity was expressed as a percentage of activity at time 0. Proteolysis of the endogenous CETP contained in HDL_3 and HDL_2 was evaluated, under the conditions described above, after incubation of each HDL subfraction with chymase.

Proteolysis of apoA-I in CETP-HDL complexes

In a preliminary experiment, we preincubated lipid-free apoA-I (50 μg) in the absence or presence of either CETP (~1 μg of CETP protein was evaluated by silver staining) or PLTP (1 μg of PLTP protein based on ELISA) (42), and in the presence of both, in 250 μl of TNE buffer at room temperature for 1 h. Then, the incubations were continued at 37°C for 6 h in the absence or presence of chymase (18 BTEE units). Aliquots of the incubation mixtures were applied to 12.5% SDS-PAGE gels, and protein bands were visualized by Western blotting of apoA-I and its fragments. The nitrocellulose membranes were treated with a polyclonal anti-apoA-I antibody raised in rabbits against purified lipid-free apoA-I in our laboratory (R 297 IgG; 1:1,000 dilution), and the bands were detected by ECL. Next, to study the susceptibility to proteolysis of lipidated apoA-I contained in the CETP complexes, 50 μg of discoidal rHDL, HDL_3, or HDL_2 was preincubated for 1 h at room temperature in the absence or presence of CETP (6.8 nmol/ml/h) in 250 μl of TNE buffer and then treated with chymase (18 BTEE units) under the conditions described above. Aliquots of the chymase-treated mixtures were applied to 12.5% SDS-PAGE, and the protein bands were subjected to Western blot analysis to visualize the degraded protein fragments. The effect of chymase on functional domains of apoA-I was further studied by Western blot analysis by the use of specific monoclonal antibodies targeted at either the N-terminus (residues 2–8) (MAb 4H1; kindly obtained from Dr. Yves Marcel) or the C-terminal region (residues 211–220) (MAb 4.1; kindly provided by Drs. Noel Fidge and Dmitri Sviridov, Prahran, Australia) of apoA-I.

Cell culture and loading of cells with cholesteryl esters

Human THP-1 monocytes (catalog No. TIB-202; American Type Culture Collection, Manassas, VA) were grown and maintained in complete RPMI 1640 medium containing 10% (v/v) FBS, 10 mM HEPES, and penicillin (100 U/ml)/streptomycin (100 μg/ml) at 37°C in 5% CO_2 and 95% air. To induce differentiation of the THP-1 cells into macrophages, the cells were plated onto 24-well plates and treated with 100 nM phorbol 12-myristate-13-acetate (Sigma-Aldrich) for 72 h before the experiments. After differentiation, the macrophages were washed twice with PBS and loaded by incubating them with 25 μg/ml [^{3}H]cholesteryl oleate-labeled acetyl-LDL in RPMI 1640 supplemented with 5% (v/v) lipoprotein-deficient serum, 10 mM HEPES, and penicillin/streptomycin for 48 h. After

loading, the macrophage foam cells were washed twice with PBS and cholesterol acceptors were added to the culture medium for evaluation of cellular cholesterol efflux.

Efflux of cellular cholesterol promoted by various acceptors

Lipid-free apoA-I, discoidal rHDL, HDL_3, and HDL_2 (100 μg each) were first preincubated in the absence or presence of CETP (5.2 nmol/ml/h) in 250 μl of TNE buffer at room temperature for 1 h. Then, incubations were continued in the absence or presence of chymase (18 BTEE units) at 37°C for 6 h. After the incubations were terminated by adding SBTI to fully inhibit chymase, aliquots of the incubation mixtures were added to the THP-1 macrophage foam cells. To evaluate cholesterol efflux, the cells were incubated in serum-free RPMI 1640 supplemented with 10 mM HEPES and antibiotics containing aliquots of the above incubation mixtures to provide the following concentrations of acceptors in medium: lipid-poor apoA-I (10 μg/ml); discoidal rHDL, HDL_3, and HDL_2 (25 μg/ml each). After incubation at 37°C for 16 h in a humidified CO_2 incubator, the media were collected and centrifuged at 2,500 rpm for 5 min to remove the detached cells. For quantification of cholesterol efflux, radioactivities in the media were determined by liquid scintillation counting (Wallac WinSpectral 1414; Wallac, Turku, Finland). The cells were then washed twice with PBS, lysed using 0.2 M NaOH, and analyzed for radioactivity. Fractional cholesterol efflux in each well was expressed as percentage radioactivity in medium relative to the total radioactivity contained in the medium and the cells. Cholesterol efflux into the incubation medium in the absence of cholesterol acceptors was considered as basal efflux and was subtracted from the efflux values in the presence of various acceptors.

Other methods

The protein content and proteolytic activity of chymase were measured using BSA as the standard and BTEE as the substrate, respectively (43, 44). Statistical significance was determined by the two-tailed Student's *t*-test.

RESULTS

Effect of chymase on the proteolytic degradation and lipid transfer activity of CETP

Because CETP in the intimal fluid has two origins (i.e., macrophage-derived or plasma-derived, the former apparently not being initially associated with HDL particles and the latter being associated), we incubated CETP with chymase either as such or after complexing CETP with various types of HDL particles. The degrees of proteolysis of CETP and of CETP-HDL complexes were compared. For complexation, CETP was incubated in the presence of lipid-free apoA-I and discoidal rHDL, HDL_3, or HDL_2 at room temperature for 1 h. Immunoblot analysis confirmed comigration of apoA-I with CETP in each case (data not shown). After a 1 h preincubation, chymase was added to the mixtures, incubations were continued for up to 6 h, and the proteins were separated by SDS-PAGE and assessed by Western blot using TP2 specific antibody against CETP. The results of representative experiments with the various complexes are shown in **Fig. 1A**, and they reveal that CETP was rapidly degraded by chymase, gen-

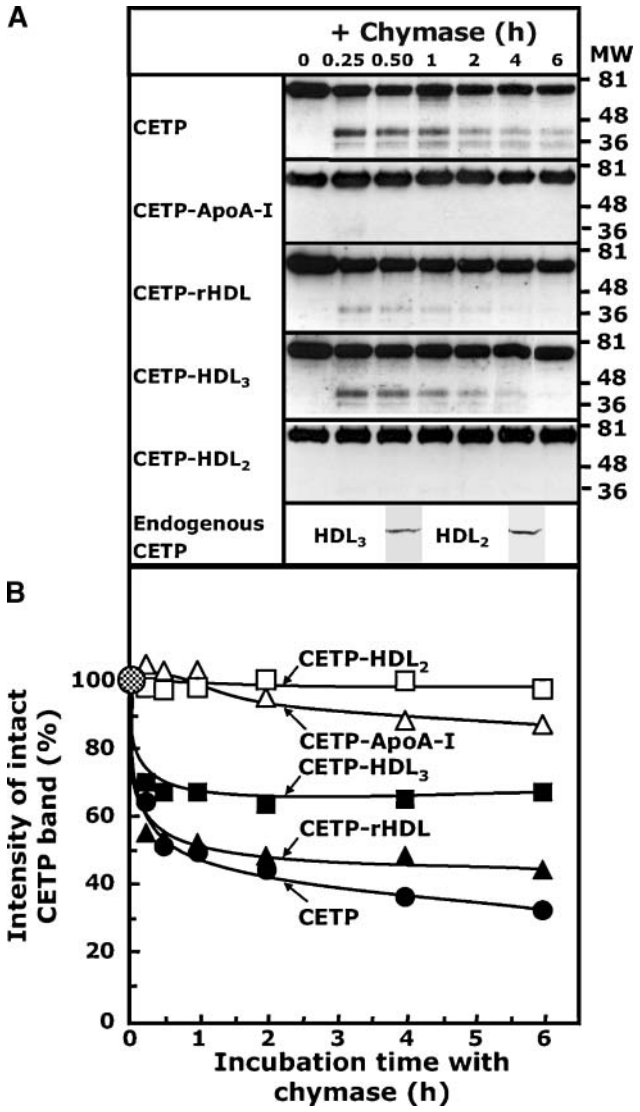

Fig. 1. Degradation of purified cholesteryl ester transfer protein (CETP) and CETP-HDL complexes by chymase. CETP was preincubated alone or with lipid-free apolipoprotein A-I (apoA-I), discoidal reconstituted high density lipoprotein (rHDL), HDL_3, or HDL_2 (50 μg each) in 120 μl of TNE buffer, pH 7.4, for 1 h at room temperature. Chymase (18 N-benzoyl-L-tyrosine ethyl ester (BTEE) units) was added to the mixtures, and incubation continued at 37°C for up to 6 h. A: Aliquots of the incubation mixtures were applied to 12.5% SDS-PAGE, proteins were transferred to nitrocellulose membranes, and CETP was detected by immunoblotting using CETP monoclonal antibody (MAb) TP2 and visualized by ECL. Endogenous CETP associated with the HDL_3 and HDL_2 preparations, corresponding to the amounts in which they were present in the incubation mixtures, are shown in the bottom panel at time 0. MW, molecular weight. B: Western blot bands were scanned from the X-ray film shown in A. Densitometric analysis was performed, and the intensity of the intact CETP band at each time interval was measured and expressed as a percentage of the control (time 0; marked with a hatched circle for all). Initial CETP activity ranged from 6.2 to 10.9 nmol/ml/h. Data shown are representative of two to five independent experiments. Open squares represent CETP preincubated with HDL_2; open triangles represent CETP preincubated with apoA-I; closed squares represent CETP preincubated with HDL_3; closed triangles represent CETP preincubated with discoidal rHDL; and closed circles represent CETP preincubated alone.

erating a specific proteolytic pattern of two major bands with approximate molecular masses of 45 and 34 kDa.

A similar pattern was observed when CETP was complexed with either discoidal rHDL or with HDL_3 particles; however, no degradation products were visible when CETP was allowed to complex with lipid-free apoA-I or HDL_2. This finding may be related to the specificity of CETP MAb TP2 toward an epitope located within the last 26 C-terminal amino acids of CETP. Thus, chymase-mediated cleavage of CETP, which is specifically complexed to either lipid-free apoA-I or HDL_2, likely occurred near the C-terminal end of the CETP. Indeed, there are 10 putative cleavage sites for chymase within the 26 C-terminal amino acid residues of CETP, according to the SWISS-PROT protein sequence data bank. Semiquantitative densitometric analysis of the gels confirmed that chymase progressively degraded CETP and that complexation of CETP with lipid-free apoA-I or HDL_2 rendered CETP more resistant against the proteolytic action of chymase (Fig. 1B). Complexes of CETP with HDL_3 also partially protected CETP from degradation. In contrast, complexation with rHDL conferred on CETP the least resistance against chymase, and the degree of proteolysis achieved was similar to that of the free CETP. Both HDL_3 and HDL_2 subclasses contained traces of immunodetectable CETP (Fig. 1A, bottom lanes). Treatment of either HDL preparation with chymase revealed that CETP associated with the HDL preparations was also degraded in a time-dependent manner (**Fig. 2**).

To assess the effect of the chymase-dependent fragmentation of CETP on its cholesteryl ester transfer activity, we next determined CETP activity promoted by isolated CETP or by CETP-HDL complexes after treatment with chymase for up to 6 h (**Fig. 3**). To render the results from the various experiments comparable, values are expressed relative to the activity at time 0, which was set at 100%. Similar to the observed fast and strong degradation of isolated CETP shown in Fig. 1B, treatment of isolated CETP with chymase caused a significant loss (50%) in activity already

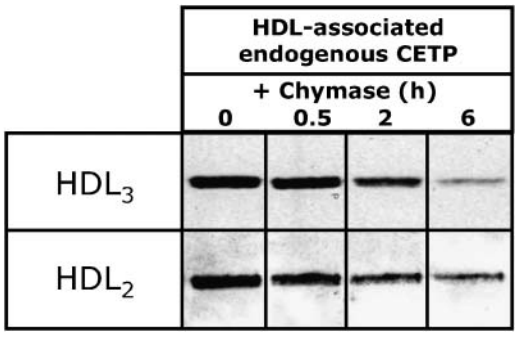

Fig. 2. Degradation of endogenous CETP by chymase. HDL_3 and HDL_2 preparations (500 μg of total protein for each) were incubated in the presence of chymase (92 BTEE units) in 500 μl of TNE buffer, pH 7.4, for up to 6 h under the conditions described for Fig. 1. From the incubation mixtures, 10 μg as total protein was loaded on the SDS-PAGE gel, and CETP was detected by immunoblotting using the CETP MAb TP2.

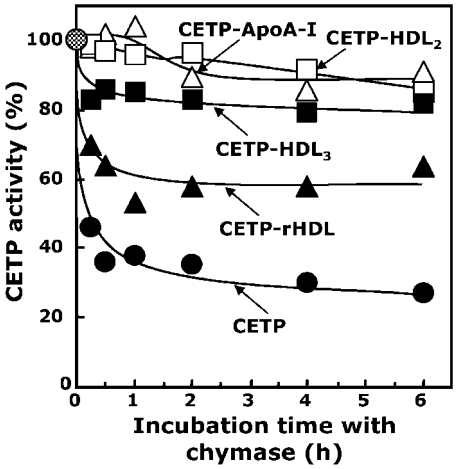

Fig. 3. Effect of chymase on CETP activity. CETP and various CETP complexes generated under the conditions described for Fig. 1 were treated with chymase as described in Materials and Methods. To measure CETP activity, aliquots of the incubation mixtures were taken at different time intervals up to 6 h. Cholesteryl ester transfer activity was expressed as a percentage of time 0 values. The absolute initial values of CETP activity (time 0; marked with a hatched circle for all) ranged from 4.1 to 14 nmol/ml/h in the different experiments. Data are means of two to five independent experiments. Isolated HDL_3 and HDL_2 contained traces of immunodetectable CETP (cholesteryl ester transfer activity of 0.71 ± 0.12 and 0.53 ± 0.08 nmol/ml/h, respectively). Open squares represent CETP preincubated with HDL_2; open triangles represent CETP preincubated with apoA-I; closed squares represent CETP preincubated with HDL_3; closed triangles represent CETP preincubated with discoidal rHDL; and closed circles represent CETP preincubated alone.

within the shortest time of incubation (15 min). Importantly, also consistent with the observed degrees of CETP degradation achieved in the various incubation mixtures (Fig. 1B), the activity of CETP was partially preserved when it was complexed with the different HDL ligands in the following order: apoA-I = HDL_2 > HDL_3 >> rHDL (Fig. 3). Although discoidal rHDL was least effective in preserving CETP activity, cholesteryl ester transfer promoted by the CETP-rHDL complexes after 6 h of incubation with chymase was still 2-fold higher than that of CETP treated with chymase in the absence of any complex-forming ligand. This observation suggests that, despite substantial proteolysis paralleling that observed with isolated CETP (Fig. 1), specific protein-protein interactions between functional domains of CETP and rHDL in the complexes may partially protect the ability of the proteolyzed CETP to transfer CE.

Effect of chymase on apoA-I degradation in CETP-HDL complexes

We previously demonstrated that lipid-poor and variously lipidated species of apoA-I serve as substrates for chymase (25). Because preincubation of CETP with lipid-free apoA-I or apoA-I-containing particles generated CETP complexes that protected CETP protein against proteolysis by chymase, we next analyzed whether the formation of such complexes would also contribute to preserving the

integrity of apoA-I present in such complexes. A preliminary experiment was conducted by incubating lipid-free apoA-I-CETP complexes in the absence or presence of chymase for 6 h at 37°C, and apoA-I was detected by Western blotting using a polyclonal anti-apoA-I antibody. Because PLTP, which shares high structural homology with CETP, also binds to apoA-I (45), we also compared the ability of PLTP to protect lipid-free apoA-I from being proteolyzed by chymase. As shown in **Fig. 4**, chymase efficiently degraded lipid-free apoA-I, leading to full loss of the intact apoA-I protein band, and after 6 h of incubation with chymase, a single 26 kDa proteolytic fragment of apoA-I was faintly visible (Fig. 4, lane 2). Again, intact apoA-I fully disappeared when apoA-I was complexed with CETP, although degradation of the formed 26 kDa band was largely prevented (Fig. 4, lane 4). Similar to CETP complexation, when apoA-I was complexed with PLTP, no intact apoA-I was visible after incubation with chymase (Fig. 4, lane 6). However, in sharp contrast to CETP, PLTP failed to prevent further degradation of the 26 kDa fragment (Fig. 4, lane 6). When CETP was present in the preincubation mixture in addition to PLTP, CETP-mediated protection of the 26 kDa fragment was again observed (Fig. 4, lane 8). These findings revealed that the protective effect against an extensive apoA-I fragmentation by chymase was attributed specifically to CETP, and the presence of PLTP did not abolish this CETP-associated protective effect. Similar results were obtained when proteolysis with chymase was extended for up to 24 h (data not shown).

Using the same approach, we next studied whether CETP, once associated with discoidal rHDL or spherical

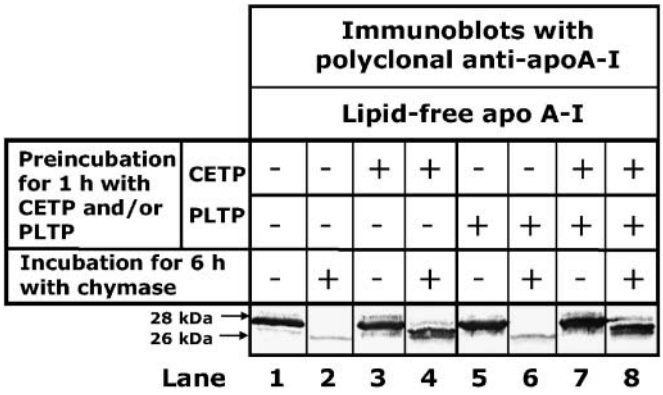

Fig. 4. Effect of CETP and phospholipid transfer protein (PLTP) on the degradation of lipid-free apoA-I by chymase. Lipid-free apoA-I (50 μg) was preincubated for 1 h at room temperature in the absence or presence of either CETP (6.8 nmol/ml/h, corresponding to 1 μg of total protein) or PLTP (600 nmol/ml/h, corresponding to 1 μg of PLTP) or both lipid transfer proteins in 250 μl of TNE buffer. Chymase (18 BTEE units) was added to the mixtures, and incubation was continued at 37°C for 6 h. Aliquots of the incubation mixtures were applied to 12.5% SDS-PAGE gels, and the protein bands were analyzed by Western blotting using a polyclonal anti-apoA-I rabbit antibody. A low molecular mass protein set was used for standardization. Lipid-free apoA-I preincubated either alone (lanes 1-2), with CETP (lanes 3-4), with PLTP (lanes 5-6), or with both CETP and PLTP (lanes 7-8) incubated in the absence or presence of chymase as indicated.

HDL, affects the proteolysis of apoA-I. In the absence of CETP, chymase degraded apoA-I in discoidal rHDL particles and also generated a large proteolytic fragment with a molecular mass of 26 kDa (**Fig. 5**, lane 2). However, the intensity of proteolysis was much weaker relative to the lipid-free apoA-I (Fig. 4, lane 2). Two additional rHDL preparations studied [apoA-I/PC/cholesterol at 1:50:12.5 and 1:100:0 (mol/mol)] behaved in a similar manner regarding chymase cleavage of apoA-I and its protection by CETP (data not shown). More importantly, complexation with CETP efficiently protected apoA-I in rHDL particles against proteolysis, and more intact apoA-I was detected in immunoblotting (Fig. 5, lane 4). In contrast, apoA-I in the spherical HDL_3 and HDL_2 particles was poorly degraded by chymase, and the formation of complexes with CETP had no further effect on such a minor degree of apoA-I degradation, which remained <10% (Fig. 5, lanes 5–8 and 9–12, respectively). These results further support our previous findings that discoidal, rather than spherical, HDL particles are the most sensitive targets for mast cell chymase (26, 28). They also suggest that CETP binding, by modifying the spatial orientation of apoA-I in discoidal HDL, restrained the access of chymase to proteolytically sensitive sites in apoA-I.

Effect of CETP on cholesterol efflux

Although it was suggested previously that CETP may have a direct role in removing cholesterol from COS cells to HDL (17), a more recent report found no effect of macrophage CETP expression on cholesterol efflux to lipid-free apoA-I (46). To gain more insight into the potential effect of CETP in this process, we performed functional experiments with cultured THP-1 macrophage foam cells. This line of human macrophages has been used extensively for lipid efflux studies, and it has been shown that modulation of the expression levels of ABCA1/G1 and scavenger receptor class B type I can be achieved by nuclear transcription factors (47). Our aim was to investigate whether the formation of complexes with CETP would affect the cholesterol acceptor abilities of apoA-I in discoidal and spherical HDL particles and whether treatment with chymase would affect their cholesterol efflux abilities. Therefore, we incubated lipid-free apoA-I and discoidal rHDL, HDL_3, and HDL_2 (100 μg each) in the absence or presence of CETP (8 nmol/ml/h) for 1 h at room temperature to generate CETP-containing complexes as described above. Incubations were continued for 6 h at 37°C in the absence or presence of chymase (18 BTEE units). Chymase was then fully inhibited with SBTI, and aliquots of the mixtures were added to cholesterol-loaded macrophages to evaluate cholesterol efflux to the medium during a 16 h incubation. We found that the addition of CETP alone (8 nmol/ml/h) to the culture medium did not affect cholesterol efflux and was similar to addition of the medium alone (4.3 ± 0.2% vs. 4.3 ± 0.4%) (data not shown). As shown in **Fig. 6**, lipid-free apoA-I strongly increased efflux (up to 27%), reflecting stimulation of the ABCA1 pathway (Fig. 6, lane 1), and the formation of CETP-apoA-I complexes had no further effect (Fig. 6,

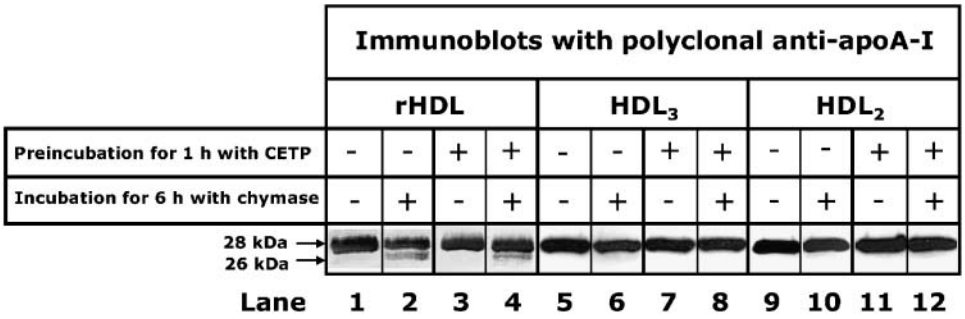

Fig. 5. Effect of CETP on the degradation of lipidated apoA-I by chymase. Discoidal rHDL, HDL_3, and HDL_2 (50 μg of protein each) were preincubated for 1 h at room temperature in the absence or presence of CETP (6.8 nmol/ml/h) in 250 μl of TNE buffer. Chymase (18 BTEE units) was added to the mixtures, and incubation was continued at 37°C for 6 h. Aliquots of the incubation mixtures were applied to SDS-PAGE gels, and apoA-I and its fragments were visualized by Western blotting using a polyclonal anti-apoA-I antibody, as described for Fig. 4. Discoidal rHDL preincubated alone (lanes 1-2) or with CETP (lanes 3-4), HDL_3 preincubated alone (lanes 5-6) or with CETP (lanes 7-8), and HDL_2 preincubated alone (lanes 9-10) or with CETP (lanes 11-12) incubated in the absence or presence of chymase as indicated.

lane 2). Discoidal rHDL, HDL_3, and HDL_2 all promoted similar efflux (~15%; Fig. 6, lanes 5, 9, 13), and complexing CETP only with HDL_3 increased efflux ($P = 0.0287$) (Fig. 6, lane 10). Because the generation of preβ-HDL is unlikely in our experimental system, our results suggest that the observed effect was produced by a subtle spatial modification of HDL_3 induced by CETP binding.

As shown above (Figs. 4, 5), the efficiency of apoA-I proteolysis by chymase was dependent on the degree of lipidation and the shape (discoidal vs. spherical) of the HDL particles. When chymase was incubated with lipid-free apoA-I, which results in complete degradation of intact apoA-I (Fig. 4, lane 2), cholesterol efflux was basically abolished (Fig. 6, lane 1 vs. lane 3), even when

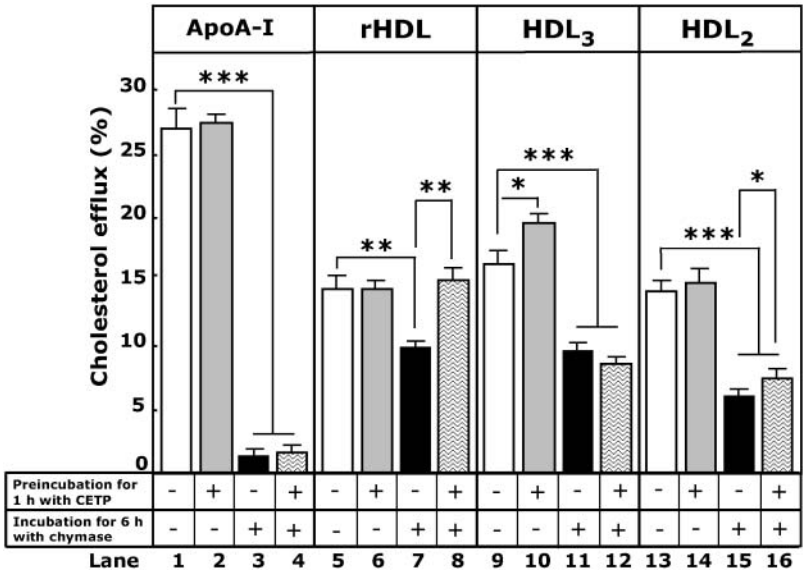

Fig. 6. Effect of chymase on cholesterol efflux promoted by CETP-HDL complexes. Lipid-free apoA-I and discoidal rHDL, HDL_3, and HDL_2 (100 μg of protein each) were preincubated in the absence or presence of CETP (5.2 ± 0.57 nmol/ml/h) at room temperature for 1 h in 250 μl of TNE buffer. Incubations were continued at 37°C for 6 h in the absence or presence of chymase (18 BTEE units). After inhibition of chymase by soybean trypsin inhibitor, aliquots of the incubation mixtures were added to THP-1 macrophage foam cells to reach the following concentrations in the culture medium: 10 μg/ml apoA-I; and 25 μg/ml rHDL, HDL_3, and HDL_2. Control wells included incubations in which the foam cells were incubated in CETP-containing medium or in culture medium only. Cholesterol efflux to medium was measured after 16 h and expressed as fractional efflux (%), as described in Materials and Methods. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$. All data are means ± SEM (n = 3). Open bars represent -CETP/-chymase; gray bars represent +CETP/-chymase; black bars represent -CETP/+chymase; and textured bars represent +CETP/+chymase.

CETP was present (Fig. 6, lane 3 vs. lane 4), reflecting the fact that the generated apoA-I-derived 26 kDa fragment was virtually unable to promote cholesterol efflux. As shown above, chymase also generated a polypeptide of 26 kDa from apoA-I in discoidal rHDL, but apoA-I in discs was more resistant to proteolysis (Fig. 5, lane 2). When such partially degraded rHDL was added to the foam cells, a 30% reduction in efflux was observed (Fig. 6, lane 5 vs. lane 7). Complexation with CETP did not modify the efflux induced by rHDL (Fig. 6, lane 6), but, in sharp contrast, it fully preserved the efflux-promoting ability of the chymase-treated CETP-rHDL complexes (Fig. 6, lane 8).

Although this finding could be partially explained by the relative protection of intact apoA-I in these complexes (Fig. 5, lane 2 vs. lane 4), we further investigated whether qualitatively different cleavages in functional domains of apoA-I in rHDL compared with that in the CETP-rHDL complexes had occurred. Indeed, there are several reports documenting that removal of the C-terminal sequence of apoA-I (e.g., the amino acid residues 187–243) inactivates apoA-I in terms of ABCA1-mediated efflux (48, 49). Thus, to determine whether truncation of apoA-I with the generation of a 26 kDa fragment had occurred in the C-terminal or the N-terminal region, we carried out Western blotting analysis using specific MAbs that recognize either a discrete epitope at the N terminus (residues 2–8; MAb 4H1) or a region close to the C terminus of apoA-I (residues 211–220; MAb 4.1). Because the 26 kDa fragment was the only apoA-I-derived proteolytic product found, we compared the effect of chymase on both unassociated lipid-free apoA-I and rHDL and on their complexes with CETP (**Fig. 7**). This analysis revealed that the 26 kDa polypeptide derived from the proteolysis of lipid-free apoA-I and its CETP complexes was immunoreactive against the N terminus, whereas the C-terminal region of apoA-I had been cleaved off, so no signal was obtained on immunoblotting (Fig. 7, lanes 3, 4). In contrast, the 26 kDa polypeptide in proteolyzed rHDL and CETP-rHDL complexes was immunoreactive against the C-terminal MAbs only (Fig. 7, lanes 7, 8), suggesting that cleavage at the N terminus had occurred.

Finally, we found that although treatment with chymase produced only minor degradation of apoA-I in HDL_3 and HDL_2 (Fig. 5, lanes 6, 10), the efflux ability was reduced by 50%, a major decrease that could not be prevented by prior complexation with CETP (Fig. 6, lane 9 vs. lanes 11, 12 and lane 12 vs. lanes 15, 16, respectively). Because other apolipoproteins of HDL, apart from a small fraction of apoA-I, are also targets of chymase (26, 50), we further analyzed the apolipoprotein profile of the chymase-treated mature HDL by further immunoblotting with monospecific anti-human apoE antibodies. Indeed, we found that the inhibitory effect of chymase was associated with extensive degradation of apoE, both in HDL_3 and HDL_2, whether complexed with CETP or not (data not shown).

DISCUSSION

The extracellular fluid of the arterial intima is a filtrate of plasma components (51) that contains, in addition to HDL particles, other proteins that participate in reverse cholesterol transport, such as CETP (10) and PLTP (14). CETP can enter the intimal fluid from the plasma compartment in association with HDL particles and can also be expressed and secreted locally by cholesterol-loaded macrophages (13) (i.e., in the intimal areas where cholesterol accumulates and inflammation prevails). Notably, in such areas, proteolytic activity is increased and, specifically, chymase-containing mast cells are activated to secrete chymase (24). Thus, in the macrophage foam cell-rich regions of the atherosclerotic lesions, chymase, HDL particles, and lipid transfer proteins may interact in multiple ways. In the present study, we evaluated some of

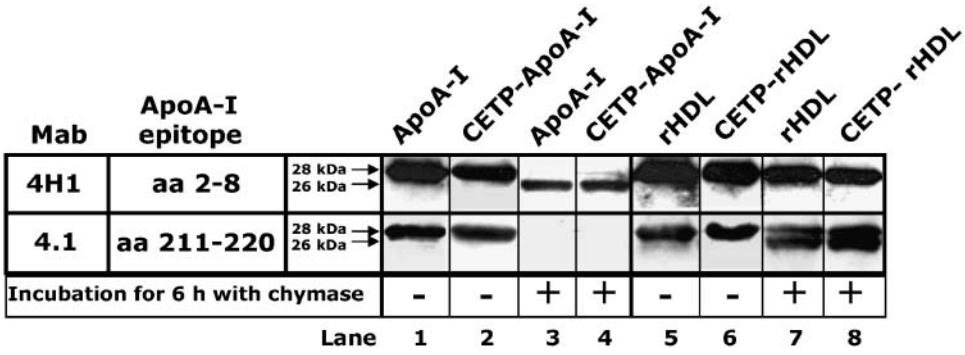

Fig. 7. Western blot analysis of the apoA-I fragments generated by chymase treatment of lipid-free apoA-I, rHDL, and their complexes with CETP. Lipid-free apoA-I and discoidal rHDL (100 μg of protein each) were preincubated in the absence or presence of CETP (10.1 nmol/ml/h) at room temperature for 1 h in 250 μl of TNE buffer. Incubations were continued at 37°C for 6 h in the absence or presence of chymase (18 BTEE units). Aliquots of the incubation mixtures were applied to 12.5% SDS-PAGE gels, and the protein bands were analyzed by Western blotting using specific MAbs recognizing the N-terminal and C-terminal regions of apoA-I. Lipid-free apoA-I (lanes 1 and 3), CETP-apoA-I complexes (lanes 2 and 4), discoidal rHDL (lanes 5 and 7), and CETP-rHDL complexes (lanes 6 and 8) incubated in the absence or presence of chymase as indicated.

these interactions using in vitro and cell culture methods. We found that chymase effectively proteolyzed CETP and impaired its cholesteryl ester transfer activity. However, when CETP was allowed first to bind to its natural ligands (i.e., to the mature HDL particles), the integrity and function of CETP were protected against the proteolytic effects of chymase. Because binding to HDL_3 also renders PLTP more resistant to chymase-mediated inactivation (29), the data in this study extend our previous notion that preserving the functions of the two lipid transfer proteins against proteolysis may be achieved via their binding to spherical α-HDL particles. Interestingly, although the formation of such CETP-HDL complexes prevented CETP from losing its cholesteryl ester transfer function by chymase, it did not prevent a major loss in the cholesterol efflux-inducing capacity of the mature HDL particles.

A previous study has shown that, although digestion by trypsin and α-chymotrypsin caused significant fragmentation of human CETP, its cholesteryl ester transfer activity remained unaffected (52). That study highlighted the fact that CETP contains a highly stabilized and complex tertiary structure necessary for cholesteryl ester transfer activity that is resistant to unfolding. Of note, the limited proteolysis of CETP with trypsin or α-chymotrypsin gave rise to a series of four to six fragments, including a C-terminal fragment of 45 kDa, that is reactive with the MAb TP2 also used in the present study, which contains a functional site possibly involved in the binding of CETP to lipoprotein interfaces (52). Here, we found that, although digestion of isolated CETP with chymase rapidly generated a major TP2-reactive fragment of 45 kDa, this proteolytic cleavage also led to a rapid loss (~70%) in its cholesteryl ester transfer activity. This result suggests that chymase, contrary to trypsin and α-chymotrypsin (52), is able to misfold the tertiary structure necessary for cholesteryl ester transfer activity. Because chymase, in contrast to α-chymotrypsin, is present in human atherosclerotic lesions, this singular cleavage pattern of chymase could be of potential pathophysiological relevance.

Most of the CETP in plasma is bound to α-HDL particles. Although CETP is not able to associate with protein-free PC liposomes, it readily forms complexes by direct binding to apoA-I/PC discs (rHDL), such as those used in this work (39). It has been suggested that, whereas CETP accommodates to the edge of HDL discs, the curvature of the surface of spherical HDL particles dictates the intercalation of CETP between phospholipid head groups (39). Data based on the crystal structure of CETP also demonstrate that the curvature of the concave surface of CETP matches the radius of the curvature observed in α-HDL particles, suggesting that potential conformational changes may occur in CETP for it to accommodate to larger non-HDL lipoproteins (7). We also found that incubation of CETP with apoA-I-containing particles of various sizes and shapes led to the formation of complexes and that, in these complexes, CETP was less exposed to chymase degradation when bound to spherical HDL than to discoidal rHDL particles. This lower degree of exposure to chymase suggests that the surface insertion of CETP may be deeper when it forms complexes with spherical than with discoidal HDL particles. The interaction of CETP with spherical HDL also protected CETP activity from proteolytic inactivation. Thus, CETP binding to HDL_3 and HDL_2 particles might have rendered the domains required for cholesteryl ester transfer less accessible to chymase.

CETP binding to lipid-free apoA-I or apoA-IV, but not apoA-II, increases its activity (38), suggesting that functionally important changes in CETP conformation are inducible by certain apolipoproteins. We found that binding to lipid-free apoA-I provided CETP both structural and functional stability against chymase action. This resistance of CETP to proteolysis in these complexes sharply diverged from the complete depletion of lipid-free apoA-I associated with CETP. In contrast, apoA-I in discoidal rHDL, although normally highly susceptible to chymase-dependent proteolysis, became very resistant against chymase when allowed first to bind to CETP. Conversely, CETP in these rHDL-complexes was extensively proteolyzed. These gains in proteolytic resistance could be explained by conformational changes in which the chymase-accessible cleavage sites of CETP or those of apoA-I became buried when complexes between CETP and lipid-free apoA-I, and between CETP and rHDL, respectively, were formed.

In contrast to the currently available information regarding the function of the lipid transfer proteins in the plasma compartment, little is known about the specific conditions that govern the remodeling of lipoproteins in the arterial intima. Recent identification of both CETP and PLTP in the human intima opens a novel road for integrating the local mechanisms of cellular cholesterol efflux and lipoprotein remodeling in atherosclerotic lesions. Here, we found that adding CETP alone to macrophage foam cells did not stimulate cholesterol efflux. Moreover, despite partial protection of CETP from chymase-dependent inactivation, complexation of apoA-I, HDL_2, or HDL_3 with CETP did not prevent the chymase-dependent impairment of cholesterol efflux promoted by these complexes. Because THP-1 macrophages were cholesterol-loaded, it is likely that the inhibited efflux promoted by the mature HDL was partially ABCG1-dependent. Importantly, by destroying an epitope located at the C-terminal region of apoA-I, chymase led to full inactivation of the ABCA1-mediated cholesterol efflux pathway induced by lipid-free apoA-I and its complexes with CETP. Of note, the CETP-rHDL complexes, but not the nonassociated rHDL, remained fully competent in their ability to promote cholesterol efflux after treatment with chymase. Although this beneficial effect may be related to the presence of more intact apoA-I in the chymase-treated CETP-rHDL complexes, the data might also indicate a qualitatively different cleavage on apoA-I in the CETP-rHDL complexes.

Unfortunately, by site-directed (N- or C-terminal) MAb analysis, we did not find any difference in apoA-I cleavage upon binding to CETP; namely, the N terminus was truncated but the C-terminal region remained immunoreactive. Cleavage of the N-terminal region does not likely result in decreased efflux, because a role for the N terminus of apoA-I in promoting cholesterol efflux has not

been reported to date. However, because of the specificity of the C-terminally oriented MAb used in the present experiments (residues 211–220), we cannot exclude the possibility that chymase had cleaved a small fragment of apoA-I (beyond amino acid 220) in the rHDL, thus reducing its cholesterol-accepting ability. In fact, we have reported previously that chymase cleaves 18 amino acids at the terminal sequences of apoA-I in discoidal rHDL of similar size as the preparation used in the present study (53). Therefore, based on the previous and present results, we consider that the association of CETP with rHDL prevents chymase-dependent functional inactivation of discoidal rHDL by preserving apoA-I intactness, and possibly also by protecting the C terminus from proteolytic attack. Thus, these results provide new insights into the interaction of CETP with discoidal HDL particles and suggest an important role for this association in maintaining efficient rates of cholesterol efflux in the atherosclerotic plaque, despite an abundance of extracellular proteases in the inflamed tissue.

Together, the present results indicate that isolated CETP and apoA-I-containing particles are highly susceptible to chymase-mediated degradation, with concomitant losses in their functions. In contrast, when present as complexes, they become protected from such degradative proteolysis to variable degrees. Surprisingly, the association of CETP with discoidal rHDL efficiently protected apoA-I from extensive proteolysis. Because the relative concentration of nascent discoidal HDL particles in the interstitial fluid of the intima fluid is relatively high (51), and they are considered to play an important role in the initial steps of reverse cholesterol transport, it is conceivable that this novel protective function of CETP could be of significance when chymase is also present in the intimal fluid. Given the high susceptibility of apoA-I in discoidal (preβ-migrating) HDL to most of the proteases present in the intimal fluid (25), it will be of great interest to examine whether the above-described protective function of CETP extends beyond chymase. Because the majority of CETP in the blood plasma is bound to mature HDL particles, most of the circulating CETP entering the intima might be protected against a local proteolytic attack by chymase. In contrast, we envision that the CETP locally synthesized and secreted by macrophages is initially unprotected and susceptible to proteolysis. Therefore, chymase secreted by activated mast cells in the vicinity of macrophages may inactivate the secreted CETP. Interestingly, it was recently shown that the expression of human CETP in the mouse liver promotes macrophage reverse cholesterol transport at the end stage of the pathway by increasing cholesterol uptake by the liver (54). These in vivo data complement our present in vitro results, which suggest that macrophage-derived CETP protects nascent discoidal HDL against proteolysis, thus assisting the maintenance of adequate efflux of cholesterol from plaque foam cells during the early steps of the reverse cholesterol transport pathway.

The findings of chymase-mediated inactivation of both PLTP (29) and CETP (present results) expand the potential role of mast cells in the subendothelial space of atherosclerotic plaques. We propose that, by inhibiting macrophage cholesterol efflux and by locally disturbing lipid transfer reactions, activated mast cells have the potential to contribute to lipid accumulation in atherosclerotic lesions in multiple ways.

This work was supported by the Finnish Foundation for Cardiovascular Research (M.J.), the Sigrid Juselius (M.L-R. and M.J.) and Aarne Koskelo (M.L-R) Foundations, the Finska Läkaresallskapet (R.V.), and the Research Council for Health, Academy of Finland, under Grant 114484 (M.J.). The Wihuri Research Institute is maintained by the Jenny and Antti Wihuri Foundation. The authors thank Sari Nuutinen for her excellent technical assistance.

REFERENCES

1. Cuchel, M., and D. J. Rader. 2006. Macrophage reverse cholesterol transport: key to the regression of atherosclerosis? *Circulation.* **113:** 2548–2555.
2. Nakamura, Y., L. Kotite, Y. Gan, T. A. Spencer, C. J. Fielding, and P. E. Fielding. 2004. Molecular mechanism of reverse cholesterol transport: reaction of pre-beta-migrating high-density lipoprotein with plasma lecithin/cholesterol acyltransferase. *Biochemistry.* **43:** 14811–14820.
3. Jiang, X. C., and H. W. Zhou. 2006. Plasma lipid transfer proteins. *Curr. Opin. Lipidol.* **17:** 302–308.
4. Oram, J. F., and A. M. Vaughan. 2006. ATP-binding cassette cholesterol transporters and cardiovascular disease. *Circ. Res.* **99:** 1031–1043.
5. Tall, A. R. 1993. Plasma cholesteryl ester transfer protein. *J. Lipid Res.* **34:** 1255–1274.
6. Marcel, Y. L., R. McPherson, M. Hogue, H. Czarnecka, Z. Zawadzki, P. K. Weech, M. E. Whitlock, A. R. Tall, and R. W. Milne. 1990. Distribution and concentration of cholesteryl ester transfer protein in plasma of normolipemic subjects. *J. Clin. Invest.* **85:** 10–17.
7. Qiu, X., A. Mistry, M. J. Ammirati, B. A. Chrunyk, R. W. Clark, Y. Cong, J. S. Culp, D. E. Danley, T. B. Freeman, K. F. Geoghegan, et al. 2007. Crystal structure of cholesteryl ester transfer protein reveals a long tunnel and four bound lipid molecules. *Nat. Struct. Mol. Biol.* **14:** 106–113.
8. de Grooth, G. J., A. H. Klerkx, E. S. Stroes, A. F. Stalenhoef, J. J. Kastelein, and J. A. Kuivenhoven. 2004. A review of CETP and its relation to atherosclerosis. *J. Lipid Res.* **45:** 1967–1974.
9. Tall, A. R., L. Yvan-Charvet, and N. Wang. 2007. The failure of torcetrapib: was it the molecule or the mechanism? *Arterioscler. Thromb. Vasc. Biol.* **27:** 257–260.
10. Ishikawa, Y., K. Ito, Y. Akasaka, T. Ishii, T. Masuda, L. Zhang, Y. Akishima, H. Kiguchi, K. Nakajima, and Y. Hata. 2001. The distribution and production of cholesteryl ester transfer protein in the human aortic wall. *Atherosclerosis.* **156:** 29–37.
11. Zhou, H., Z. Li, M. R. Hojjati, D. Jang, T. P. Beyer, G. Cao, A. R. Tall, and X. C. Jiang. 2006. Adipose tissue-specific CETP expression in mice: impact on plasma lipoprotein metabolism. *J. Lipid Res.* **47:** 2011–2019.
12. Luo, Y., C. P. Liang, and A. R. Tall. 2001. The orphan nuclear receptor LRH-1 potentiates the sterol-mediated induction of the human CETP gene by liver X receptor. *J. Biol. Chem.* **276:** 24767–24773.
13. Faust, R. A., J. H. Tollefson, A. Chait, and J. J. Albers. 1990. Regulation of LTP-I secretion from human monocyte-derived macrophages by differentiation and cholesterol accumulation in vitro. *Biochim. Biophys. Acta.* **1042:** 404–409.
14. Desrumaux, C. M., P. A. Mak, W. A. Boisvert, D. Masson, D. Stupack, M. Jauhiainen, C. Ehnholm, and L. K. Curtiss. 2003. Phospholipid transfer protein is present in human atherosclerotic lesions and is expressed by macrophages and foam cells. *J. Lipid Res.* **44:** 1453–1461.
15. Oram, J. F., G. Wolfbauer, A. M. Vaughan, C. Tang, and J. J. Albers. 2003. Phospholipid transfer protein interacts with and stabilizes ATP-binding cassette transporter A1 and enhances cholesterol efflux from cells. *J. Biol. Chem.* **278:** 52379–52385.
16. Lee-Rueckert, M., R. Vikstedt, J. Metso, C. Ehnholm, P. T. Kovanen,

and M. Jauhiainen. 2006. Absence of endogenous phospholipid transfer protein impairs ABCA1-dependent efflux of cholesterol from macrophage foam cells. *J. Lipid Res.* **47:** 1725–1732.
17. Zhang, Z., S. Yamashita, K. Hirano, Y. Nakagawa-Toyama, A. Matsuyama, M. Nishida, N. Sakai, M. Fukasawa, H. Arai, J. Miyagawa, et al. 2001. Expression of cholesteryl ester transfer protein in human atherosclerotic lesions and its implication in reverse cholesterol transport. *Atherosclerosis.* **159:** 67–75.
18. Liu, R., M. R. Hojjati, C. M. Devlin, I. H. Hansen, and X. C. Jiang. 2006. Macrophage phospholipid transfer protein deficiency and apoE secretion. impact on mouse plasma cholesterol levels and atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* **27:** 190–196.
19. Valenta, D. T., N. Ogier, G. Bradshaw, A. S. Black, D. J. Bonnet, L. Lagrost, L. K. Curtiss, and C. M. Desrumaux. 2006. Atheroprotective potential of macrophage-derived phospholipid transfer protein in low-density lipoprotein receptor-deficient mice is overcome by apolipoprotein AI overexpression. *Arterioscler. Thromb. Vasc. Biol.* **26:** 1572–1578.
20. Vikstedt, R., D. Ye, J. Metso, R. B. Hildebrand, T. J. Van Berkel, C. Ehnholm, M. Jauhiainen, and E. M. Van. 2007. Macrophage phospholipid transfer protein contributes significantly to total plasma phospholipid transfer activity and its deficiency leads to diminished atherosclerotic lesion development. *Arterioscler. Thromb. Vasc. Biol.* **27:** 578–586.
21. Lindstedt, K. A., M. J. Leskinen, and P. T. Kovanen. 2004. Proteolysis of the pericellular matrix: a novel element determining cell survival and death in the pathogenesis of plaque erosion and rupture. *Arterioscler. Thromb. Vasc. Biol.* **24:** 1350–1358.
22. Kaartinen, M., A. Penttilä, and P. T. Kovanen. 1994. Mast cells of two types differing in neutral protease composition in the human aortic intima. *Arterioscler. Thromb.* **14:** 966–972.
23. Lindstedt, L., M. Lee, and P. T. Kovanen. 2001. Chymase bound to heparin is resistant to its natural inhibitors and capable of proteolyzing high density lipoproteins in aortic intimal fluid. *Atherosclerosis.* **155:** 87–97.
24. Kovanen, P. T. 2007. Mast cells: multipotent local effector cells in atherothrombosis. *Immunol. Rev.* **217:** 105–122.
25. Lee-Rueckert, M., and P. T. Kovanen. 2006. Mast cell proteases: physiological tools to study functional significance of high density lipoproteins in the initiation of reverse cholesterol transport. *Atherosclerosis.* **189:** 8–18.
26. Lee, M., A. von Eckardstein, L. Lindstedt, G. Assmann, and P. T. Kovanen. 1999. Depletion of preβ_1LpA1 and LpA4 particles by mast cell chymase reduces cholesterol efflux from macrophage foam cells induced by plasma. *Arterioscler. Thromb. Vasc. Biol.* **19:** 1066–1074.
27. Favari, E., M. Lee, L. Calabresi, G. Franceschini, F. Zimetti, F. Bernini, and P. T. Kovanen. 2004. Depletion of prebeta-HDL by human chymase impairs ATP-binding cassette transporter A1- but not SR-B1-mediated lipid efflux to HDL. *J. Biol. Chem.* **279:** 9930–9936.
28. Lee, M., P. Uboldi, D. Giudice, A. L. Catapano, and P. T. Kovanen. 2000. Identification of domains in apoA-I susceptible to proteolysis by mast cell chymase. Implications for HDL function. *J. Lipid Res.* **41:** 975–984.
29. Lee, M., J. Metso, M. Jauhiainen, and P. T. Kovanen. 2003. Degradation of phospholipid transfer protein (PLTP) and PLTP-generated pre-beta-high density lipoprotein by mast cell chymase impairs high affinity efflux of cholesterol from macrophage foam cells. *J. Biol. Chem.* **278:** 13539–13545.
30. Ohnishi, T., S. Yokoyama, and A. Yamamoto. 1990. Rapid purification of human plasma lipid transfer proteins. *J. Lipid Res.* **31:** 397–406.
31. Tollefson, J. H., S. Ravnik, and J. J. Albers. 1988. Isolation and characterization of a phospholipid transfer protein (LTP-II) from human plasma. *J. Lipid Res.* **29:** 1593–1602.
32. Basu, S. K., J. L. Goldstein, R. G. W. Anderson, and M. S. Brown. 1976. Degradation of cationized low density lipoprotein and regulation of cholesterol metabolism in homozygous familial hypercholesterolemia fibroblasts. *Proc. Natl. Acad. Sci. USA.* **73:** 3178–3182.
33. Brown, M. S., S. E. Dana, and J. L. Goldstein. 1975. Receptor-dependent hydrolysis of cholesterol esters contained in plasma low density lipoprotein. *Proc. Natl. Acad. Sci. USA.* **72:** 2925–2929.
34. Setala, N. L., J. M. Holopainen, J. Metso, S. K. Wiedmer, G. Yohannes, P. K. Kinnunen, C. Ehnholm, and M. Jauhiainen. 2007. Interfacial and lipid transfer properties of human phospholipid transfer protein: implications for the transfer mechanism of phospholipids. *Biochemistry.* **46:** 1312–1319.
35. Blanche, P. J., E. L. Gong, T. M. Forte, and A. V. Nichols. 1981. Characterization of human high-density lipoproteins by gradient gel electrophoresis. *Biochim. Biophys. Acta.* **665:** 408–419.
36. Groener, J. E., R. W. Pelton, and G. M. Kostner. 1986. Improved estimation of cholesteryl ester transfer/exchange activity in serum or plasma. *Clin. Chem.* **32:** 283–286.
37. Speijer, H., J. E. Groener, E. van Ramshorst, and A. van Tol. 1991. Different locations of cholesteryl ester transfer protein and phospholipid transfer protein activities in plasma. *Atherosclerosis.* **90:** 159–168.
38. Guyard-Dangremont, V., L. Lagrost, and P. Gambert. 1994. Comparative effects of purified apolipoproteins A-I, A-II, and A-IV on cholesteryl ester transfer protein activity. *J. Lipid Res.* **35:** 982–992.
39. Bruce, C., W. S. Davidson, P. Kussie, S. Lund-Katz, M. C. Phillips, R. Ghosh, and A. R. Tall. 1995. Molecular determinants of plasma cholesteryl ester transfer protein binding to high density lipoproteins. *J. Biol. Chem.* **270:** 11532–11542.
40. Pussinen, P., M. Jauhiainen, J. Metso, J. Tyynelä, and C. Ehnholm. 1995. Pig plasma phospholipid transfer protein facilitates HDL interconversion. *J. Lipid Res.* **36:** 975–985.
41. Swenson, T. L., C. B. Hesler, M. L. Brown, E. Quinet, P. P. Trotta, M. F. Haslanger, F. C. Gaeta, Y. L. Marcel, R. W. Milne, and A. R. Tall. 1989. Mechanism of cholesteryl ester transfer protein inhibition by a neutralizing monoclonal antibody and mapping of the monoclonal antibody epitope. *J. Biol. Chem.* **264:** 14318–14326.
42. Jauhiainen, M., and C. Ehnholm. 2005. Determination of human plasma phospholipid transfer protein mass and activity. *Methods.* **36:** 97–101.
43. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
44. Woodbury, R. G., M. T. Everitt, and H. Neurath. 1981. Mast cell proteases. *Methods Enzymol.* **80:** 588–609.
45. Pussinen, P. J., M. Jauhiainen, J. Metso, L. E. Pyle, Y. L. Marcel, N. H. Fidge, and C. Ehnholm. 1998. Binding of phospholipid transfer protein (PLTP) to apolipoproteins A-I and A-II: location of a PLTP binding domain in the amino terminal region of apoA-I. *J. Lipid Res.* **39:** 152–161.
46. Van Eck, M., D. Ye, R. B. Hildebrand, J. Kar Kruijt, W. de Haan, M. Hoekstra, P. C. Rensen, C. Ehnholm, M. Jauhiainen, and T. J. Van Berkel. 2007. Important role for bone marrow-derived cholesteryl ester transfer protein in lipoprotein cholesterol redistribution and atherosclerotic lesion development in LDL receptor knockout mice. *Circ. Res.* **100:** 678–685.
47. Nakaya, K., M. Ayaori, T. Hisada, S. Sawada, N. Tanaka, N. Iwamoto, M. Ogura, E. Yakushiji, M. Kusuhara, H. Nakamura, et al. 2007. Telmisartan enhances cholesterol efflux from THP-1 macrophages by activating PPARgamma. *J. Atheroscler. Thromb.* **14:** 133–141.
48. Burgess, J. W., R. S. Kiss, H. Zheng, S. Zachariah, and Y. L. Marcel. 2002. Trypsin-sensitive and lipid-containing sites of the macrophage extracellular matrix bind apolipoprotein A-I and participate in ABCA1-dependent cholesterol efflux. *J. Biol. Chem.* **277:** 31318–31326.
49. Vedhachalam, C., L. Liu, M. Nickel, P. Dhanasekaran, G. M. Anantharamaiah, S. Lund-Katz, G. H. Rothblat, and M. C. Phillips. 2004. Influence of apoA-I structure on the ABCA1-mediated efflux of cellular lipids. *J. Biol. Chem.* **279:** 49931–49939.
50. Lee, M., L. Calabresi, G. Chiesa, G. Franceschini, and P. T. Kovanen. 2002. Mast cell chymase degrades apoE and apoA-II in apoA-I-knockout mouse plasma and reduces its ability to promote cellular cholesterol efflux. *Arterioscler. Thromb. Vasc. Biol.* **22:** 1475–1481.
51. Smith, E. B. 1990. Transport, interactions and retention of plasma proteins in the intima: the barrier function of the internal elastic lamina. *Eur. Heart J.* **11 (Suppl. E):** 72–81.
52. Hesler, C. B., M. L. Brown, D. S. Feuer, Y. L. Marcel, R. W. Milne, and A. R. Tall. 1989. Structure-function analysis of plasma cholesteryl ester transfer protein by protease digestion and expression of cDNA fragments in *Escherichia coli*. *J. Biol. Chem.* **264:** 11317–11325.
53. Lee, M., P. T. Kovanen, G. Tedeschi, E. Oungre, G. Franceschini, and L. Calabresi. 2003. Apolipoprotein composition and particle size affect HDL degradation by chymase: effect on cellular cholesterol efflux. *J. Lipid Res.* **44:** 539–546.
54. Tanigawa, H., J. T. Billheimer, J. I. Tohyama, Y. Zhang, G. Rothblat, and D. J. Rader. 2007. Expression of cholesteryl ester transfer protein in mice promotes macrophage reverse cholesterol transport. *Circulation.* **116:** 1267–1273.