Structural and Functional Determinants of Human Plasma Phospholipid Transfer Protein Activity As Revealed by Site-Directed Mutagenesis of Charged Amino Acids†

Gabriel Ponsin,‡ Shi-Jing Qu, Hui-Zhen Fan,§ and Henry J. Pownall*

Department of Medicine, Baylor College of Medicine, Houston, Texas 77030

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ABSTRACT: Human plasma phospholipid transfer protein (PLTP) exchanges phospholipids between lipoproteins and remodels high-density lipoproteins (HDLs). We determined phospholipid transfer activity and HDL binding ability in wild-type PLTP and in 16 PLTP variants created by replacing 12 charged amino acids by site-directed mutagenesis. The data were analyzed in relation to the structure of a member of the same gene family, bactericidal/permeability-increasing protein, which is a boomerang-shaped molecule containing two symmetrical, hydrophobic pockets that bind phospholipid molecules. When expressed in COS-7 cells, wild-type and all mutant PLTPs accumulated intracellularly to nearly the same extent. Relative to wild-type PLTP, substitution(s) for amino acids with a lateral position totally exposed to the solvent produced reductions in transfer activity proportional to the reductions in the level of HDL binding. Variants containing substitutions for charged amino acids on the concave surface of PLTP did not affect binding to HDL or specific transfer activity. A mutation in the C-terminal pocket (E270R) led to a decrease in both the specific transfer activity and the level of binding to HDLs, whereas mutations in the N-terminal pocket (R25E and D231R) resulted in a large decrease in specific transfer activity without affecting HDL binding. The data support a model of transfer in which N- and C-terminal pockets have different roles in HDL binding and transfer activity. The N-terminal pocket may be critical to PLTP transfer activity but may have no involvement in binding to lipoproteins, whereas amino acid substitutions in the C-terminal pocket might reduce PLTP activity by decreasing PLTP's affinity for HDLs.

High-density lipoprotein (HDL)[1] cholesterol, which is a negative risk factor for cardiovascular disease, transports cholesterol from peripheral tissue to the liver through reverse cholesterol transport (*1*−*3*). In plasma, HDL is remodeled by two enzymes, lecithin:cholesterol acyltransferase and hepatic lipase, and by two lipid transfer proteins, cholesteryl ester transfer protein (CETP) and phospholipid transfer protein (PLTP; *1*, *4*−*6*). CETP is the exclusive carrier of neutral lipids between lipoproteins, but also transports phospholipids; PLTP transfers sphingo- and glycerolipids, especially phosphatidylcholine.

PLTP may be an important agent in reverse cholesterol transport (*7*, *8*). Extracellular PLTP enhances intracellular mobilization and efflux of phospholipids to plasma lipoproteins (*9*); adenovirus-mediated overexpression of PLTP increases serum PLTP activity and stimulates HDL turnover, and decreases the levels of serum lipids and HDL (*10*, *11*). Conversely, mice in which the PLTP gene has been disrupted have no plasma phospholipid transfer activity and have reduced plasma HDL concentrations (*12*). PLTP mediates the disproportionation of normal HDL into larger particles and smaller, pre-β HDLs that efficiently accept cholesterol from peripheral cells (*7*, *13*−*19*). Although phospholipid transfer precedes HDL disproportionation (*20*), the mechanistic connection between these two processes is not known (*21*, *22*).

PLTP belongs to a gene family of lipid transfer proteins that includes CETP, lipopolysaccharide-binding protein, and bactericidal/permeability-increasing protein (*23*, *24*). PLTP has a two-domain architecture; each domain contains a lipid-binding pocket, essential for transfer activity (*25*, *26*). A disulfide bridge between cysteines 146 and 185 is important for structural integrity, while the 30 C-terminal amino acid residues are not required for secretion or activity (*26*, *27*).

Interaction of PLTP with lipoprotein surfaces is an obligatory component of lipid transfer. Therefore, PLTP must contain structural determinants for binding to lipid surfaces; the determinants may (or may not) be independent of those for transfer activity. It has been suggested that PLTP binding to lipoproteins might have an electrostatic component. Modifications of the electrostatic charge of lipoproteins have been shown to affect the PLTP-mediated transfer of phospholipids (*28*), and the activity of CETP, which is homolo-

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* To whom correspondence should be addressed: MS A-601, Department of Medicine, Baylor College of Medicine, 6565 Fannin St., Houston, TX 77030. Telephone: (713) 798-4160. Fax: (713) 798-5134. E-mail: hpownall@bcm.tmc.edu.

‡ Current address: INSERM Unité 352, Biochimie et Pharmacologie, Institut National des Sciences Appliquées de Lyon, 20 avenue Albert Einstein, 69100 Villeurbanne, France.

§ Current address: The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030.

[1] Abbreviations: HDL, high-density lipoprotein; CETP, cholesteryl ester transfer protein; PLTP, phospholipid transfer protein; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay.

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Table 1: Primers for Mutant PLTPs

mutant PLTP	mutation primer[a]
R25E	5′-GGC-TGC-AAG-ATC-GAG-GTC-ACC-TCC-GAC-3′
E37R	5′-CTG-GTG-AAG-CAG-AGG-GGG-CTG-CGC-TTT-3′
R235E	5′-CTG-GAC-ATG-GAC-TTC-GAG-GGG-GCC-TTC-TTC-CCC-3′
R235I	5′-CTG-GAC-ATG-GAC-TTC-ATC-GGG-GCC-TTC-TTC-CCC-3′
R262E	5′-CTG-CAG-GAG-GAA-GAG-GAG-ATG-GTG-TAT-GTG-GCC-3′
R262I	5′-CTG-CAG-GAG-GAA-GAG-ATC-ATG-GTG-TAT-GTG-GCC-3′
R235/262E	two-site mutation using two primers, R235E and R262E
R235/262I	two-site mutation using two primers, R235I and R262I
E270R	5′-GTG-GCC-TTC-TCT-AGG-TTC-TTC-TTC-GAC-3′
D231R	5′-ACC-AGC-AAC-CTG-CGG-ATG-GAC-TTC-CGG-3′
E259/260/261R	5′-GAG-CCC-CAG-CTG-CAG-AGG-AGA-AGG-CGG-ATG-GTG-TAT-3′
E259/260/261/270R	four-site mutation using primers for E259/260/261R and E270R
E428R	5′-CCC-ATG-CTC-AAT-AGG-CGG-ACC-TGG-CGT-3′
K382E	5′-GCT-CTC-CGG-GGG-GAG-GCC-CTG-CGC-ACG-3′
R380E	5′-AAG-ATG-GCT-CTC-GAG-GGG-AAG-GCC-CTG-3′
R380E/K382E	5′-AAG-ATG-GCT-CTC-GAG-GGG-GAG-GCC-CTG-CGC-ACG-3′

[a] Codons used for substitutions were GAG(E), ATC(I), ACG(R), CGG(R), and AGH(R).

gous with PLTP, is determined in part by electrostatic interactions (*29*−*31*). Lipid transfer studies of CETP mutants indicate that Lys233 and Arg259 are essential for interaction with charged lipid surfaces (*32*). These two amino acids correspond to Arg235 and Arg262 in PLTP, respectively, likely essential for PLTP binding (*33*). Using site-directed mutagenesis, we expressed in COS cells PLTP mutants in which 11 conserved charged amino acids were replaced. The synthesis, secretion, phospholipid transfer activity, and HDL binding of each were compared with those of wild-type PLTP.

MATERIALS AND METHODS

Materials. Restriction enzymes were purchased from Boehringer Mannheim (Indianapolis, IN). The Sequenase version 2.0 sequencing kit and [α-^{35}S]dATP were from Amersham (Arlington Heights, IL). [^{3}H]-1,2-Dipalmitoylglycerol-3-phosphorylcholine was from DuPont NEN (Wilmington, DE), and phospholipid was from Avanti Polar Lipids (Birmingham, AL). Rabbit anti-goat IgG and the Immun-Blot kit were from Bio-Rad (Hercules, CA). The cell culture medium, fetal bovine serum, and pSV-SPORT1 vector were from Gibco BRL (Gaithersburg, MD). Egg phosphatidylcholine was from Sigma Chemical Co. (St. Louis, MO). HDL was isolated from fresh human plasma by sequential ultracentrifugation between *d* values of 1.063 and 1.21 g/mL (*34*).

Construction of the Wild-Type Expression Vector. A prototypic PLTP cDNA was obtained by polymerase chain reaction and inserted into the serum response factor site of a phagemid, pCR-Script SK(+), with a 3′−5′ orientation. Prototypic PLTP cDNA (1.65 kb) was cut from the phagemid by *Eco*RI and *Not*I and subcloned into a mammalian expression vector, pSV·SPORT1, for expression; the expression product from prototypic PLTP pSV·SPORT1 was inactive in COS-6 cells. Direct sequencing and comparison with the sequence of Day et al. (*35*) revealed three point mutations, which were sequentially replaced by site-directed mutagenesis to yield our wild-type PLTP cDNA (pSV·SPORT1-PLTP WT), which had the same primary structure as that reported by Day et al. (*35*). COS-7 cells transfected with pSV·SPORT1-PLTP WT efficiently expressed PLTP.

Site-Directed Mutagenesis. Starting with pSV·SPORT1-PLTP WT as a template, we conducted site-directed mutagenesis according to the method of Deng and Nicholoff (*36*), which permits site-directed mutagenesis of virtually any plasmid by elimination of a unique site. Using a unique *Kpn*I restriction site within pSV·SPORT1 as a marker for successful mutagenesis, the observed success rate exceeded 80%. Once a mutation was formed, the *Kpn*I site was converted to an *Eco*RV site and reversed during a subsequent mutation. Sixteen primers for mutagenesis within the coding region were synthesized (Table 1). Mutagenesis was performed using the Transformer site-directed mutagenesis kit from Clontech (Palo Alto, CA). Ten transformants were chosen for each of 16 charged amino acid mutations. After digestion with either *Kpn*I or *Eco*RV, at least two positive mutant vectors were selected and verified by direct sequencing. The final candidate mutant cDNAs were processed to large-scale purification of plasmid (∼500 μg) with a Qiagen tip.

Ex Vivo Expression of PLTP. A modified DEAE-dextran method (*37*) was used to transfect wild-type and mutant PLTP cDNAs into COS-7 cells. Cells were grown at 37 °C in 75 cm^2 flasks, in duplicate or triplicate, in high-glucose Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal bovine serum and a 10× antibiotic/antimycotic mixture. The cells at >90% confluence were subcultured at ratios between 1:4 and 1:6 for an additional 24 h period before transfection. Mutant PLTP cDNAs were transfected into COS-7 cells (20 μg per flask), incubated with DNA-DEAE stock and chloroquine for 3 h, and shocked with 10% dimethyl sulfoxide for 2 min. Control flasks containing only COS-7 cells or COS-7 cells with pSV·SPORT1 plasmids were treated in the same manner as those containing the PLTP insert. After incubation at 37 °C in DMEM containing 10% fetal bovine serum for 72 h, the cultures were changed to nonserum DMEM and incubated for 72 h before harvesting. The medium was collected and concentrated 20-fold by using a Centriprep-30 apparatus (Amicon, Beverly, MA). Cells were lysed with 1.2 mL of a buffer containing 250 mM Tris-HCl (pH 8.0), 1% NP-40, 150 mM NaCl, 100 μg/mL phenylmethanesulfonyl fluoride, and 1 μg/mL pepstatin A. Samples were stored at −20 °C until they were tested for PLTP mass and activity.

PLTP Activity. The PLTP-stimulated transfer of phospholipids was assessed using single-bilayer vesicles of egg phosphatidylcholine labeled with [^{3}H]dipalmitoylphosphatidylcholine as the donors and human plasma HDLs as the

acceptors (*38*). In brief, labeled single-bilayer vesicles were prepared by drying 12.5 μmol of egg phosphatidylcholine and 3.7 μCi of [^{3}H]dipalmitoylphosphatidylcholine under nitrogen, and dissolving the residue in 0.3 mL of absolute ethanol. The resulting preparation was rapidly injected through a 26 gauge needle into 4.7 mL of 10 mM Tris-buffered saline. Donors and acceptors were extensively dialyzed at 4 °C. Labeled single-bilayer vesicles (90 μg of phospholipids), 200 μg of HDL, and 10 μL of culture medium or lysate were mixed in a final volume of 280 μL. After incubation for 1 h at 37 °C, single-bilayer vesicles were precipitated by addition of 40 μL of 200 mM $MgCl_2$ and of 80 μL of 0.0085% dextran sulfate. The samples were cooled on ice and centrifuged at 20000*g* for 10 min. The supernatants were collected, and the precipitates were dissolved in 200 μL of 0.1 M NaOH. Radioactivity was determined in both the supernatants and precipitates. The activity of PLTP was calculated as the amount of phospholipids transferred per hour and per milliliter of either medium or cell lysate.

Enzyme-Linked Immunosorbent Assay (ELISA) of PLTP. A 3.15 kDa, truncated recombinant His-tagged PLTP was produced in *Escherichia coli.* In brief, a 0.82 kb PLTP fragment from the 3′ end was cut by *Bam*HI and *Sal*I from 1.43 kb cDNA already inserted into pQE-30 (Qiagen). The 0.82 kb PLTP cDNA was subcloned into pQE-31 (pQE-31-0.82 PLTP) and expressed in *E. coli*. PLTP was transformed into M15[pREP4] or JM109, cultured overnight, and inoculated at a 1:10 to 1:20 ratio into LB broth containing 25 μg/mL kanamycin or 100 μg/mL ampicillin at 37 °C for 1 h. Isopropyl thio-β-D-galactoside was added to bring the solution to 0.5 mM, and the sample was incubated for an additional 5 h. The cells were harvested, and the 3.15 kDa recombinant PLTP was purified using Ni−NTA resin (Qiagen) under denaturing conditions. The resulting truncated recombinant PLTP was used to raise a polyclonal goat anti-PLTP antiserum that served for an ELISA and Western blot analysis.

An indirect ELISA was used to assay the PLTP concentrations in transfected COS-7 cells, using purified, 3.15 kDa recombinant PLTP as the standard. The entire procedure was performed at room temperature. Aliquots of either 20-fold concentrated medium or cell lysate were diluted in carbonate/bicarbonate buffer (pH 9.2) to give a final volume of 100 μL and incubated for 2 h in ELISA plates. These were then blocked by 1 h incubations with a phosphate-buffered saline solution (pH 7.4) and Tween 20 (0.005%) containing 0.5% gelatin. After being washed with phosphate-buffered saline and Tween 20 (0.005%), the wells were treated with a 1:50 dilution of goat anti-PLTP IgG for 2 h. Finally, a 1:1000 dilution of rabbit anti-goat IgG conjugated with horseradish peroxidase was added to the wells, and the color was developed using *o*-phenylenediamine dihydrochloride.

Western Blot Analysis of PLTP. Western blot analysis of PLTP in cell lysate and medium of transfected COS-7 cells was performed as previously described (*39*). Medium was collected and concentrated 20-fold with a Centriprep-30 apparatus. The cells were detached with trypsin, washed with phosphate-buffered saline, pelleted by centrifugation at 1500*g* for 5 min, and resuspended in 1 mL of 250 mM Tris (pH 8.0). The samples were then frozen in a dry ice/ethanol bath and thawed at 37 °C. This procedure was repeated, and the resulting lysates were clarified by centrifugation at 19 000 rpm for 10 min, using a Beckman J21 rotor. Aliquots of cell medium and lysate were submitted to SDS−PAGE using 12% gels. The resulting protein bands were transferred to a nitrocellulose membrane and incubated in diluted goat anti-PLTP serum. Finally, PLTP was visualized by incubation with rabbit anti-goat IgG (H + L) horseradish peroxidase-conjugated serum (Bethyl Lab, Montgomery, TX) and use of the Immuno-Blot assay kit (Bio-Rad).

Binding of PLTP Mutants to HDL. Nitrocellulose membranes (1 cm × 1 cm) were incubated overnight at room temperature in a solution of HDL (2.5 mM phospholipid). After the membranes had been washed, the HDL-coated pieces were incubated for 2 h at 37 °C with each PLTP mutant (50 μg) in 100 μL of buffer. Bovine serum albumin-coated pieces of nitrocellulose were used as negative controls. After the pieces had been washed, both the HDL- and bovine serum albumin-coated pieces were analyzed for bound PLTP by using goat anti-PLTP antiserum and horseradish peroxidase-conjugated anti-goat IgG, as described above for the Western blot procedure. After color had developed, the nitrocellulose sheets were incubated at room temperature in 1 mL of acetate buffer (pH 4) to disrupt the antigen−antibody complex and release the color from the nitrocellulose sheets into the medium. The absorbance at 570 nm was measured. Comparison of the results for the two experiments, expressed as (OD of mutants/OD of WT) × 100, gave a correlation between experiments that was good ($r^2 = 0.76$) but not perfect due slow fading of the color and incomplete release of the color from the nitrocellulose sheets. The binding of each PLTP mutant was compared to that of WT PLTP in the same experiment, using the same HDL preparation. The entire experiment was conducted for two completely independent times.

Statistical Methods. First-order lines of regression and coefficients of determination were calculated by using Sigma Plot.

RESULTS

To modify the electrostatic charge on PLTP, five positive and seven negative amino acids were replaced by site-directed mutagenesis. Through single or combined mutations, 16 mutants were generated, resulting in charge modifications ranging from −4 to +8 with respect to wild-type PLTP

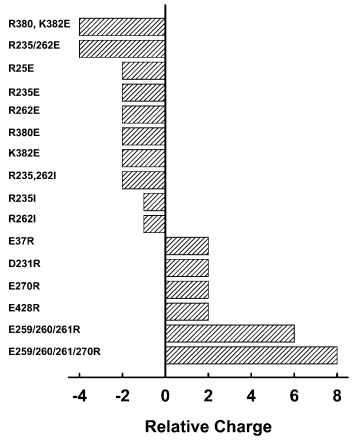

FIGURE 1: PLTP electrostatic charges, mutants relative to the wild type. Five positive and seven negative amino acids were replaced by site-directed mutagenesis to generate the PLTP mutants.

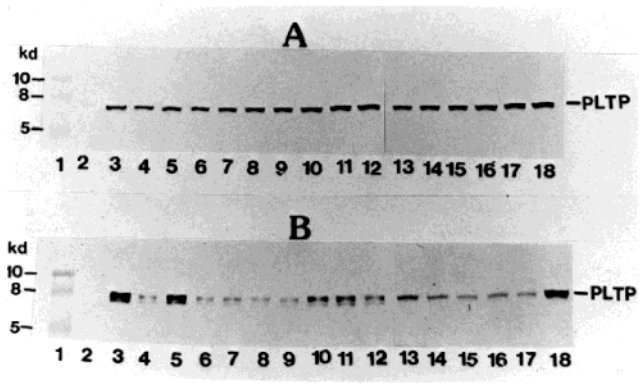

FIGURE 2: Accumulation of PLTP mutants in transfected COS-7 cells. Aliquots of (A) cell lysate and (B) medium were submitted to SDS−PAGE using 12% gels. After being electrotransferred onto a nitrocellulose membrane, the samples were analyzed for PLTP content by Western blotting: lane 1, molecular mass markers; lane 2, nontransfected COS-7 cells; lane 3, wild-type PLTP; lane 4, R235E; lane 5, R235I; lane 6, R262E; lane 7, R262I; lane 8, R235/262E; lane 9, R25E; lane 10, R380E; lane 11, K382E; lane 12, R380E/K382E; lane 13, E37R; lane 14, E259/260/261R; lane 15, E270R; lane 16, E259/260/261/270R; lane 17, D231R; and lane 18, E428R. R235/262I is not shown.

Table 2: ELISA-Quantitated Intracellular (Lysate) and Extracellular (Medium) Expression and Calculated Secretion Efficiency of Wild-Type and Mutant PLTPs

PLTP	concentration (ng/mL) (% of that of wild type)		secretion efficiency[b]
	intracellular[a]	extracellular	
wild type	54.5 (100)	38.8 (100)	0.71
R235E	53.5 (98)	18.7 (48)	0.35
R235I	45.8 (84)	39.5 (101)	0.86
R262E	51.5 (95)	5.5 (14)	0.11
R262I	41.1 (75)	1.8 (5)	0.04
R235/262E	48.6 (89)	6.8 (18)	0.14
R235/262I	41.1 (75)	1.1 (3)	0.03
R25E	55.4 (102)	4.7 (12)	0.08
R380E	58.3 (107)	37.1 (96)	0.64
K382E	52.6 (97)	36.6 (94)	0.70
R380E/K382E	60.2 (110)	38.6 (99)	0.64
E37R	54.3 (99)	25.5 (66)	0.47
E259/260/261R	48.4 (89)	1.5 (4)	0.03
E270R	52.3 (96)	14.7 (38)	0.28
E259/260/261/270R	55.6 (102)	4.5 (12)	0.08
D231R	47.4 (87)	14.0 (36)	0.30
E428R	55.2 (101)	34.8 (90)	0.63

[a] Value obtained from one assay; similar values were obtained in another independent assay. [b] Ratio of extracellular to intracellular PLTP concentration.

(Figure 1). The wild-type and mutant PLTPs were expressed in COS-7 cells. According to Western blot analysis, intracellular concentrations were similar for mutant and wild-type PLTPs (Figure 2A); a quantitative ELISA showed that intracellular PLTP concentrations range from 75 to 110% of that of the wild type (Table 2). In contrast, both PLTP concentrations and transfer activities in extracellular medium were highly variable (Figure 2B and Tables 2 and 3). The secretion efficiency calculated as the ratio of extracellular to intracellular PLTP mass (Table 2) varied from almost nil to ~120% of that of the wild type as the control (Table 2). The biologic activity of all PLTP mutants was determined from the in vitro transfer of phospholipids from single-bilayer vesicles to HDL. Intracellular specific transfer activity was calculated for wild-type PLTP and for all PLTP mutants; if detectable, the PLTP specific transfer activity in medium was calculated as well (Table 3). The secretion efficiency and intracellular specific transfer activity of the mutants were highly positively correlated (Figure 3, $R = 0.92$), suggesting the existence of an intracellular mechanism that prevents secretion of an inactive form of PLTP; the mechanism is probably proteolysis because large amounts of inactive protein did not accumulate intracellularly. There was a striking positive relation between the intracellular and extracellular specific transfer activities of PLTP mutants, indicating that the intracellular and extracellular forms are structurally similar (Figure 4, $R = 0.96$).

Although analysis of the relation between the mutant PLTP intracellular specific transfer activity and relative electrostatic charge revealed no simple relation (Figure 5), the mutants were distributed into three well-defined clusters. Two of the clusters comprised the relatively electronegative mutants; the mutants in one of the clusters were similar to wild-type PLTP in intracellular transfer activity (see Table 3), whereas in the other electronegative group, there was little or no transfer activity. With the exception of E428R, the relatively electropositive mutants formed a third cluster, in which intracellular specific transfer activity ranged from 25 to 40% of that of wild-type PLTP.

According to the binding to HDL relative to wild-type PLTP, each of the 10 mutants was assigned to one of three groups: normal binding (similar to that of the wild type), medium binding (clearly lower but still quantifiable), and low binding (nil or nonquantifiable) (Figure 6 and Table 3). There was no relation between the relative affinity of the mutants for HDL and their relative electrostatic charge (Table 4). However, there was a positive relation ($R = 0.94$) between mutant PLTP intracellular specific activity and HDL binding capability (Figure 6) with two notable exceptions. R25E had a specific transfer activity close to nil but binding that was nearly 60% of that of WT. Similarly, the specific transfer activity of D231R was lower that than predicted by the line of regression for the remainder of the mutants, but its HDL binding was similar to that of WT.

DISCUSSION

PLTP belongs to a gene family that includes CETP, lipopolysaccharide-binding protein, and bactericidal/permeability-increasing protein (*23*, *24*). Conservation of charged amino acids in this family suggests that they are required for structural integrity, lipid binding, and transfer activity (*31*−*33*). To address this question, 12 conserved charged amino acids in PLTP, five positive and seven negative, were replaced by site-directed mutagenesis (see Table 1). The mutants were expressed in COS-7 cells, and the biosynthesis, secretion, transfer activity, and binding to HDL were determined for each PLTP mutant.

Intracellular concentrations of all mutants were similar to that of wild-type PLTP (see Table 2), indicating that the synthesis rate was likely not affected by the mutagenesis. In contrast, mutant PLTP extracellular transfer activities varied greatly, ranging from undetectable to ~100% of wild-type PLTP transfer activity (see Table 3). Mutant PLTP specific transfer activity and PLTP secretion into the extracellular medium were highly correlated (see Figure 3). Inactive PLTP mutants were not secreted, whereas all PLTP mutants

Table 3: Phospholipid Transfer Activity, Specific Activity, and HDL Binding Capability of Wild-Type and Mutant PLTPs

PLTP	transfer activity (nmol/mL)		specific activity[a] (nmol/ng)		HDL binding capability
	intracellular	extracellular	intracellular	extracellular	
wild type	2771 ± 33	2998 ± 70	50.8 ± 0.6	77.3 ± 1.8	normal
R235E	257 ± 40	380 ± 24	4.8 ± 0.07	20.3 ± 1.3	low
R235I	2058 ± 1	2536 ± 261	44.9 ± 0.1	64.2 ± 6.6	NM[c]
R262E	ND[b]	ND[b]	0.0	−	low
R262I	13 ± 1	ND[b]	0.33 ± 0.01	−	NM[c]
R235/262E	ND[b]	ND[b]	0.0	−	NM[c]
R235/262I	20 ± 1	ND[b]	0.49 ± 0.01	−	NM[c]
R25E	ND[b]	ND[b]	0.0	−	normal
R380E	2845 ± 10	2846 ± 43	48.9 ± 0.2	76.7 ± 1.2	normal
K382E	2568 ± 14	2561 ± 195	48.8 ± 0.3	70.0 ± 5.3	normal
R380E/K382E	2703 ± 18	2789 ± 49	45.7 ± 0.3	72.3 ± 1.3	NM[c]
E37R	948 ± 6	904 ± 9	17.5 ± 0.1	35.3 ± 0.3	low
E259/260/261R	729 ± 36	ND[b]	15.1 ± 0.7	−	medium
E270R	945 ± 43	538 ± 92	18.1 ± 0.8	36.6 ± 6.3	medium
E259/260/261/270R	706 ± 18	ND[b]	12.7 ± 0.3	−	NM[c]
D231R	933 ± 166	198 ± 23	19.7 ± 3.5	14.1 ± 1.7	normal
E428R	2945 ± 291	2158 ± 50	53.4 ± 5.3	62 ± 1.4	normal

[a] Ratio of transfer activity to the corresponding PLTP concentration shown in Table 2. [b] Not detectable. [c] Not measured.

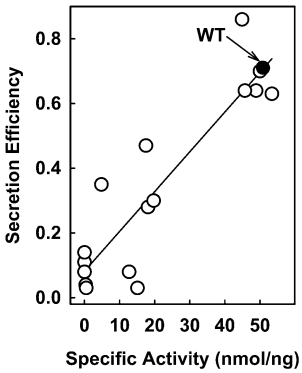

FIGURE 3: Correlation between the secretion efficiency (see Table 2) and intracellular specific activity (see Table 3) of PLTP mutants ($r^2 = 0.83$): (●) wild-type PLTP and (○) mutant PLTPs.

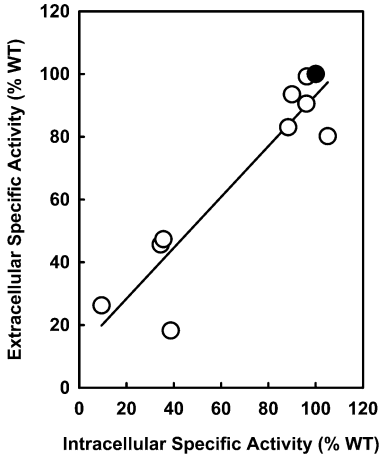

FIGURE 4: Correlation for PLTP mutants between calculated extracellular and intracellular specific activities (see Table 3) expressed as percentages of wild-type PLTP ($r^2 = 0.89$): (●) wild-type PLTP and (○) mutant PLTPs.

exhibiting intracellular transfer activity were secretion competent. Moreover, the specific transfer activities of mutants assessed extracellularly, when activity was measurable, appeared to be quite similar to those of mutants assessed intracellularly, suggesting that additional processing of PLTP between synthesis and secretion did not greatly affect specific transfer activity.

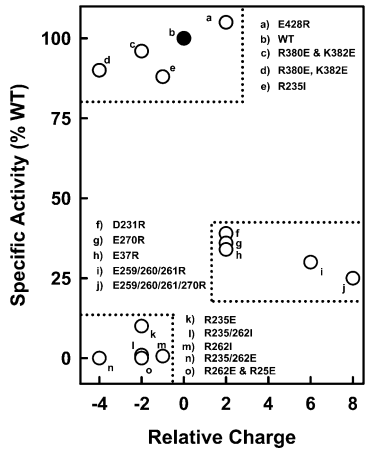

FIGURE 5: Relation for PLTP mutants between intracellular specific activity (see Table 3) expressed as a percentage of wild-type PLTP and electrostatic charge relative to that of wild-type PLTP (see Figure 1). Points c and o each represent overlapping values for pairs of mutants.

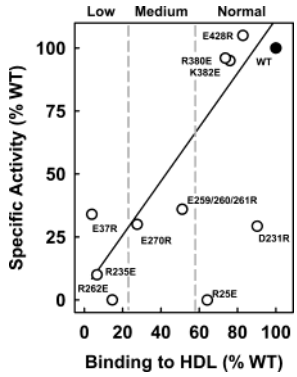

FIGURE 6: Relation between mutant PLTP intracellular specific activity expressed as a percentage of that of wild-type PLTP and ability relative to wild-type PLTP to bind to HDL (see Table 3) ($r^2 = 0.85$): (●) wild-type PLTP and (○) mutant PLTPs. The line of regression does not include points corresponding to R25E and D231R.

While there was no linear correlation between the intracellular specific transfer activity and relative electrostatic charge for PLTP mutants, nearly all of the mutants that were assessed clustered into three distinct groups. Mutants with a positive charge relative to wild-type PLTP had specific

Table 4: Distribution of Mutant PLTPs According to HDL Binding Capability and Electrostatic Charge Relative to Those of Wild-Type PLTP

HDL binding[a]	negative relative electrostatic charge	positive relative electrostatic charge
low	R235E R262E	E37R
medium		E259/260/261R E270R
normal	R25E R380E K382E	D231R E428R

[a] HDL binding capability was not assessed in six of the 16 mutant PLTPs (see Table 3).

transfer activities that were 25−40% of wild-type activity. Second, relative electronegativity was associated with an "all or nothing" effect. Some mutants in this cluster resembled wild-type PLTP in specific transfer activity, whereas the others had little or no activity.

To understand these differences, we assessed HDL binding affinity according to intracellular specific transfer activity for some of the mutants (see Figure 6). Although the molecular mechanism for lipid transfer by PLTP remains to be fully characterized, we and others have proposed the involvement of PLTP binding to lipoprotein surfaces (*16*, *21*, *40*). In principle, a decrease in PLTP specific transfer activity may result from a defect in its transfer ability per se, or from a decrease in its binding affinity for lipoprotein surfaces. There is a qualitative positive relation between HDL binding and specific transfer activity with two exceptions (Figure 6). This suggests that the decreased specific transfer activity of some of the mutants was due in part to a reduced affinity for lipoprotein surfaces. D231R and R25E resembled wild-type PLTP in HDL binding but were dramatically lower in specific transfer activity than the wild type. These substitutions affected PLTP's phospholipid transfer activity independent of binding to HDL.

Isostructural with bactericidal/permeability-increasing protein (*25*, *26*), PLTP is a boomerang-shaped molecule composed of two symmetrical barrels separated by a linker region. Both N- and C-terminal barrels contain a hydrophobic pocket that can accommodate a phospholipid molecule. According to an analysis by various programs based on the Chou−Fasman algorithm (*41*), wild-type and mutant PLTPs are similar in secondary structure. On that basis, further analysis of the effects of these PLTP mutations on HDL binding and on specific transfer activity can be interpreted within the context of the Beamer−Huuskonen three-dimensional structure (*25*, *26*). None of the substitutions was found to induce any significant change (data not shown), indicating that they should not greatly affect the overall three-dimensional integrity. This permits us to use the PLTP structural model with reasonable confidence and to assign the intramolecular position of each substituted amino acid.

The PLTP mutants were distributed into three well-defined clusters of transfer activity and HDL binding (Figure 7). One group included E259/260/261R, R262E, and R235I. Residues 259−262 form a basolateral buckle that is totally exposed to the solvent, while the latter has a similar disposition on the symmetrically opposite side of the molecule. Mutations of these amino acids led to a decreased level of PLTP binding

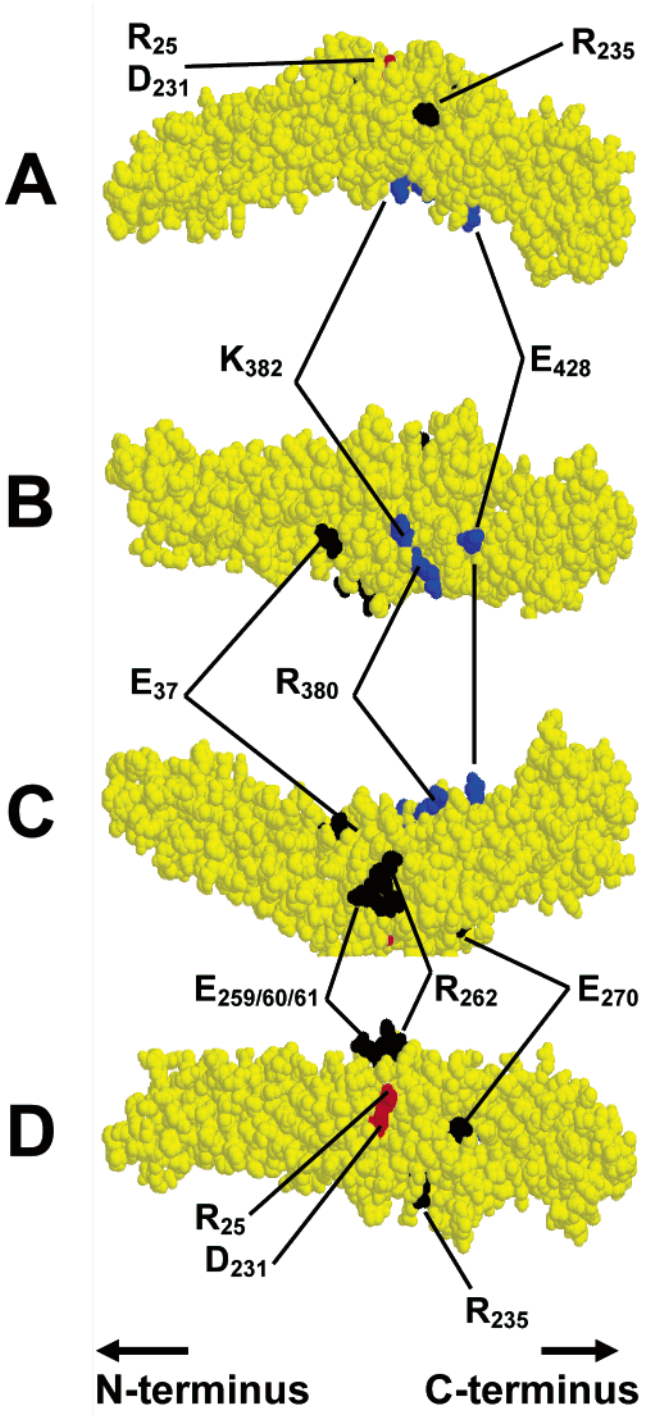

FIGURE 7: Three-dimensional structural model of human PLTP shown in space-filling representation. Opposite lateral views of the molecule are depicted in panels A and C. Views of the concave and convex surfaces are given in B and D, respectively. The substitutions for amino acids shown in black induced a decrease in both the specific activity of PLTP and its level of binding to HDL. The mutations of amino acids shown in red induced a decrease in the specific activity of PLTP without affecting binding to HDL. The mutations of amino acids shown in blue had no effect on either the specific activity of PLTP or its binding to HDL.

to HDL, accompanied by a proportional decrease in the specific transfer activity. The greatest effect was observed with R262E and R235E, thus confirming that these two amino acids are critical for optimal binding of PLTP to HDL. This finding is consistent with previous reports suggesting that R262 and R235 in PLTP are the equivalents of K233 and

R259 in CETP, respectively, which are essential for the interaction of CETP with charged lipid surfaces (*32*, *33*).

The second group of mutations are clustered within the concave surface of the PLTP molecule. These (R380E, K382E, R380E/K382E, and E428R) did not affect binding to HDL or specific transfer activity. However, E428R is at the end of helix B of the C-terminal pocket and at the interface of the pocket with the more exposed part of the protein. Thus, E428R might also have been assigned to the third group of mutations, which affect the two pocket zones. E270 is located on helix A in the very bottom of the C-terminal pocket, while E37 belongs to helix B of the N-terminal pocket. R25 and D231 form a paired ion structure with their lateral chains in close contact and merging at the convex surface of the molecule. These two amino acids belong to two parallel β-strands forming part of the bottom of the N-terminal pocket. The mutations in the deep parts of the pockets were associated with very different effects. Mutation of E270 in the C-terminal pocket led to a decrease in both specific transfer activity and the level of binding to HDL (see Tables 3 and 4). In contrast, mutation of R25 or D231 in the N-terminal pocket resulted in a large decrease in PLTP specific transfer activity even though the binding of the protein to HDL was similar to that of the wild type.

These findings are of particular interest in light of the previous work of Huuskonen et al. (*26*), who, after studying the effects of a series of mutations of PLTP, proposed that despite their apparent symmetry the two pockets might have distinct and independent roles in PLTP function. The N-terminal pocket would be important for PLTP transfer activity without being critical to the binding of the protein to lipoproteins. In contrast, the inactivation of PLTP containing mutations in the C-terminal pocket on PLTP specific transfer activity would be the consequence of a decrease in the lipoprotein binding ability of the protein. Our data concerning the mutations on E270, R25, and D231 agree with this concept. However, the E37R mutation at the entrance to the N-terminal pocket decreased both the level of binding and activity. This apparent discrepancy can be understood. Within the three-dimensional structure of PLTP, E37 is close to F464 and the F464E mutation is known to destabilize the normal folding of the C-terminal tail of PLTP (*26*). One can therefore consider that any modification of the charges in this particular part of PLTP might induce a folding defect that inactivates the protein.

Collective consideration of our data leads to two conclusions. First, although the substitutions were selected on the basis of only conservation of charged amino acids within the gene family of lipid transfer proteins, most mutations were in surface-exposed regions of the PLTP molecule predicted by the boomerang-shaped structural model. That finding provides additional support for the model. Second, our studies provide direct evidence to support the concept (*26*) that the N- and C-terminal pockets have different roles with respect to PLTP activity.

REFERENCES

1. Fielding, C. J., and Fielding, P. E. (1995) Molecular physiology of reverse cholesterol transport, *J. Lipid Res. 36*, 211−228.
2. Fielding, C. J., and Fielding, P. E. (1997) Intracellular cholesterol transport, *J. Lipid Res. 38*, 1503−1521.
3. Jonas, A. (1998) Regulation of lecithin cholesterol acyltransferase activity, *Prog. Lipid Res. 37*, 209−234.
4. Tall, A. (1995) Plasma lipid transfer proteins, *Annu. Rev. Biochem. 64*, 235−257.
5. Bruce, C., Chouinard, R. A., Jr., and Tall, A. R. (1998) Plasma lipid transfer proteins, high-density lipoproteins, and reverse cholesterol transport, *Annu. Rev. Nutr. 18*, 297−330.
6. van Tol, A. (2002) Phospholipid transfer protein, *Curr. Opin. Lipidol. 13*, 135−139.
7. Nishida, H. I., and Nishida, T. (1997) Phospholipid transfer protein mediates transfer of not only phosphatidylcholine but also cholesterol from phosphatidylcholine-cholesterol vesicles to high-density lipoproteins, *J. Biol. Chem. 272*, 6959−6964.
8. Jiang, X. C. (2002) The effect of phospholipid transfer protein on lipoprotein metabolism and atherosclerosis, *Front. Biosci. 7*, d1634−d1641.
9. Wolfbauer, G., Albers, J. J., and Oram, J. F. (1999) Phospholipid transfer protein enhances removal of cellular cholesterol and phospholipids by high-density lipoprotein apolipoproteins, *Biochim. Biophys. Acta 1439*, 65−76.
10. Föger, B., Santamarina-Fojo, S., Shamburek, R. D., Parrot, C. L., Talley, G. D., and Brewer, H. B., Jr. (1997) Plasma phospholipid transfer protein. Adenovirus-mediated overexpression in mice leads to decreased plasma high-density lipoprotein (HDL) and enhanced hepatic uptake of phospholipids and cholesteryl esters from HDL, *J. Biol. Chem. 272*, 27393−27400.
11. Ehnholm, S., van Dijk, K. W., van't Hof, B., van der Zee, A., Olkkonen, V. M., Jauhiainen, M., Hofker, M., Havekes, L., and Ehnholm, C. (1998) Adenovirus mediated overexpression of human phospholipid transfer protein alters plasma HDL levels in mice, *J. Lipid Res. 39*, 1248−1253.
12. Jiang, X. C., Bruce, C., Mar, J., Lin, M., Ji, Y., Francone, O. L., and Tall, A. R. (1999) Targeted mutation of plasma phospholipid transfer protein gene markedly reduces high-density lipoprotein levels, *J. Clin. Invest. 103*, 907−914.
13. Jauhiainen, M., Metso, J., Pahlman, R., Blomqvist, S., van Tol, A., and Ehnholm, C. (1993) Human plasma phospholipid transfer protein causes high-density lipoprotein conversion, *J. Biol. Chem. 268*, 4032−4036.
14. Tu, A. Y., Nishida, H. I., and Nishida, T. (1993) High-density lipoprotein conversion mediated by human plasma phospholipid transfer protein, *J. Biol. Chem. 268*, 23098−23105.
15. Pulcini, T., Terru, P., Sparrow, J. T., Pownall, H. J., and Ponsin, G. (1995) Plasma factors affecting the in vitro conversion of high-density lipoproteins labeled with a non-transferable marker, *Biochim. Biophys. Acta 1254*, 13−21.
16. Rao, R., Albers, J. J., Wolfbauer, G., and Pownall, H. J. (1997) Molecular and macromolecular specificity of human plasma phospholipid transfer protein, *Biochemistry 36*, 3645−3653.
17. Korhonen, A., Jauhiainen, M., Ehnholm, C., Kovanen, P. T., and Ala-Korpela, M. (1998) Remodeling of HDL by phospholipid transfer protein: demonstration of particle fusion by ^{1}H NMR spectroscopy, *Biochem. Biophys. Res. Commun. 249*, 910−916.
18. Lusa, S., Jauhiainen, M., Metso, J., Somerharju, P., and Ehnholm, C. (1996) The mechanism of human plasma phospholipid transfer protein-induced enlargement of high-density lipoprotein particles: evidence for particle fusion, *Biochem. J. 313*, 275−282.
19. Castro, G. R., and Fielding, C. J. (1988) Early incorporation of cell-derived cholesterol into pre-β migrating high-density lipoprotein, *Biochemistry 27*, 25−29.
20. Huuskonen, J., Olkkonen, V. M., Ehnholm, C., Metso, J., Julkunen, I., and Jauhiainen, M. (2000) Phospholipid transfer is a prerequisite for PLTP-mediated HDL conversion, *Biochemistry 39*, 16092−16098.
21. Lalanne, F., and Ponsin, G. (2000) Mechanism of the phospholipid transfer protein-mediated transfer of phospholipids from model lipid vesicles to high-density lipoproteins, *Biochim. Biophys. Acta 1487*, 82−91.
22. Kawano, K., Qin, S.-C., Lin, M., Tall, A. R., and Jiang X.-C. (2000) Cholesteryl ester transfer protein and phospholipid transfer protein have nonoverlapping functions *in vivo*, *J. Biol. Chem. 275*, 29477−29481.
23. Tu, A.-Y., Deeb, S. S., Iwasaki, L., Day, J. R., and Albers, J. J. (1995) Organization of human phospholipid transfer protein gene, *Biochem. Biophys. Res. Commun. 207*, 552−558.
24. Kirschning, C. J., Au-Young, J., Lamping, N., Reuter, D., Pfeil, D., Seilhamer, J. J., and Schumann, R. R. (1997) Similar organization of the lipopolysaccharide-binding protein (LBP) and phospholipid transfer protein (PLTP) genes suggests a common gene family of lipid-binding proteins, *Genomics 46*, 416−425.

25. Beamer, L. J., Carroll, S. F., and Eisenberg, D. (1997) Crystal structure of human BPI and two bound phospholipids at 2.4 Å resolution, *Science 276*, 1861−1864.
26. Huuskonen, J., Wohlfahrt, G., Jauhiainen, M., Ehnholm, C., Teleman, O., and Olkkonen, V. M. (1999) Structure and phospholipid transfer activity of human PLTP: analysis by molecular modeling and site-directed mutagenesis, *J. Lipid Res. 40*, 1123−1130.
27. Qu, S. J., Fan, H. Z., Kilinc, C., and Pownall, H. J. (1999) Role of cysteine residues in human plasma phospholipid transfer protein, *J. Protein Chem. 18*, 193−198.
28. Desrumaux, C., Athias, A., Masson, D., Gambert, P., Lallemant, C., and Lagrost, L. (1998) Influence of the electrostatic charge of lipoprotein particles on the activity of the human plasma phospholipid transfer protein, *J. Lipid Res. 39*, 131−142.
29. Nishida, H. I., Arai, H., and Nishida, T. (1993) Cholesterol ester transfer mediated by lipid transfer protein as influenced by changes in the charge characteristics of plasma lipoproteins, *J. Biol. Chem. 268*, 16352−16360.
30. Masson, D., Athias, A., and Lagrost, L. (1996) Evidence for electronegativity of plasma high-density lipoprotein-3 as one major determinant of human cholesterol ester transfer protein activity, *J. Lipid Res. 37*, 1579−1590.
31. Jiang, X. C., Bruce, C., Cocke, T., Wang, S., Boguski, M., and Tall, A. R. (1995) Point mutagenesis of positively charged amino acids of cholesteryl ester transfer protein: conserved residues within the lipid transfer/lipopolysaccharide binding protein gene family essential for function, *Biochemistry 34*, 7258−7263.
32. Wang, S., Wang, X., Deng, L., Rassart, E., Milne, R. W., and Tall, A. R. (1993) Point mutagenesis of carboxyl-terminal amino acids of cholesteryl ester transfer protein. Opposite faces of an amphipathic helix important for cholesteryl ester transfer or for binding neutralizing antibody, *J. Biol. Chem. 268*, 1955−1959.
33. Lagrost, L., Desrumaux, C., Masson, D., Deckert, V., and Gambert, P. (1998) Structure and function of the plasma phospholipid transfer protein, *Curr. Opin. Lipidol. 9*, 203−209.
34. Havel, R. J., Eder, H. A., and Bragdon, J. H. (1955) The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum, *J. Clin. Invest. 34*, 1345−1353.
35. Day, J. R., Albers, J. J., Lofton-Day, C. E., Gilbert, T. L., Ching, A. F., Grant, F. J., O'Hara, P. J., Marcovina, S. M., and Adolphson, J. L. (1994) Complete cDNA encoding human phospholipid transfer protein from human endothelial cells, *J. Biol. Chem. 269*, 9388−9391.
36. Deng, W. P., and Nickoloff, J. A. (1992) Site-directed mutagenesis of virtually any plasmid by eliminating a unique site, *Anal. Biochem. 200*, 81−88.
37. Selden, R. F., Howie, K. B., Rowe, M. E., Goodman, H. M., and Moore, D. D. (1986) Human growth hormone as a reporter gene in regulation studies employing transient gene expression, *Mol. Cell. Biol. 6*, 3173−3179.
38. Damen, J., Regts, J., and Scherphof, G. (1982) Transfer of [^{14}C]-phosphatidylcholine between liposomes and human plasma high-density lipoprotein. Partial purification of a transfer-stimulating plasma factor using a rapid transfer assay, *Biochim. Biophys. Acta 712*, 444−452.
39. Qu, S. J., Fan, H. Z., Blanco-Vaca, F., and Pownall, H. J. (1993) Roles of cysteines in human lecithin:cholesterol acyltransferase, *Biochemistry 32*, 3089−3094.
40. Tollefson, J. H., Ravnik, S., and Albers, J. J. (1988) Isolation and characterization of a phospholipid transfer protein (LTP-II) from human plasma, *J. Lipid Res. 29*, 1593−1602.
41. Chou, P. Y., and Fasman, G. D. (1978) Empirical predictions of protein conformation, *Annu. Rev. Biochem. 47*, 251−276.

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