Cholesteryl Ester Transfer Protein Is Secreted by Hep G2 Cells and Contains Asparagine-linked Carbohydrate and Sialic Acid*

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A cholesteryl ester transfer protein (CETP) of apparent M, 74,000 has recently been purified from human plasma. Cholesteryl ester transfer activity was found to accumulate in the medium of cultured Hep G2 cells. The transfer activity was removed by immunoprecipitation with specific antibodies to the plasma CETP. Sodium dodecyl sulfate gel electrophoresis of immunoprecipitates prepared from the medium of cells pulsed with \(^{35}\)S)methionine revealed a broad specific band of protein of M, 72,000 to 76,000; by contrast, immunoprecipitates of cellular homogenates showed a sharp specific band of M, 58,000. The M, 72,000 to 76,000 band disappears, concomitant with the appearance of lower M, products, upon neuraminidase or glycopeptidase F treatment of medium immunoprecipitates or of purified CETP. The results indicate that liver cells have the capacity to synthesize and secrete CETP. The CETP peptide acquires asparagine-linked carbohydrate and sialic acid during intracellular processing.

One or more plasma proteins facilitate the transfer and exchange of cholesteryl esters (CE), triglycerides, and phospholipids among the lipoproteins (1-5). Recently, a plasma cholesteryl ester transfer protein (CETP) has been purified to homogeneity and has been found to consist of a single polypeptide of apparent M, 74,000 in SDS-polyacrylamide gels (1). Monospecific antibodies to this protein remove all of the cholesteryl ester transfer activity of a partially purified plasma fraction (1). However, relatively little is known concerning potential tissue sites of synthesis of CETP, or the molecular form of newly secreted CETP. The goals of the present study were to determine if the CETP is secreted by a model liver cell, the Hep G2 cell, and to examine the molecular form of the newly secreted CETP.

**MATERIALS AND METHODS**

**Cholesteryl Ester Transfer Activity in Hep G2 Cell Medium—**Hep G2 cells were grown on 100-mm Petri dishes as described above, washed twice with phosphate-buffered saline (PBS), washed once with DMEM, and then incubated with 10 ml of medium containing one of the following: (a) anti-CETP rabbit IgG (84 µg/ml) prepared by ammonium sulfate precipitation and DEAE-cellulose chromatography as previously described (1), (b) nonimmune rabbit IgG (84 µg/ml), (c) goat anti-rabbit IgG, or (d) no IgG. After 48 h, the medium was harvested and a 30-fold excess of goat anti-rabbit IgG or an equal volume of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA was added. This solution was reincubated for 24 h at 4°C. Immunoprecipitates were precipitated in a microfuge at top speed and 0.5 ml of the supernatant was assayed for CE transfer activity.

\(^{35}\)S\)Methionine Labeling of Newly Synthesized Protein—Hep G2 cells were grown until 65% confluent, washed three times with PBS and incubated for 8 h in 10 ml of methionine-free DMEM. Cells were then pulsed with \(^{35}\)S) methionine (Du Pont-New England Nuclear, 1986 CI/mmol) for 20 h. Seventy percent of the radiolabeled protein was incorporated into secreted protein at this time. Labeled protein was immunoprecipitated, as described (6, 7). The medium was cleared using inactivated Staphylococcus aureus. Either 100 µg of anti-CETP IgG or 100 µg of nonimmune IgG was added per 0.5 ml of medium for 24 h at 4°C. Immunoprecipitated protein was dissociated from immune complexes by solubilization in 0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 1% 2-mercaptoethanol, and 0.5% bromphenol blue and heating at 100°C for 5 min. Samples were analyzed by electrophoresis on a 6-16% polyacrylamide gel slab gel and subjected to autoradiography. To prepare Hep G2 cell homogenates, cells from a confluent 100-mm dish that had been pulsed with \(^{35}\)S)methionine were washed three times with PBS and harvested by scraping into 2 ml of 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, containing 2 mM phenylmethylsulfonyl fluoride and 2 mM benzamidine. Five hundred-µl aliquots were treated as described above to immunoprecipitate newly synthesized protein.

**Digestion of Purified CETP with Glycohydrolases—**Two-µg samples of purified CETP (1) were digested with glycopeptidase F or neuraminidase, \(\beta\)-galactosidase, and \(\beta\)-galactosidase (Genzyme). The glycopeptidase F incubation mixture consisted of 1 unit of glycopeptidase F in 100 mM sodium phosphate, pH 8. Neuraminidase digestions were performed with 60 or 180 milliunits of neuraminidase in 50 mM sodium acetate, pH 5.5, 150 mM NaCl, 4 mM CaCl\(_2\), \(\beta\)-galactosidase incubation consisted of 40 units of \(\beta\)-galactosidase in 60 mM sodium acetate.
Hep G2 Cells Secrete Cholesteryl Ester Transfer Protein

RESULTS

There was a time-dependent accumulation of cholesteryl ester transfer activity in the medium of cultured Hep G2 cells, as shown for a representative experiment in Fig. 1. A similar secretion of CE transfer activity by Hep G2 cells was found in five separate experiments. The transfer activity was demonstrated in an assay which measures the transfer of radio-labeled cholesteryl esters from HDL to LDL. The results of this assay can be influenced by several nonspecific variables. To determine if the cholesteryl ester transfer activity was in fact due to the previously described CETP (1), the medium was subjected to immunoprecipitation, using specific IgG prepared by immunization of a rabbit with purified CETP (1). This resulted in removal of more than 80% of the CE transfer activity from the medium (Fig. 2). By contrast, nonimmune IgG or second antibody alone had no significant effect on cholesteryl ester transfer activity in the medium. Thus, there is specific immunoprecipitation of cholesteryl ester transfer activity from the medium of Hep G2 cells by CETP-specific antibodies.

In order to visualize CETP in the immunoprecipitates, Hep G2 cells were pulsed with [3H]methionine to label newly synthesized proteins, the medium was precipitated with CETP antibodies, and the immunoprecipitates were analyzed by SDS-PAGE and autoradiography. There was specific immunoprecipitation of a protein of M, approximately 72,000–76,000 (Fig. 3, lane D), identical in electrophoretic mobility to the previously purified CETP (1). Although both nonimmune and immune IgG caused precipitation of several other lesser bands, only the band of M, 72,000–76,000 was specifically immunoprecipitated (cf. Fig. 3, lanes C and D). In contrast to the results obtained with the cellular media, immunoprecipitates of cellular homogenates showed a sharp specific band of average M, 58,000 (Fig. 3, lane B, range of M, 54,000–62,000, n = 3). To verify further the identity of the specifically immunoprecipitated bands with the plasma CETP, immunoprecipitates were formed in the presence of 1 µg of purified plasma CETP. This resulted in disappearance of only the M, 72,000–76,000 in cellular media and of only the M, 58,000 band in cellular homogenates (not shown).

The broadness of the band of CETP in SDS gels, as well as its high M, (72,000–76,000), relative to a probable intracellular precursor (M, 58,000), suggested that the mobility of CETP in SDS gels might be influenced by the presence of carbohydrate. Accordingly, the purified CETP was treated with a variety of enzymes, which remove carbohydrate, and then analyzed by SDS gel electrophoresis and immunoblotting with specific CETP antibodies. Treatment of CETP with glyco-

![Fig. 1. Accumulation of CE transfer activity in Hep G2 cell medium. Media was removed at each time point and assayed for CE transfer activity by the ultracentrifugation method, as described under "Materials and Methods."](image)

![Fig. 2. Immunoprecipitation of cholesteryl ester transfer activity in Hep G2 cell medium by anti-CETP IgG. Hep G2 cells were grown in the presence of 84 µg of DEAE-purified anti-CETP IgG, nonimmune IgG, or no IgG (control) per ml of media for 48 h. The IgG was precipitated by adding a 30-fold excess of second antibody, and 0.5 ml of the media was then assayed for activity.](image)

![Fig. 3. Immunoprecipitation of newly synthesized [3H]methionine-labeled CETP from Hep G2 cells, as shown by SDS-PAGE and autoradiography. Hep G2 cell homogenates were used for lanes A and B, and cell media for lanes C and D. Lanes A and C, nonimmune IgG; lanes B and D, anti-CETP IgG. The position of molecular weight markers run simultaneously on the gel are indicated.](image)
peptidase F (to remove asparagine (N)-linked sugars) resulted in the formation of three sharp bands of M, 64,000, 62,000, and 60,000 (Fig. 4, lane B). When the amount of glycopeptidase F was increased (3-fold), the lowest of the bands became most pronounced, but there was no further decrease in M, (not shown). This result suggests that the three bands resulted from partial digestion of CETP, with a limiting M, of about 60,000 resulting from complete removal of N-linked sugars.

Digestion of purified CETP with neuraminidase resulted in a smaller change in M, to be similar to that produced by a number of proteases acting on CETP with higher doses of neuraminidase resulting in formation of two additional products of much lower M, (53,000 and 45,000, Fig. 4, lane M). However, this pattern was noted to be similar to that produced by a number of proteases acting on CETP, including trypsin, chymotrypsin, and an endogenous protease copurifying with the CETP, with the latter producing initial degradation fragments of M, 56,000 and 48,000, which can be seen in the heavily loaded lane of control CETP in Fig. 4, lane J. When an inhibitor of serine proteases (2 mM E 600) was included, the major product of neuraminidase digestion still had M, 67,000 but the lower M, degradation fragments were much reduced in amount (Fig. 4, lane L). Thus, removal of sialic acid produced a protein of apparent M, 67,000, and the lower M, fragments resulted from a protease contaminating the neuraminidase preparation. By contrast, the product of glycopeptidase F digestion was not influenced by the presence of protease inhibitors. Sequential treatments with neuraminidase and then glycopeptidase F or glycopeptidase F and then neuraminidase produced no further change in M, compared to glycopeptidase F alone, implying that the sialic acid of CETP is attached to the N-linked sugar. Also, treatment of purified CETP with neuraminidase and then O-glycanase (to remove core saccharides of structure Galβ1,3GalNAc linked to either serine or threonine) resulted in no change in mobility (Fig. 4, lanes E and F) compared to neuraminidase alone (Fig. 4, lane G). Treatment of purified CETP with β-galactosidase (to remove terminal galactose) resulted in no change in mobility; the CETP band was diffuse after this treatment because of the high salt content in the buffer (Fig. 4, lane K) but a similar effect was produced by adding the buffer alone to control CETP. Similar results to Fig. 4 were obtained when immunoprecipitates of newly synthesized CETP were treated with glycopeptidase F (M, of product 59,000), neuraminidase (68,000) and β-galactosidase (M, 74,000). To see if N-linked sugars were necessary for secretion of CETP, cells were treated with tunicamycin (2 µg/ml). This resulted in a pronounced (>90%) reduction in the amount of the 74-kDa band present in the medium; no lower M, forms of CETP were immunoprecipitated from the medium of tunicamycin-treated cells (not shown).

In further experiments the activity of purified CETP was measured following removal of carbohydrate. The removal of carbohydrate resulted in relatively little change in cholesteryl ester transfer activity which was 94% of control (neuraminidase) or 84% of control (glycopeptidase F at maximum dose). In this set of experiments, complete digestion of the parent CETP molecule was verified by SDS-PAGE.

**DISCUSSION**

The present study demonstrates the secretion of cholesteryl ester transfer activity by cultured Hep G2 cells. The removal of activity by precipitation with specific CETP antibodies, along with the appearance of a single specific band corresponding to CETP in immunoprecipitates, shows that the transfer activity was due to secretion of CETP by Hep G2 cells. The coincidence in M, of the secreted form of CETP with that of CETP purified from plasma (1) indicates that the latter is not itself a breakdown fragment formed during the purification procedure. Faust and Albers (8) have also recently reported the secretion of cholesteryl ester transfer activity by Hep G2 cells; however, this report did not provide molecular identification of the CETP.

The broadness of the band of CETP precipitated from medium (Fig. 3) or isolated from plasma (1) suggested the possibility of protein microheterogeneity. Neuraminidase or glycopeptidase F treatment of the CETP resulted in an increase in mobility and sharpening of the CETP band in SDS-polyacrylamide gels, forming a peptide of similar M, to a CETP precursor (M, 58,000) specifically immunoprecipitated from cellular homogenates. These results suggest that the CETP peptide (M, <58,000) acquires N-linked carbohydrate and sialic acid during intracellular processing. Consistent with this suggestion, a cDNA to the CETP, obtained from a human liver library, encodes an M, 53,000 protein which displays four potential asparagine-linked glycosylation sites (9). Since glycopeptidase F cleaves at the N-glycosidic linkage (10), the presence of three distinct bands in partial digests with glycopeptidase F (Fig. 4, lane B) suggests that at least three of the four sites are glycosylated. Despite treatments of CETP with neuraminidase, glycopeptidase F, β-galactosidase, and O-gly-

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2 In an attempt to demonstrate a higher M, form of CETP within cells, cells were pulsed for 1 h with [35S]methionine and then chased for 1 h with cold methionine. However, no specific bands between 53 and 74 kDa were seen, suggesting rapid secretion of CETP following the addition of sialic acid in the Golgi apparatus.
canase, in increasing doses or in combinations, we were not able to produce a peptide of M, 53,000. Several possibilities could explain the residual difference of about 6 kDa between the glycopeptidase F-digested protein and the cDNA-encoded peptide (8). For example, there could be O-linked sugar, not susceptible to digestion by neuraminidase and O-glycanase, or additional post-translational modifications such as phosphorylation or addition of fatty acids.

Although the addition of N-linked sugars seemed to be necessary for the normal secretion of CETP (as shown by lack of secretion of CETP by tunicamycin-treated cells), the removal of carbohydrate from purified CETP resulted in only minor reductions of cholesteryl transfer activity. Thus, despite the unusually high content of hydrophobic amino acid residues in CETP (1), the N-linked carbohydrate and sialic acid are not essential for activity of purified CETP. By contrast, small amounts of phospholipid bound to purified CETP are needed to maintain cholesteryl ester transfer activity.\textsuperscript{3}

Previously, cholesteryl ester transfer activity has been shown to accumulate in the perfusate of rabbit liver, suggesting hepatic synthesis and secretion of a cholesteryl ester transfer protein (11). The present investigation indicates that hepatocytes have the capacity to synthesize and secrete CETP. The CETP can increase the transfer of CE from HDL into cultured Hep G2 cells (12), raising the possibility that CETP secreted by the liver cell might enhance the cellular uptake of CE in the same tissue microenvironment. Cultured monocyte macrophages have been shown to synthesize and secrete a lipid transfer activity with some characteristics of CETP (13), and the mRNA for CETP is present in spleen and other peripheral tissues (9). Thus, it is likely that CETP synthesis is not confined to the liver and intestine, like most of the apoproteins, but rather that it shows a more widespread tissue distribution, and perhaps, like apoE (14), participates in the localized redistribution of cholesterol within tissues.

\textsuperscript{3} T. Swenson, R. Brocia, and A. Tall, unpublished results.