Cyclosporine Binds to the Neutral Lipid and Potentially Other Binding Sites of Lipid Transfer Protein I

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Purpose. The objective of this study was to determine if cyclosporine (CSA) binds directly to the neutral lipid-binding site of lipid transfer protein I (LTP I).

Methods. This was accomplished by determining LTP I concentrations and cholesteryl esters (CE) and CSA radioactivity of eluted fast protein liquid chromatography (FPLC) fractions following an injection of different treatment groups (i.e., LTP I alone, ^{3}H-CE liposomes alone, ^{3}H-CSA liposomes alone, ^{3}H-CE liposomes + LTP I, and ^{3}H-CSA liposomes + LTP I) onto an FPLC column. Our hypothesis is that CSA will bind to the neutral lipid-binding site of LTP I because of its high solubility/interaction with cholesterol and triglycerides.

Results. Coincubation of LTP I with ^{3}H-CE liposomes resulted in a significant decrease in the LTP I peak reported at fraction 10 and the appearance of a broad LTP I peak at fractions 30–34 compared to control. Coincubation of LTP I with ^{3}H-CSA liposomes resulted in a significant decrease in the LTP I peak reported at fraction 10 and fraction 30 compared to control. In addition, 30% of the original radioactivity associated with ^{3}H-CSA liposomes was found coeluted with the unbound LTP I peak at fraction 10. Taken together, these findings suggest that CSA does bind to the neutral lipid-binding site of LTP I but may also bind to other regions along the LTP I molecule.

Conclusions. We have determined that LTP I mediated transfer of CSA between lipoproteins may be a result of the direct binding of CSA to LTP I at both its neutral binding site and potentially other binding sites along the molecule. These findings provide further evidence that the distribution/redistribution of drugs among plasma lipoproteins facilitated by LTP I may serve as a possible mechanism for determining the ultimate fate of drug compounds

KEY WORDS: cyclosporine; lipid transfer protein I; neutral lipid-binding site; lipoproteins.

INTRODUCTION

Lipid transfer protein I (LTP I) (1), also known as cholesteryl ester transfer protein, is a 476–amino acid glycoprotein with a molecular weight of 74,000. LTP I has been shown to be responsible for all of the facilitated transfer of core lipoprotein lipid cholesteryl esters (CE) and triglyceride (TG), about one-half of the coat lipoprotein lipid phosphatidylcholine (PC) transfer (2–4), and a portion of transfer of several water-insoluble drugs (5–8) between different plasma lipoprotein particles. Several investigators have demonstrated that LTP I has a unique binding site for CE, TG, and PC found at the carboxyl-terminus end, which is responsible for the transfer of these lipids through a carrier-mediated mechanism. Our laboratory has recently reported that facilitated transfer of one of these water-insoluble compounds, cyclosporine (CSA), by LTP I between high-density lipoproteins (HDL) and low-density lipoproteins (LDL) was partially dependent on LTP I's CE (7) and TG transfer activities (8) but not on its PC transfer activity (18). In addition, our laboratory has recently reported that changes in plasma lipoprotein core lipid concentration and composition influence the lipoprotein association of CSA (9). These findings suggest that CSA binding to LTP I's neutral lipid binding site, either directly or complexed with CE or TG, may facilitate its transfer between lipoproteins. However, to date exactly how CSA is transferred by LTP I between lipoproteins has not been determined.

One of the proposed biologic consequences of CSA association to lipoproteins is the change in the drug's pharmacologic effect. Lemaire and co-workers have suggested that the drug's availability to tissue and, hence, its pharmacologic (or toxic) effects may depend on which lipoprotein the drug is bound to (13). They have observed an enhanced antiproliferative effect of CSA when it was bound to LDL that was not evident when the drug was bound to either very-low density lipoproteins (VLDL) or HDL (13,14). Furthermore, transplantation patients who are administered CSA exhibit plasma dyslipidemias (i.e., lipid disturbances) including hypocholesterolemia and hypertriglyceridemia (15,16) that have elevated LTP I levels (17). Thus, determining if LTP I facilitates the binding of CSA to certain lipoproteins and by what mechanism(s) may help to explain differences in CSA's pharmacologic behavior following administration to hypocholesterolemic (12) and/or hypertriglyceridemic patients (10,11).

The objective of this study was to determine if CSA binds directly to the neutral lipid-binding site of LTP I. This was accomplished by determining LTP I concentrations and CE and CSA radioactivity of eluted fast protein liquid chromatography (FPLC) fractions following an injection of different treatment groups (i.e., LTP I alone, ^{3}H-CE liposomes alone, ^{3}H-CSA liposomes alone, ^{3}H-CE liposomes + LTP I, or ^{3}H-CSA liposomes + LTP I) onto an FPLC column. Our hypothesis is that CSA will bind to the neutral lipid-binding site of LTP I because of its high solubility/interaction with cholesterol and triglycerides.

MATERIALS AND METHODS

Chemical Reagents and Lipid Assay Kits

Radiolabeled CSA (*mebmt*-β-^{3}H-cyclosporine, specific activity 5.82 mCi/mg) and radiolabeled CE [1α,2α(*n*)-^{3}H-cholesteryl oleate, specific activity 73.4 mCi/mg], both dissolved in 100% ethanol solution, were purchased from Amersham Pharmacia Biotech (Oakville, Ontario, Canada). Tris-HCl, sodium azide, ethylenediaminetetraacetate (EDTA), sodium bromide, sodium chloride, sodium carbonate, sodium bicarbonate, sodium phosphate (anhydrous), potassium phosphate monobasic, potassium chloride, bovine serum albumin, cholesteryl oleate, sodium hydroxide, *p*-nitrophenyl phosphate, triton X-100, and cold CSA were purchased from Sigma Chemical Company (St. Louis, MO).

LTP I (1,690 μg protein/mL, 30 μg LTP I/mL), stabilized with albumin in a buffer, was purified from human lipopro-

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tein-deficient plasma as previously described (2–4). Phospholipids were bought from Avanti Lipids (Alabaster, AL). Affinity-purified goat antimouse phosphatase conjugate was purchased from Promega (Madison, WI). Normolipidemic fasted pooled human plasmas (total plasma cholesterol concentrations in the range 135–150 mg/dL) were obtained from Canadian Blood Services (Vancouver, BC, Canada) and Bioreclamation (Hicksville, NY). TP2, a monoclonal antibody directed against the neutral lipid-binding site of LTP I (19), was obtained from the Ottawa Heart Institute (Ottawa, ON, Canada). Total plasma and lipoprotein triglycerides, cholesterol (free and cholesteryl ester), phospholipids, and protein concentrations were determined by enzymatic assays purchased from Sigma Diagnostics (St. Louis, MO), Hoffmann-La Roche Diagnostics (Laval, QC, Canada), and Biorad Laboratories (Mississauga, ON, Canada). Bovine serum albumin (BSA) protein standards were purchased from Sigma Diagnostics (St. Louis, MO). Sterile 0.2-μm syringe filters were purchased from Millipore (Bedford, MA). An XK column (18 mm × 20 cm) and Sephacryl HR 100 packing were obtained from Amersham Pharmacia Biotech (Oakville, ON, Canada). Chloroform and Nunc Maxisorp brand 96-well plates were obtained from Fisher Scientific (Nepean, ON, Canada).

Total Protein, Lipid Transfer Protein I, Cyclosporine, and Cholesteryl Ester Quantification

Eluted fractions obtained from the FPLC column following injections of different treatment groups were analyzed for total protein and LTP I content utilizing a modified Lowry assay (20,21) and an LTP I ELISA methodology that utilizes a monoclonal antibody, TP2, that binds to the neutral lipid-binding site of LTP I (22), as previously described. The ^{3}H-CSA and ^{3}H-CE were quantified by radioactivity.

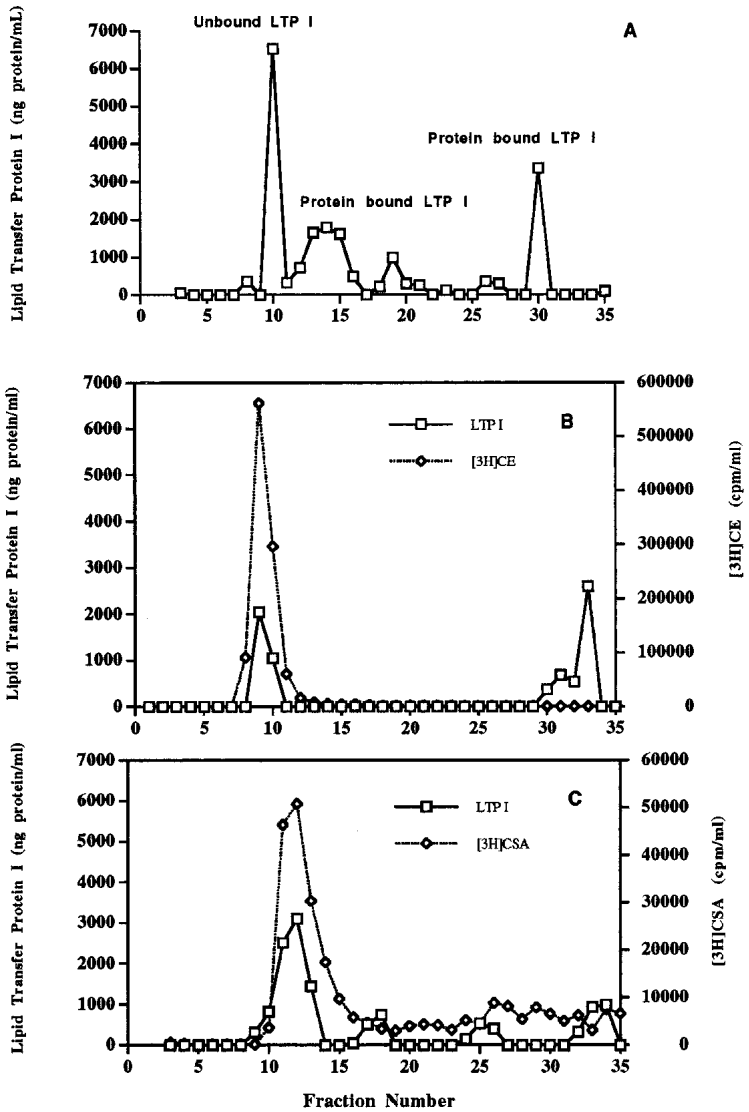

Fig. 1. Chromatograph of LTP I concentration (ng/mL) and ^{3}H-CE and ^{3}H-CSA radioactivity (cpm/mL) following injections onto a FPLC system with a Sephacryl HR 100 XK column (18 mm × 20 cm) of (a) 9 μg LTP I alone, (b) 9 μg LTP I + ^{3}H-CE liposomes (egg PC 590 nmol, CE 9.1 nmol), and (c) 9 μg LTP I + ^{3}H-CSA liposomes (egg PC 590 nmol, CSA 8.1 nmol). Data are presented as mean ± standard deviation of three independent runs (n = 3).

Cholesteryl Ester and Cyclosporine Binding to Lipid Transfer Protein I

Egg phosphatidylcholine (PC; 590 nmol) liposomes composed of cholesteryl oleate and ^{3}H-cholesteryl oleate (9.1 nmol total) or unlabeled CSA and ^{3}H-CSA (8.1 nmol total) were made as previously described (23) and incubated with purified LTP I (9 μg protein) for 1 h at 37°C. Following incubation, the mixture (0.5 ml injection volume) was loaded onto a Sephacryl HR 100 XK FPLC column (18 mm × 20 cm), and 1-ml fractions were collected and assayed for ^{3}H-CE, ^{3}H-CSA, and LTP I protein concentrations. The mobile phase consisted of 10 mM Tris Cl and 1 mM EDTA buffer, pH 7.4, and the flow rate was set at 0.25 mL/min for a 3-min load. The sample loop used was 0.5 mL. The column was washed with 300 mL of buffer after each run.

Chromatographs (n = 3 for each treatment group) for LTP I, ^{3}H-CE, and ^{3}H-CSA liposomes injected alone onto the FPLC column were obtained and served as controls. However, when free ^{3}H-CSA was injected onto the column, less than 5% of the original radioactivity was recovered because of nonspecific binding to the column; thus, this approach was not used in further studies.

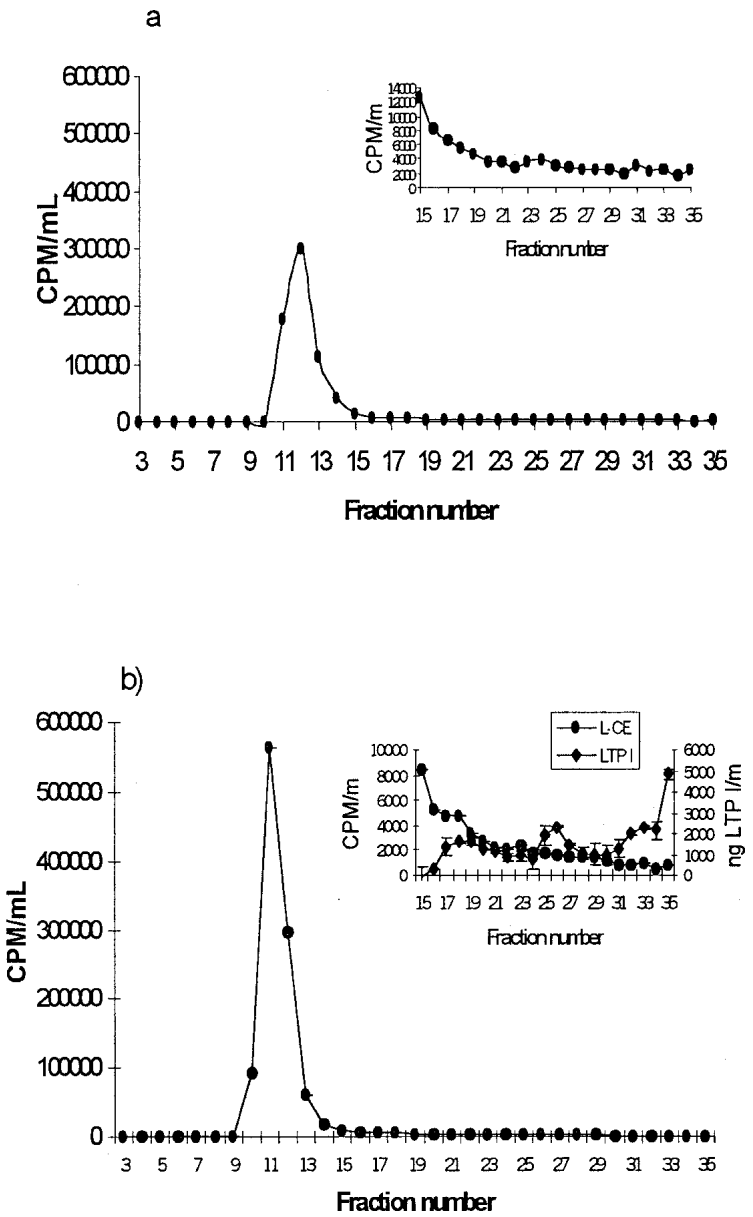

Fig. 2. Chromatograph of ^{3}H-CE radioactivity (cpm/mL) following injections onto a FPLC system with a Sephacryl HR 100 XK column (18 mm × 20 cm) of (a) ^{3}H-CE liposomes (egg PC 590 nmol, CE 9.1 nmol) and (b) 9 μg LTP I + ^{3}H-CE liposomal vesicles (egg PC 590 nmol, CE 9.1 nmol). Insets show fractions 15 to 35 with an additional y axis showing amount of LTP I in the eluted fractions. Data are presented as mean ± standard deviation of three independent runs (n = 3).

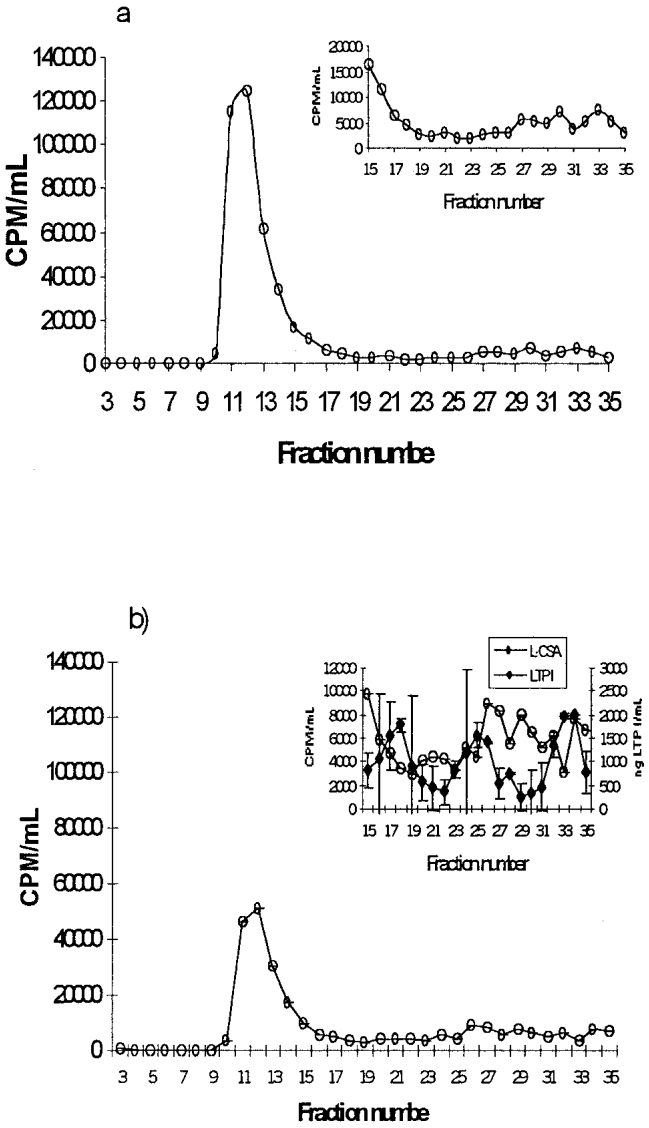

Fig. 3. Chromatograph of ^{3}H-CSA radioactivity (cpm/mL) following injections onto a FPLC system with a Sephacryl HR 100 XK column (18 mm × 20 cm) of (a) ^{3}H-CSA liposomes (egg PC 590 nmol, CSA 8.1 nmol) and (b) 9 μg LTP I + ^{3}H-CSA liposomes (egg PC 590 nmol, CSA 8.10 nmol). Insets show fractions 15 to 35 with an additional y axis showing amount of LTP I in the eluted fractions. Data are presented as mean ± standard deviation of three independent runs (n = 3).

STATISTICAL ANALYSIS

Differences in the amount of CE and CSA radioactivity recovered in each fraction obtained from the FPLC column in the absence and presence of LTP I were compared (PCANOVA; Human Systems Dynamics). Critical differences were assessed by Tukey *post hoc* tests. A difference was considered significant if the probability of chance explaining the results was reduced to less than 5% ($p < 0.05$). Data were presented as a representative chromatograph of three separate injections with CV less than 8%.

RESULTS AND DISCUSSION

The objective of this study was to determine if CSA directly binds to LTP I at its neutral lipid-binding site located at the carboxyl terminus. Our data suggests that CSA binds directly to LTP I not only at the neutral lipid binding but potentially at other sites along the molecule.

We have previously demonstrated that increases in LTP I concentration resulted in an increased percentage of CSA recovered in the HDL/lipoprotein-deficient plasma fraction during short-term incubations (8,10). Furthermore, we observed that the transfer of CSA between HDL and LDL appears to be partially facilitated through LTP I CE and TG transfer activities. As LTP I is the protein which catalyzes the transfer exchange of CE from CE-rich lipoproteins (HDL and LDL) for TG from TG-rich lipoproteins (VLDL), these findings suggest that CSA plasma distribution could be partially explained by its lipoprotein CE and TG content. Since the neutral lipids, CE and TG, are found in the lipid core of lipoproteins and are transferred between lipoproteins by binding to the neutral lipid binding site of LTP I suggests that LTP I may facilitate CSA's lipoprotein transfer by directly binding the drug to it's neutral lipid binding site.

The binding of CE, TG and phospholipid (PL) to the neutral lipid binding site of LTP I has been assessed by others using monoclonal antibodies raised up against the neutral lipid binding site (23). In our initial studies investigating the interaction of CSA with lipoproteins (9,10) we have hypothesized that lipophilic compounds, such as CSA, would be recognized as lipid-like particle. Thus, in order to determine if CSA binds to LTP I we have modified the procedures used to determine lipoprotein lipid binding to LTP I to assess the interaction of CSA with LTP I.

When LTP I alone was injected onto the FPLC column the following elution profile was observed (Fig. 1A). Fraction 10 represents unbound LTP I while fractions 14 and 30 represents albumin bound LTP I as previously reported by others (22). This was further confirmed by quantifying the total protein recovered in each fraction. The greatest concentration of total protein recovered was found in fractions 14 and 30 (data not shown) corresponding to the two additional LTP I peaks.

To confirm the validity of our FPLC procedure, CE binding to LTP I was determined by a methodology previously published (22). Co-incubation of LTP I with [^{3}H]-CE liposomes resulted in a significant decrease in the LTP I peak reported at fraction 10 (unbound LTP I) and the appearance of a broad LTP I peak at fractions 30-34 (Fig. 1B) compared to control (Fig. 1A). In addition, the majority of radioactivity associated with [^{3}H]-CE was found co-eluted with the unbound LTP I peak at fraction 10 (Fig. 1B) and was higher than when [^{3}H]-CE liposomes were injected alone onto the FPLC column (Fig. 2). Taken together these findings confirm that [^{3}H]-CE binds to the neutral lipid binding site of LTP I.

Co-incubation of LTP I with [^{3}H]-CSA liposomes resulted in a significant decrease in the LTP I peak reported at fraction 10 (unbound LTP I) and fraction 30 (protein bound LTP I) (Fig. 1C) compared to control (Fig. 1A). In addition, 30% of the original radioactivity associated with [^{3}H]-CSA liposomes was found co-eluted with the unbound LTP I peak at fraction 10 (Fig. 1C) and was lower than when [^{3}H]-CSA liposomes were injected alone onto the FPLC column (Fig. 3). Taken together these findings suggest that CSA does bind to the neutral lipid binding site of LTP I, but may also bind to

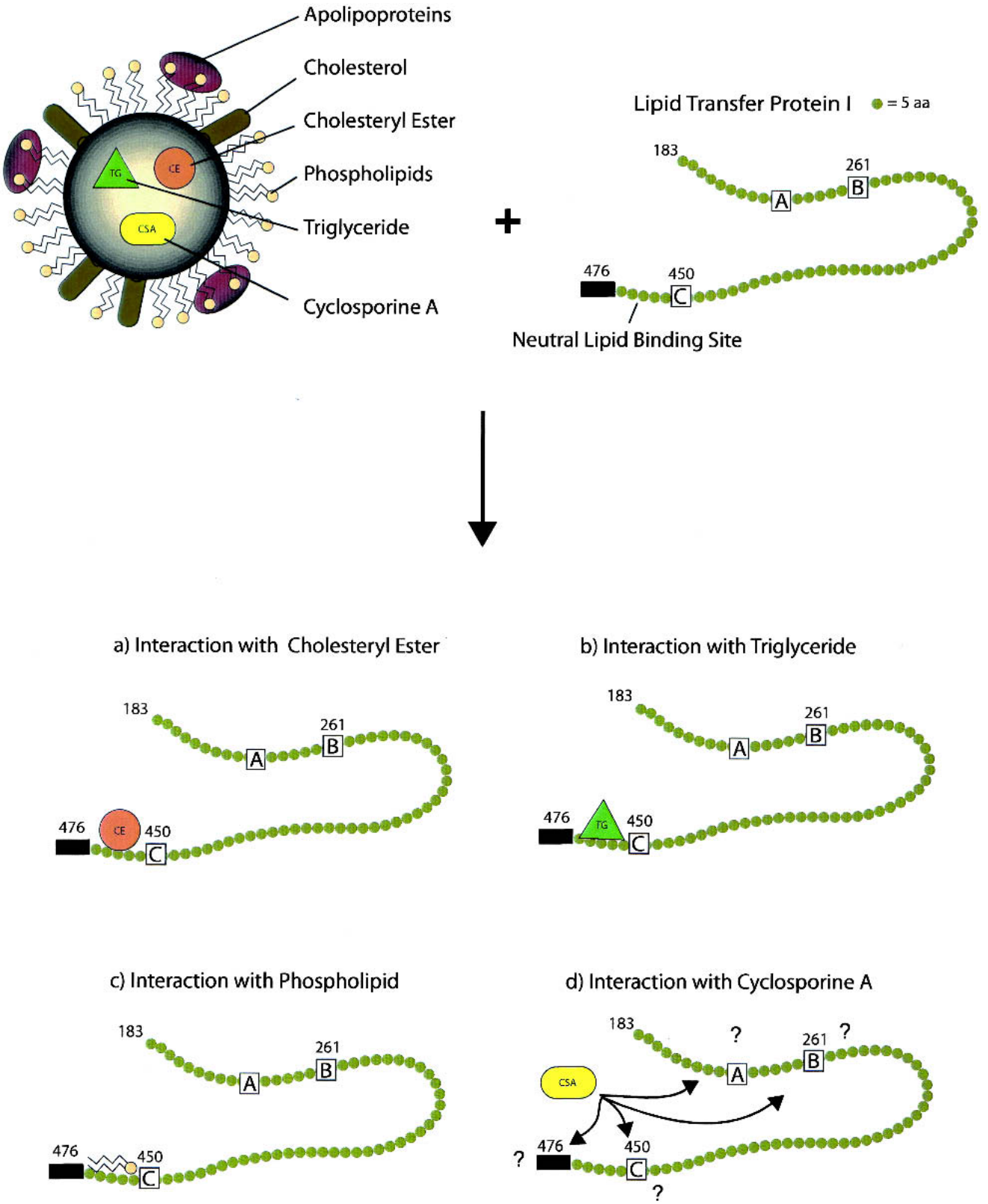

Fig. 4. Model of lipid transfer protein I (LTP I) interactions with cholesteryl esters (CE), triglycerides (TG), phosphatidylcholine (PC) and cyclosporine A (CSA).

other regions along the LTP I molecule (Fig. 4), albumin found in the LTP I preparation or non-specifically to the FPLC column. Studies to confirm these hypotheses are required.

In conclusion we have determined that LTP I mediated transfer of CSA between lipoproteins may be a result of the direct binding of CSA to LTP I at both it's neutral binding and potentially other binding sites along the molecule. These

findings provide further evidence that the distribution/redistribution of drugs among plasma lipoproteins facilitated by LTP I may serve as a possible mechanism for determining the ultimate fate of drug compounds (24). LTP I may have potential application in the field of drug delivery since it can facilitate the transfer of lipophilic drugs such as halofantrine, amphotericin B, and CSA between different lipoprotein subclasses (9). A recent paper (25) suggests a potential application of plant lipid transfer proteins for drug delivery. This group reports that skin lipids such as sphingosine, sphingomyelin and cerebroside (an azole derivative with antitumoral and/or antileishmania properties), and amphotericin B were shown to bind to plant lipid transfer protein I. These binding studies suggest the potential for the development of LTP I-mediated transport and controlled release of low molecular weight drugs.

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