An Amphipathic Helical Region of the N-terminal Barrel of Phospholipid Transfer Protein Is Critical for ABCA1-dependent Cholesterol Efflux*

John F. Oram†1, Gertrud Wolfbauer†, Chongren Tang‡, W. Sean Davidson§, and John J. Albers‡2

From the †Division of Metabolism, Endocrinology and Nutrition, Department of Medicine, Box 356426, University of Washington, Seattle, Washington 98195 and the §Department of Pathology and Laboratory Medicine, University of Cincinnati, Cincinnati, Ohio 45237-0507

Phospholipid transfer protein (PLTP) mimics high-density lipoprotein apolipoproteins in removing cholesterol and phospholipids from cells through the ATP-binding cassette transporter A1 (ABCA1). Because amphipathic α-helices are the structural determinants for ABCA1 interactions, we examined the ability of synthetic peptides corresponding to helices in PLTP to remove cellular cholesterol by the ABCA1 pathway. Of the seven helices tested, only one containing PLTP residues 144–163 (p144), located at the tip of the N-terminal barrel, promoted ABCA1-dependent cholesterol efflux and stabilized ABCA1 protein. Mutating methionine 159 (Met-159) in this helix in PLTP to aspartate (M159D) or glutamate (M159E) nearly abolished the ability of PLTP to remove cellular cholesterol and dramatically reduced PLTP binding to phospholipid vesicles and its phospholipid transfer activity. These mutations impaired PLTP binding to ABCA1-generated lipid domains and PLTP-mediated stabilization of ABCA1 but increased PLTP binding to ABCA1. PLTP interactions with ABCA1 also mimicked apolipoproteins in activating Janus kinase 2; however, the M159D/E mutants were also able to activate this kinase. Structural analyses showed that the M159D/E mutations had only minor effects on PLTP conformation. These findings indicate that PLTP helix 144–163 is critical for removing lipid domains formed by ABCA1, stabilizing ABCA1 protein, interacting with phospholipids, and promoting phospholipid transfer. Direct interactions with ABCA1 and activation of signaling pathways likely involve other structural determinants of PLTP.

Phospholipid transfer protein (PLTP) plays important and diverse roles in lipoprotein metabolism (1, 2). Plasma PLTP transfers phospholipids between lipoproteins and remodels HDL to generate lipid-poor particles (3–7), and hepatic PLTP facilitates the production of triglyceride-rich apoB-containing particles in mice (8, 9). PLTP is expressed ubiquitously in human and mouse tissues (10, 11), suggesting that it may locally modulate lipid metabolism in peripheral tissues. Little is known, however, about the interactions of PLTP with peripheral cells and its effects on cellular lipid metabolism.

One possible function of peripheral PLTP is to transport lipids from cells to lipoproteins. An important mediator of lipid export from cells is ATP-binding cassette transporter A-1 (ABCA1), a membrane protein that transports cholesterol and phospholipids from cells to HDL apolipoproteins, such as apoA-I (12). Human and mouse model studies have shown that ABCA1 is cardioprotective (13, 14). We showed previously that PLTP can mimic HDL apolipoproteins in binding to ABCA1, removing cellular cholesterol and phospholipids, and stabilizing ABCA1 protein (15). Because sterol loading of macrophages induces both ABCA1 and PLTP (16–18), the interaction of these two proteins may serve to prevent the accumulation of cholesterol in arterial macrophages that accelerates atherosclerosis (19). Indeed, it was shown that macrophages from PLTP-deficient mice have higher levels of cholesterol (20) and reduced ABCA1-dependent cholesterol efflux (21) compared with cells expressing PLTP. Although there is conflicting data, three mouse bone marrow transplantation studies have provided evidence that macrophage-derived PLTP reduces atherosclerosis (22–24).

The observation that PLTP mimics apolipoproteins in binding to ABCA1 and removing cellular lipids implies that PLTP and apolipoproteins share structural features that mediate ABCA1 interactions. Studies of mutant apolipoproteins and mimetic synthetic peptides revealed that the major structural determinant responsible for ABCA1 interactions is the amphipathic α-helix (25–29). Although PLTP does not have structural elements homologous to those in apolipoproteins, it is predicted to contain seven amphipathic helical domains. In the current study, we used synthetic peptides and PLTP mutants to identify a phospholipid-binding helical region near the tip of the N-terminal barrel of PLTP that is critical for promoting ABCA1-dependent cholesterol efflux and stabilizing ABCA1 protein. These studies provide further insight into the structural properties of proteins that play a role in the ABCA1 lipid export pathway.
A PLTP Helix Involved in ABCA1-dependent Cholesterol Efflux

EXPERIMENTAL PROCEDURES

Lipoproteins, ApoA-I, Recombinant PLTP, and Peptides—HDL was prepared by sequential ultracentrifugation in the density range 1.125–1.21 g/ml and depleted of apoE and apoB by heparin-agarose chromatography (30). Recombinant wild-type and mutant PLTP was isolated by Ni-nitrotriacetic acid resin column chromatography from serum-free conditioned culture media collected from BHK cells transfected with a His-tagged human PLTP cDNA using methotrexate as a selection agent (5, 31). The isolated PLTP fractions were assayed for phospholipid transfer activity and for purity by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The mutant PLTP containing amino acid substitutions (K146L, M159D, and M159E) were prepared using the GeneEditor in vitro Site-Directed Mutagenesis System (Promega). PLTP was labeled with $^{125}$I using the Iodo-Beads iodination reagent (Pierce), and $^{125}$I-labeled wild-type and mutant PLTP were adjusted to the same specific activity (400–500 cpm/ng protein) by dilution with unlabeled PLTP. The integrity of the $^{125}$I-PLTP was verified by autoradiography and mutant PLTP was subjected to denaturing SDS-PAGE in 2–20% gradient gels and to non-denaturing PAGE in 3–33% gels. To measure ABCA1 protein levels, microsomal membranes were isolated from homogenized J774 macrophages by ultracentrifugation, membrane proteins were solubilized in SDS buffer and resolved by SDS-PAGE, and ABCA1 was identified by immunoblot analysis (35). ABCA1 protein levels were quantified using OptiQuant computer software (Packard Instruments). Cellular contents of JAK2 and phosphorylated JAK2 were measured by immunoblot analyses using antibodies to JAK2 (Santa Cruz Biotechnology) and tyrosine-phosphorylated JAK2 (BIOSOURCE International) (29, 37). Equal amounts of membrane or cell protein were added per gel lane.

Lipid Binding—The propensity for lipid association was measured using the biotin-capture lipid affinity (BCLA) assay as detailed previously (38). Briefly, a constant mass of small unilamellar POPC vesicles (SUVs) containing small amounts of biotinylated lipid and fluorescently labeled lipid were mixed with increasing amounts of apolipoprotein in a microplate setup. After binding reached equilibrium, vesicle-bound protein was separated from unbound protein in a 96-well streptavidin column plate.

PLTP Phospholipid Transfer Activity—The indicated amounts of PLTP were assayed at 37 °C in a shaking water bath for their ability to transfer $^{14}$C-labeled phospholipid from donor liposome particles to acceptor HDL$_3$ particles (39). After 20 min, the reaction was stopped by immersion of the tubes into ice water; 500 μl of 2% outdated plasma were added, and donor particles were precipitated with 100 μl of dextran sulfate/magnesium chloride for 30 min and separated from acceptor substrate by centrifugation (40). Radioactivity in the supernatant was measured and corrected for the background transfer, and phospholipid transfer activity was expressed as percent phospholipid transferred per aliquot PLTP per 20 min.

PLTP Cross-linking—To determine the degree of self-association of PLTP in solution, chemical cross-linking was performed using bis(sulfosuccinimidyl) suberate (BS3) as described (40). Briefly, PLTP in phosphate-buffered saline at a concentration of about 1.0 mg/ml was treated with a final concentration of 0.2 or 0.5 mM cross-linker. The samples were incubated at room temperature for 6 h, and the reaction was quenched by the addition of one-sixth volume of 50 mM ethanolamine in PBS. The samples were then run on 8–25% SDS-PAGE gels and compared with noncross-linked PLTP and molecular weight standards.

Circular Dichroism—PLTP samples were freshly dialyzed against PBS (pH 7.4). Proteins were then diluted to 100 μg/ml, and spectra were collected on a Jasco J-715 spectropolarimeter in a 1-mm cell as an average of four scans. The scans were from 260 to 190 nm at 50 nm/min with a 0.5-nm step size and 0.5-s response. Bandwidth was set to 1 mm and the slit width was 500 μm. To confirm the accuracy of dilution, protein concentration was verified by $A_{280}$ on the diluted samples. Mean residual ellipticity (MRE) was calculated as described by Woody (41) using 111.0 Da as the mean residual weight for PLTP. The fractional helical content was calculated using the formula of Chen et al. (42) and the MRE

Immunoblot Analyses—PLTP was subjected to denaturing SDS-PAGE in 2–20% gradient gels and to non-denaturing PAGE in 3–33% gels. To measure ABCA1 protein levels, microsomal membranes were isolated from homogenized J774 macrophages by ultracentrifugation, membrane proteins were solubilized in SDS buffer and resolved by SDS-PAGE, and ABCA1 was identified by immunoblot analysis (35). ABCA1 protein levels were quantified using OptiQuant computer software (Packard Instruments). Cellular contents of JAK2 and phosphorylated JAK2 were measured by immunoblot analyses using antibodies to JAK2 (Santa Cruz Biotechnology) and tyrosine-phosphorylated JAK2 (BIOSOURCE International) (29, 37). Equal amounts of membrane or cell protein were added per gel lane.

Cholesterol Efflux—Baby hamster kidney (BHK) cells expressing mifepristone-inducible human ABCA1 were radiolabeled for 24 h with $[^3]$H]cholesterol added to the 10% fetal bovine growth medium (32). ABCA1 expression was then induced by incubating cells for 20 h with DMEM containing 1 mg/ml bovine serum albumin (DMEM/BSA) and 10 mM mifepristone. Efflux of $[^3]$H]cholesterol was measured after a 2–6-h incubation with DMEM/BSA without or with PLTP or peptides (32). Cholesterol efflux mediated by PLTP/peptides was calculated as the percentage of total $[^3]$H]cholesterol (medium plus cell) released into the medium after subtracting the value obtained with DMEM/BSA alone.

Cell Surface and ABCA1 Binding—For cell surface binding assay (28, 33, 34), control or mifepristone-treated BHK cells were incubated for 2 h with 2 μg/ml $^{125}$I-PLTP minus or plus 40 μg/ml unlabeled PLTP, chilled on ice, washed twice at 0 °C with PBS/BSA and twice with PBS, and digested with 0.1 N NaOH. Cell-associated radioactivity and cell protein were measured, and results were expressed as ng of PLTP per mg of cell protein. For the ABCA1 binding studies, cells were incubated for 2 h with 5 μg/ml $^{125}$I-PLTP, treated for 30 min at room temperature with PBS containing 1 mg/ml DSP (cross-linking agent), and washed twice with cold PBS containing 20 mM glycine (35, 36). ABCA1 was isolated from detergent extracts by immunoprecipitation and reduced SDS-PAGE, and $^{125}$I-labeled PLTP was visualized by PhosphorImaging.

ABCA1 Stabilization—J774 macrophages were cholesterol-loaded by 24-h incubations with 50 μg/ml acetylated LDL and incubated with DMEM/BSA plus 0.5 mM 8-Br-cAMP for 20 h to induce ABCA1. Cells were washed twice with PBS/BSA, incubated for 4 h with DMEM/BSA with or without 8-Br-cAMP plus or minus 10 μg/ml apoA-I, PLTP, or peptides, and membrane ABCA1 protein levels were measured by immunoblot analysis (29, 35).
at 222 nm. Each experiment was repeated three times with a similar pattern observed consistently.

**Tryptophan Fluorescence**—PLTP samples prepared for the circular dichroism analysis were subjected to fluorescence excitation at 295 nm in a Photon Technology International Quan-tamaster fluorescent spectrophotometer, and emission was scanned from 300–380 nm in a 0.5-cm cuvette at room temperature. The spectra from samples were corrected for buffer blanks. The wavelength of maximum fluorescence was determined at the highest value of detector counts in the scan range. Each sample was scanned three times with 2 min of dark time between scans to prevent photobleaching.

**RESULTS**

We showed previously that PLTP can mimic HDL apolipoproteins in binding to ABCA1, removing cellular cholesterol and phospholipids, and stabilizing ABCA1 protein. This implies that PLTP and apolipoproteins share structural features that mediate ABCA1 interactions. Studies of small apolipoprotein-mimetic peptides have shown that the major structural determinant for binding ABCA1 and removing lipids is the amphipathic α-helix (26, 28, 29, 43). A structural model of PLTP has been formulated from x-ray crystallography of a close PLTP homolog, bactericidal permeability increasing protein, and from sequence alignments with other members of this transfer protein family (44). This model predicts that PLTP forms two barrel-shaped domains linked by a β-sheeted interface (Fig. 1A). Each barrel contains two amphipathic helical regions proximal to the interface and one near the barrel tip, with an additional helix extending from the interface. The two medial helices in each barrel form hydrophobic pockets that bind phospholipid molecules.

We synthesized peptides corresponding to each of these seven helices (Table 1, denoted by the location of their N-terminal amino acids) and tested them for their abilities to promote [3H]cholesterol efflux from ABCA1-transfected BHK cells. Of these peptides, only p144 had any substantial ability to promote ABCA1-dependent cholesterol efflux, and this was comparable to that observed with full-length PLTP (Fig. 2A). The effects of p144 were concentration-dependent and saturated at ~10 μg/ml (Fig. 2B). This saturable component was markedly reduced in mock-transfected cells that lack ABCA1.

The ABCA1 protein is highly unstable in cultured macrophages (half-life ~ 1.5 h) and is rapidly degraded when cells which are first incubated with an ABCA1 inducer, such as 8-Br-cAMP, are subsequently incubated without inducer (35). Apolipoproteins or their small mimetic peptides stabilize ABCA1 protein by inhibiting its proteolysis (29, 43, 45). We therefore tested the PLTP helical peptides for their abilities to stabilize ABCA1 protein. When J774 macrophages were first incubated with 8-Br-cAMP to induce ABCA1 to high levels and subsequently incubated with medium lacking this cAMP analog, ABCA1 almost completely disappeared after 4 h (Fig. 3A, left lane). Adding apoA-I to this chase medium prevented this dis-

**FIGURE 1. Structural models for PLTP and its amphipathic α-helices.** A, PLTP structural model showing the predicted locations of the seven amphipathic helices. B, close-up of the tip of the N-terminal barrel showing the amino acids in the 144–163 helix targeted for mutations and the amino acids in the hydrophobic cluster. C, helical wheel representation of helix 144–163 showing the amino acid substitutions in PLTP mutants.

**TABLE 1**

Sequences of synthetic predicted amphipathic helical peptides in PLTP

| Peptide | Sequence                  |
|---------|---------------------------|
| p9      | Ac–VTSEXNLEKLVQEGLAFLE–NH₂ |
| p144    | Ac–FKKVVDGLSTPTGFLNFL–NH₂ |
| p169    | Ac–PLKTVHAGTVLNLN–NH₂    |
| p247    | Ac–VYVAPKSEFFDSAMESYFR–NH₂ |
| p281    | Ac–DLMLRATTQGTVLSS–NH₂   |
| p396    | Ac–PLKTMLQGUMPNLNRK–NH₂  |
| p445    | Ac–LHTAKGLREVIEK–NH₂     |
A PLTP Helix Involved in ABCA1-dependent Cholesterol Efflux

The only PLTP peptide that stabilized ABCA1 significantly was p144 (Fig. 3A). Thus, this peptide mimics apolipoproteins and PLTP in both promoting cholesterol efflux and stabilizing the ABCA1 protein. The sequence of p144 predicts that it is a highly hydrophobic amphipathic helix with a broad non-polar face rich in phenylalanines and a narrow polar face containing only 4 charged residues (Fig. 1C). To determine if this helix in intact PLTP plays a role in removing cholesterol from cells by the ABCA1 pathway, we prepared PLTP mutants with amino acid substitutions in the 144–163 region of PLTP. The K146L mutant has the hydrophobic amino acid leucine substituted for the positively charged lysine located at the polar and non-polar helical interface and near the tip of the N-terminal barrel (Fig. 1, B and C). The M159D and M159E mutants have the negatively charged aspartate or glutamate substituted for the lipophilic methionine residue located at the polar and non-polar helical interface (Fig. 1C) and oriented into the chamber of the N-terminal barrel (Fig. 1B). The purpose of these mutations was to change the charge or hydrophobicity at the polar/non-polar interface of the helix and to substitute charged residues for a methionine that may affect lipid interactions in the barrel.

When incubated with ABCA1-transfected BHK cells, PLTP with the K146L mutation had only a slightly reduced cholesterol efflux activity compared with wild type PLTP, but the M159D and M159E mutants were almost completely inactive (Fig. 4A). A similar pattern was observed when HDL was included in the medium (Fig. 4B). Thus, substituting a negatively charged amino acid for the methionine in the 144–163 helix of PLTP nearly abolished ABCA1-dependent cholesterol efflux activity.

We also examined the effects of these mutations on the ability of PLTP to stabilize ABCA1 protein in J774 macrophages. As we also reported previously (15), the disappearance of the ABCA1 protein following cAMP removal was overcome in the presence of either apoA-I or wild-type PLTP (Fig. 3B). The K146L mutation had little effect on the ABCA1-stabilizing appearance. As with cholesterol efflux, the only PLTP peptide that stabilized ABCA1 significantly was p144 (Fig. 3A). Thus, this peptide mimics apolipoproteins and PLTP in both promoting cholesterol efflux and stabilizing the ABCA1 protein.

The sequence of p144 predicts that it is a highly hydrophobic amphipathic helix with a broad non-polar face rich in phenylalanines and a narrow polar face containing only 4 charged residues (Fig. 1C). To determine if this helix in intact PLTP plays a role in removing cholesterol from cells by the ABCA1 pathway, we prepared PLTP mutants with amino acid substitutions in the 144–163 region of PLTP. The K146L mutant has the hydrophobic amino acid leucine substituted for the positively charged lysine located at the polar and non-polar helical interface and near the tip of the N-terminal barrel (Fig. 1, B and C). The M159D and M159E mutants have the negatively charged aspartate or glutamate substituted for the lipophilic methionine residue located at the polar and non-polar helical interface (Fig. 1C) and oriented into the chamber of the N-terminal barrel (Fig. 1B). The purpose of these mutations was to change the charge or hydrophobicity at the polar/non-polar interface of the helix and to substitute charged residues for a methionine that may affect lipid interactions in the barrel.

When incubated with ABCA1-transfected BHK cells, PLTP with the K146L mutation had only a slightly reduced cholesterol efflux activity compared with wild type PLTP, but the M159D and M159E mutants were almost completely inactive (Fig. 4A). A similar pattern was observed when HDL was included in the medium (Fig. 4B). Thus, substituting a negatively charged amino acid for the methionine in the 144–163 helix of PLTP nearly abolished ABCA1-dependent cholesterol efflux activity.

We also examined the effects of these mutations on the ability of PLTP to stabilize ABCA1 protein in J774 macrophages. As we also reported previously (15), the disappearance of the ABCA1 protein following cAMP removal was overcome in the presence of either apoA-I or wild-type PLTP (Fig. 3B). The K146L mutation had little effect on the ABCA1-stabilizing
effect of PLTP, but both the M159E and M159D mutations abolished it. These results show that substituting an acidic residue for the methionine in helix 144–163 severely impairs the ability of PLTP to both promote ABCA1-dependent cholesterol eflux and stabilize ABCA1 protein.

We showed previously that PLTP can be chemically cross-linked to ABCA1 (15), consistent with a direct interaction between these two proteins. To determine if the mutations in helix 144–163 affect the binding of PLTP to ABCA1, we incubated mock- and ABCA1-transfected BHK cells with wild-type and mutant 125I-PLTP, treated cells with the cross-linking agent DSP, immunoprecipitated ABCA1 from detergent-solubilized cells, and identified recovered 125I-PLTP by SDS-PAGE. The specific activities of the wild-type and mutant PLTPs were adjusted to the same value by dilutions with unlabeled PLTP. Results are representative of two experiments.

The interaction of apoA-I with ABCA1 elicits intracellular signals, including autophosphorylation of Janus kinase 2 (JAK2) (29, 37). This signaling pathway promotes the binding of apoA-I and its mimetic peptides to ABCA1 required for lipid removal (29). Incubating ABCA1-expressing BHK cells with apoA-I, wild-type PLTP, or mutant PLTP dramatically increased phosphorylation of JAK2 (Fig. 5B). Therefore, consistent with the ABCA1 binding assay, none of these mutations impaired the ability of PLTP to activate JAK2. Neither apoA-I nor wild-type or mutant PLTP had any effect on the total expression of JAK2 (not shown). These results suggest that the 144–163 region in PLTP, despite being involved in lipid removal, does not play a role in activating JAK2.

Most of the apolipoprotein binding sites on the surface of ABCA1-expressing cells appear to be phospholipid domains formed by the action of ABCA1 rather than ABCA1 itself (46, 47). We therefore examined the effects of the helix 144–163 mutations on binding of 125I-PLTP to mock- and ABCA1-transfected BHK cells. Overexpressing ABCA1 in BHK cells dramatically increased cell-surface binding of wild-type 125I-PLTP (Fig. 6). As with cholesterol eflux, the K146L mutation slightly reduced PLTP binding to ABCA1-expressing cells, but the M159D/E mutations markedly decreased binding. Thus, although the M159D/E mutations enhanced direct binding of PLTP to ABCA1, which would be only a minor fraction of total cell surface PLTP binding, these mutations severely impaired binding to the lipid domains formed by ABCA1 on the cell surface.

The cell surface binding data suggest that the M159D/E mutations impair the interaction of PLTP with phospholipids. We tested this possibility by measuring binding of wild-type and mutant PLTP to phosphatidylethanolamine vesicles. With increasing mass ratios of protein to phospholipid, wild-type PLTP progressively bound to vesicles, which was unsaturable up to a protein/lipid mass ratio of 1:1 (Fig. 7A). This is in contrast to the behavior of other well known lipid-binding proteins, such as apoA-I, which saturates the vesicle binding sites at ~0.2 μg of added protein per μg of vesicles. Because the reaction did not reach a saturation point, it was not possible to calculate equilibrium binding parameters for PLTP. The M159E and M159D mutants were much less efficient.
than wild-type PLTP in interacting with the vesicles. This confirms that the M159D/E mutations impair protein ability to bind phospholipids.

Because phospholipid binding is required for the lipid transfer activity of PLTP, we measured the effects of the PLTP mutations on the ability of PLTP to transfer liposomal phosphatidylcholine to HDL particles. Concentration curves showed that wild-type PLTP increased phospholipid transfer to HDL with saturable kinetics, and that the K146L mutation had no effect on this activity (Fig. 7B). In contrast, this phospholipid transfer activity was greatly reduced by the M159D/E mutations. With increasing concentrations, however, the M159D/E eventually reached the same transfer activity as wild-type PLTP, with about a 20-fold shift in EC50. Thus, the M159D/E mutations severely impaired but did not abolish the phospholipid transfer activity of PLTP.

We then investigated the possibility that conformational changes in PLTP could account for the impaired ability of the methionine mutants to interact with phospholipids and remove ABCA1-transported cholesterol from cells. Neither the K146L nor the M159D/E mutations had any effect on electrophoretic migration of PLTP in SDS-denaturing (Fig. 8A) or non-denaturing (Fig. 8B) polyacrylamide gels, suggesting that these mutations had only minor effects on the secondary and tertiary structure of PLTP. On the non-denaturing gels, PLTP appeared as bands with apparent molecular mass greater than 200 kDa, consistent with forming oligomers. This idea was confirmed by results showing that incubating 1.0 mg/ml wild-type PLTP for 6 h with the homobifunctional cross-linking agent BS3 readily cross-linked PLTP, forming large oligomers that failed to enter the separating region of the SDS-PAGE gel (data not shown).

To determine the impact of the methionine mutations on structural elements in PLTP, wild-type and M159D/E mutants
Protein. Wild-type PLTP exhibits a single minimum at around 222 nm, which is highly sensitive to changes in the secondary structure of the protein. Conformational changes in mutant PLTPs were monitored.

Wild-type PLTP was dialyzed into phosphate-buffered saline, and the samples were diluted to 0.1 mg/ml. Mutant PLTP was dialyzed into phosphate-buffered saline, and the samples were excited at 295 nm (to minimize the contribution of Tyr) in a 0.5-cm path length cuvette at room temperature, and fluorescence spectra were recorded. The spectra shown are averaged from two independent runs.

The wavelength of maximum fluorescence (WMF) was blue-shifted (335 ± 1 nm), indicating that the tryptophan residues exist in a relatively hydrophobic environment. Thus, the residues are most likely protected from aqueous solution and are sequestered into hydrophobic regions of the folded protein. The two methionine mutants demonstrated an increased overall fluorescence intensity compared with the wild-type. In addition, these both showed a small but reproducible blue shift relative to wild-type, with both PLTP M159D and M159E exhibiting a WMF of 332.5 nm. Both the difference in intensity and WMF are consistent with the circular dichroism data in suggesting a minor conformational shift when an acidic residue is substituted for Met-159.

**DISCUSSION**

PLTP mimics HDL apolipoproteins in removing cholesterol and phospholipids from cells by the ABCA1 pathway (15). ABCA1-dependent lipid export occurs by a cascade of events involving direct apolipoprotein binding to ABCA1, activation of signaling pathways, apolipoprotein binding to lipid domains formed by ABCA1, and solubilization of these lipids (12, 26, 46, 48, 49). In addition, the interaction of apolipoproteins with cells stabilizes ABCA1 protein and dramatically reduces its degradation rate (29, 43, 45). Our previous studies showed that PLTP can also mediate most of these events (15), implying that PLTP and apolipoproteins share structural features that promote ABCA1 interactions.

Because the amphipathic $\alpha$-helix appears to be the structural determinant for ABCA1 interactions (26, 28, 29, 43), we prepared synthetic peptides corresponding to each of the seven predicted amphipathic helices in PLTP and tested them for their abilities to remove cellular cholesterol. We found that the only peptide that could promote cholesterol efflux from ABCA1-expressing cells corresponded to amino acids 144–163 in PLTP (p144), which is an amphipathic helix predicted to be located near the tip of the N-terminal barrel of PLTP (Fig. 1, A and B). We then prepared mutants in this PLTP helix with a hydrophilic leucine substituted for a positively-charged lysine (K146L) or negatively charged aspartate or glutamate substituted for a hydrophobic methionine (M159D/E). The rationale for this approach was to change the charge or hydrophobicity at the polar/non-polar interface of the helix and to substitute charged residues for a methionine oriented into the core of the N-terminal barrel, where it may affect lipid interactions (see Fig. 1, B and C). We showed previously that oxidative modification of methionines in apoA-I impairs ABCA1 interactions (50).

Whereas the K146L mutation had little effect, the M159D/E mutations almost completely abolished the ability of PLTP to remove cholesterol from ABCA1-expressing cells. Moreover, the M159D/E mutations dramatically reduced PLTP binding to the cell surface lipid domains formed by ABCA1. These results suggest that the 144–163 helix in PLTP plays a critical role in mediating PLTP interactions with ABCA1-generated lipid domains and the solubilization of these lipids. A role for this helix in lipid interactions was supported by results showing that the M159D/E mutations also severely impaired PLTP binding to phospholipid vesicles and its phospholipid transfer activity. These effects were associated with only minor changes in PLTP secondary structure.

**FIGURE 9.** Conformational changes in mutant PLTPs. A, wild-type or mutant PLTP was dialyzed into phosphate-buffered saline, and the samples were diluted to 0.1 mg/ml. A, far-UV circular dichroism spectra were recorded in a 0.01-cm path cell at room temperature for an average of four scans. The mean residual ellipticity was calculated using a mean residual weight of 111.0 Da. The relatively high salt content was required to maintain protein solubility. Therefore, data below 190 nm could not be reliably recorded. The data shown are averaged spectra from three independent runs. B, PLTP samples were excited at 295 nm (to minimize the contribution of Tyr) in a 0.5-cm path length cuvette at room temperature, and fluorescence spectra were recorded. The spectra shown are averaged from two independent runs.

were analyzed by far UV circular dichroism. This method is highly sensitive to changes in the secondary structure of the protein. Wild-type PLTP exhibits a single minimum at around 215 nm (Fig. 9A), consistent with a protein that is dominated by $\beta$-sheet secondary structural elements. Based on the ellipticity at 222 nm (see "Experimental Procedures"), we estimate the total helical content of the protein to be about 17%, which is slightly below the 20% helical content predicted from computer models. Interestingly, both methionine mutations resulted in a similar and more pronounced minimum at 215 nm. This suggests an altered secondary structure compared with the wild-type. PLTP M159E and M159D were determined to be about 19% helical and likely exhibit slightly increased $\beta$-sheet structure. These data indicate that the substitution of negatively charged residues for Met-159 was sufficient to produce a small but clear conformational change.

To further probe the impact of the mutations on the structure of PLTP, we monitored the chemical environment of the three endogenous tryptophan residues in the protein by fluorescence. Tryptophan emission scans showed that wild-type PLTP exhibited a uniform bell-shaped fluorescence emission spectrum between 300 and 380 nm when excited at 295 nm (Fig. 9B). The wavelength of maximum fluorescence (WMF) was blue-shifted (335 ± 1 nm), indicating that the tryptophan residues exist in a relatively hydrophobic environment. Thus, these residues are most likely protected from aqueous solution and are sequestered into hydrophobic regions of the folded protein. The two methionine mutants demonstrated an increased overall fluorescence intensity compared with the wild-type. In addition, these both showed a small but reproducible blue shift relative to wild-type, with both PLTP M159D and M159E exhibiting a WMF of 332.5 nm. Both the difference in intensity and WMF are consistent with the circular dichroism data in suggesting a minor conformational shift when an acidic residue is substituted for Met-159.
A PLTP Helix Involved in ABCA1-dependent Cholesterol Efflux

conformation, making it less likely that they impared function by altering structures outside of helix 144–163.

Cross-linking studies suggested that the M159D/E mutations in the 144–163 helix of ABCA1 do not impair direct binding to ABCA1. These mutations doubled the amount of PLTP that could be cross-linked to ABCA1, implying that these substitutions actually increased direct PLTP/ABCA1 interactions. Because most of the apolipoprotein binding to ABCA1 expressing cells is to the lipid domains formed by ABCA1 (46, 47), this increased ABCA1 binding would not be detected by a cell surface binding assay, especially since lipid binding was markedly reduced by these mutations. Our results suggest that these PLTP mutants retain their ABCA1 binding activity but lose their ability to remove lipids, which supports the idea that these two activities depend on different structural properties.

We were unable to directly test whether peptide p144 binds to ABCA1 or the lipid domains formed by ABCA1. With competitive binding assays, p144 actually increased cell surface binding and ABCA1 cross-linking of radiolabeled PLTP, possibly because it is very hydrophobic and interacts directly with PLTP. Non-denaturing PAGE and cross-linking assays revealed that PLTP self-associates in solution, and perhaps the addition of helix 144–163 enhances PLTP self-association.

Exposing ABCA1-expressing cells to apoA-I or its mimetic peptides activates the tyrosine kinase JAK2 (29, 37). Inhibiting or ablating JAK2 dramatically reduces binding of amphipathic helices to ABCA1 (29, 37), indicating that optimum apolipoprotein binding to ABCA1 requires an active JAK2. We found that PLTP also mimics apolipoproteins in stimulating JAK2 phosphorylation, but the M159D/E mutations did not impair this effect. Thus, activation of JAK2 is associated with direct binding of PLTP to ABCA1 and is independent of lipid removal activity.

We showed previously that PLTP can mimic apolipoproteins in blocking degradation of ABCA1 protein (15). Here we show that p144 was the only synthetic PLTP helical peptide that significantly stabilized ABCA1 protein and that the M159D/E mutations impaired the ABCA1-stabilizing effects of PLTP. These results show an association of ABCA1 stabilization with binding to lipid domains rather than to ABCA1. This supports our previous work showing that inhibiting ABCA1 binding of apoA-I or its mimetic peptides has no effect on ABCA1 stabilization (29). It is possible that binding of amphipathic helices to lipid domains in close proximity to ABCA1 are responsible for stabilizing the protein (51).

Taken together, these studies suggest that helix 144–163 in PLTP is involved in mediating binding and solubilization of lipid domains formed by ABCA1 and stabilizing ABCA1 protein. Inducing ABCA1 in cells generates cell surface cholesterol domains accessible for subsequent apolipoprotein removal, even when ABCA1-interacting proteins are absent from the medium, indicating that protein binding to ABCA1 is not required for forming these lipid domains. Lipid removal, however, does appear to require binding of apolipoproteins or their mimetic peptides to ABCA1, as inhibiting ABCA1 binding also reduces lipid interactions and cholesterol efflux. Our results also show that it is possible to generate mutations in ABCA1-interacting proteins, such as PLTP, that impair lipid removal and ABCA1 stabilization but not binding to ABCA1 or activating the JAK2 signaling pathway.

The structural model of PLTP suggests a possible mechanism by which PLTP helix 144–163 facilitates lipid interactions and transport. PLTP contains a hydrophobic cluster of amino acids at the tip of the N-terminal barrel that promotes the phospholipid transfer interactions with HDL particles (Fig. 1A) (52). It is possible that this cluster interacts with the phospholipid/cholesterol domains formed by ABCA1, which appear to be exovesiculated lipids that protrude from the cell surface. Helix 144–163, which extends down the barrel chamber from the hydrophobic cluster, may facilitate solubilization of these lipids and their transfer between membrane surfaces and the lipid-binding pocket. Substituting a negatively charged amino acid for methionine, which is predicted to be within the barrel chamber, could disrupt the hydrophobic face needed for these lipid interactions and distort the chamber structure. Circular dichroism and tryptophan fluorescence assays showed that these mutations caused a minor but discernable change in PLTP conformation.

The current and previous studies show that both HDL apolipoproteins and PLTP have the potential of removing lipids from cells by the ABCA1 pathway. Whereas apolipoproteins become lipidated to form nascent particles that eventually mature into HDL, PLTP may function to shuttle lipids between cells and existing HDL particles, as has been described for PLTP-mediated interlipoprotein transfer (53). In support of this shuttle model are our previous results showing that when ABCA1 was induced in fibroblasts or macrophages, PLTP-mediated lipid efflux required the presence of HDL or phospholipid vesicles as acceptors (15, 54). PLTP alone promoted lipid efflux when ABCA1 was hyperexpressed in transfected cells (15), as is also shown here.

A role for PLTP in macrophage cholesterol efflux is suggested by studies showing that the sterol-responsive liver X receptor induces macrophage PLTP in concert with other proteins involved in cholesterol efflux, including ABCA1 and apoE (16, 17, 55). Macrophages from PLTP-deficient mice have higher levels of cholesterol (20) and reduced ABCA1-dependent cholesterol efflux (21) compared with cells expressing PLTP. Macrophage-derived PLTP has the potential to remove cellular cholesterol by multiple mechanisms, including remodeling of HDL particle to generate cholesterol acceptors (54, 56), modulating macropage production of apoE (22), and interacting with ABCA1 (15, 56). An understanding of the structural features in PLTP involved in the ABCA1 pathway may help determine the contribution of PLTP/ABCA1 interactions to whole body lipoprotein metabolism and cardiovascular disease.

Acknowledgments—We thank An-Yue Tu for generating the PLTP mutants and Hal Kennedy for preparing the PLTP structural model.

REFERENCES
1. Huuskonen, J., and Ehnholm, C. (2000) Curr. Opin. Lipidol. 11, 285–289
2. van Tol, A. (2002) Curr. Opin. Lipidol. 13, 135–139
3. Jauhiainen, M., Metso, J., Pahlman, R., Blomqvist, S., van Tol, A., and Ehnholm, C. (1993) J. Biol. Chem. 268, 4032–4036
