Changes in helical content or net charge of apolipoprotein C-I alter its affinity for lipid/water interfaces

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Abstract Amphipathic α-helices mediate binding of exchangeable apolipoproteins to lipoproteins. To probe the role of α-helical structure in protein-lipid interactions, we used oil-drop tensiometry to characterize the interfacial behavior of apolipoprotein C-I (apoC-I) variants at triolein/water (TO/W) and 1-palmitoyl-2-oleoylphosphatidylcholine/tri olein/water (POPC/TO/W) interfaces. ApoC-I, the smallest apolipoprotein, has two amphipathic α-helices. Mutants had single Pro or Ala substitutions that resulted in large differences in helical content in solution and on phospholipids. The ability of apoC-I to bind TO/W and POPC/TO/W interfaces correlated strongly with phospholipid-bound helical content. On compression of these interfaces, peptides with higher helical content were ejected at higher pressures. Substitution of Arg for Pro in the N-terminal α-helix altered net charge and reduced apoC-I affinity for POPC/TO/W interfaces. Our results suggest that peptide-lipid interactions drive α-helix binding to and retention on lipoproteins. Point mutations in small apolipoproteins could significantly change α-helical propensity or charge, thereby disrupting protein-lipid interactions and preventing the proteins from regulating lipid-protein catabolism at high surface pressures.—Meyers, N. L., L. Wang, O. Gursky, and D. M. Small. Changes in helical content or net charge of apolipoprotein C-I alter its affinity for lipid/water interfaces. J. Lipid Res. 2013. 54: 1927–1938.

Supplementary key words protein-lipid interaction • surface chemistry • drop tensiometry

Lipoproteins are macromolecular assemblies of varying lipid and protein content that transport lipids in the body (1, 2). High density lipoproteins (HDL) remove excess cholesterol from peripheral tissue for excretion, and low density lipoproteins (LDL) mediate cholesterol delivery to cells. The probability of developing atherosclerosis correlates with the balance between HDL and LDL (1–3). Exchangeable apolipoproteins transfer among various lipoprotein classes, including HDL and triacylglycerol (TAG)-rich very low density lipoproteins (VLDL) and chylomicrons (CM) (2). Once bound to lipoprotein surfaces, these proteins regulate HDL and LDL levels by serving as cofactors or inhibitors for many lipophilic enzymes or ligands for cellular receptors (4–11).

The secondary structure motif of apolipoproteins responsible for reversible binding to lipid/water interfaces is the class A, amphipathic α-helix (12, 13). This α-helix has a large (30–50%) apolar face subtending less than 180°, positively charged residues at its polar/nonpolar interface, and negatively charged residues along its polar face (Fig. 1) (12–14). Hydrophobic residues interact with apolar lipid moieties, while charged residues form ionic interactions with solvent molecules and phospholipid head groups (15, 16).

In solution, amphipathic α-helices can be largely unfolded [in apoA-II or apoCs (17–20)] or folded in helix bundles [in the N-terminal domains of apoE and apoA-I (21, 22)]. Studies of apoE, apoLp-III, and their mutant forms showed that the rates of liposome clearance decreased with greater protein size, thermodynamic stability, or degree of self-association, i.e., with increased tertiary or quaternary structure (23–26). These results suggest that helix-helix interactions decrease lipid-binding ability as helix bundles must open to expose their apolar helical faces to lipid.

The dependency of lipid surface binding on the tertiary structure and self-association of apolipoproteins makes it

Abbreviations: CD, circular dichroism; CM, chylomicron; DMPC, 1, 2-dimyristoylphosphatidylcholine; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; POPC/TO/W, palmitoyloleoylphosphatidylcholine/tri olein/water; SUV, small unilamellar vesicle; TAG, triacylglycerol; TO, triolein; TO/W, triolein/water; WT, wild-type.

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lipid-binding proteins, such as apoA-I and α-synuclein, the α-helices in apoC-I are shown as 11/3 helices (3.67 residues per turn) that are slightly unwound compared with the canonical α-helix (3.6 residues per turn), which optimizes the hydrophobic face for lipid binding (13). Due to the analogous position of Glu19 in the N-terminal helix of apoC-I to glutamate residues in 22-residue amphipathic α-helices of apoA-I that activate lecithin:cholesterol acyltransferase (29), the apolar face of the N-terminal helix was extended to include Glu19 and Leu8. The phospholipid-bound structure of apoC-I in proline mutagenesis studies is more consistent with the C-terminal helix, spanning residues 34–47 (38, 39) instead of residues 38–52 predicted via NMR (18).

ApoC-I monomers in solution lack substantial tertiary structure but adopt a fluctuating helix-turn-helix conformation with an average helical content of 31% (18, 37). On binding to phospholipid, the average helical content of apoC-I increases to 65–75%. The helical content of apoC-I in solution and on phospholipid can be altered by single Ala or Pro substitutions in either α-helix (Table 1) (37, 38). We speculate that this is unique to small apolipoproteins, as point mutations in single helices of larger proteins would likely have little effect on protein helical content. For these reasons, apoC-I is well suited for probing the role of secondary structure in lipid binding.

Studies using apoC-I point mutants and discoidal lipoproteins reconstituted from 1,2-dimyristoylphosphatidylcholine (DMPC) showed that, as the aqueous α-helical content of apoC-I increased, the rate and temperature range of DMPC clearance increased (37–41). However, these studies were limited by a nonphysiologic interface (DMPC is found only in trace amounts on lipoproteins (42, 43)) and the inability to monitor real-time surface modifications induced by protein binding. In the current work, we used wild-type and four point mutants of human apoC-I that differ in α-helical content in solution and on DMPC (Table 1). Of the three residues difficult to understand the role of preexisting secondary structure in lipid binding. One approach to analyze the effects of helical propensity on protein-lipid interactions is to use large proteins, such as apoA-I, from which individual helices have been deleted (27). However, these deletions change helical content, protein size, hydrophobicity, and tertiary interactions, which all contribute to lipid binding.

Alternatively, apolipoprotein-like peptides of 18–22 residues have been used to determine the effects of helical properties on the phospholipid affinity of α-helices (15, 16, 28–34). Peptide-lipid interactions were analyzed by many techniques, including intrinsic tryptophan fluorescence, differential scanning calorimetry, equilibrium dialysis against HDL, and surface chemistry techniques. These studies showed that changing the charge distribution, hydrophobicity, or helical propensity of α-helices altered phospholipid affinity. One limitation of these approaches is that short peptides are fully unfolded in solution, such that the role of preexisting helical structure on phospholipid affinity cannot be determined. In addition, these studies often used phospholipids that are found in low abundance on lipoproteins or failed to incorporate other lipoprotein constituents (triacylglycerol, cholesterol, fatty acids, etc.).

Here, we utilize a midway approach to determine the dependence of lipid affinity on secondary structure by using the smallest human apolipoprotein C-I (57 amino acids, 6.6 kDa) and its point mutants. ApoC-I is a secondary activator of lecithin:cholesterol acyltransferase and an inhibitor of cholesterol ester transfer protein, aiding in the synthesis of mature HDL (6–8). ApoC-I inhibits lipoprotein lipase and apoE-mediated uptake by cellular receptors, retarding catabolism of TAG-rich lipoproteins (9, 35, 36).

ApoC-I is predicted to contain two class A, amphipathic α-helices spanning the residues 7–29 and 34–47 (18, 37, 38). Fig. 1 shows the N- (left) and C-terminal (right) α-helical wheel diagrams. Similar to the class A helices in other lipid-binding proteins, such as apoA-I and α-synuclein, the α-helices in apoC-I are shown as 11/3 helices (3.67 residues per turn) that are slightly unwound compared with the canonical α-helix (3.6 residues per turn), which optimizes the hydrophobic face for lipid binding (13). Due to the analogous position of Glu19 in the N-terminal helix of apoC-I to glutamate residues in 22-residue amphipathic α-helices of apoA-I that activate lecithin:cholesterol acyltransferase (29), the apolar face of the N-terminal helix was extended to include Glu19 and Leu8. The phospholipid-bound structure of apoC-I in proline mutagenesis studies is more consistent with the C-terminal helix, spanning residues 34–47 (38, 39) instead of residues 38–52 predicted via NMR (18).

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In the current work, we used wild-type and four point mutants of human apoC-I that differ in α-helical content in solution and on DMPC (Table 1). Of the three residues

![Fig. 1. Helical wheel representations of the two predicted apoC-I amphipathic α-helices. Helices are modeled as 11/3 helices (13). By convention, apolar residues are colored in yellow, polar in gray, basic in blue, acidic in red, and glycine in pink. The hydrophobic face of each helix is indicated by dotted lines. Amino acids substituted for Ala or Pro are shown by red circles.](image-url)
mutated (Gly15, Arg23, and Met38), only Glycine 15 is conserved (44). Point mutants with similar hydrophobic moments as WT apoC-I (Table 1) were selected to correlate differences in lipid binding with the helical propensity of the peptides. G15P and M38P were selected to probe the contribution of the C- and N-terminal α-helices, respectively, to lipid binding. R23P was selected to mimic the acidic charge of the unexpressed human apoC-I pseudogene (44) and to probe the role of net charge in lipid binding.

To determine the role of helical content in apoC-I binding to interfaces that mimic lipoprotein surfaces, we used oil-drop tensiometry (45, 46) to characterize the behavior of the peptides at triolein/water (TO/W) and 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC)/TO/W interfaces. TO is a common TAG, and POPC is the most abundant phospholipid on lipoproteins (43). The TO/W interface provides a model for protein interactions with a TAG core, a component of all TAG-rich lipoproteins, while POPC/TO/W interfaces more closely resemble the surface of TAG-rich lipoproteins. Oil-drop tensiometry revealed the ability of each peptide to modify the surface pressure of these interfaces, mirroring protein ability to remodel lipid surfaces (46, 47). Expansion and compression of these interfaces revealed the pressure at which each peptide was ejected, reflective of protein retention on lipoproteins (46, 47).

**Materials and Methods**

**Materials**

Wild-type (WT) and mutant forms of full-size human apoC-I with single Pro or Ala substitutions at selected sites (M38P, R23P, G15P, and G15A) were produced via solid-state synthesis and purified by HPLC to 95–98% purity as described (37, 41). Peptide termini were not chemically blocked. Protein purity was confirmed by SDS gel electrophoresis and mass spectrometry (data not shown). Stock solutions were prepared by dissolving lyophilized peptides in 2 mM sodium phosphate buffer, pH 7.4 (standard buffer), to a peptide concentration of 1.0 mg/ml, as measured by Lowry assays. The helical content of aqueous peptides was verified by far-UV circular dichroism (CD) (38).

Triolein (>99% pure) was purchased from NU-CHEK PREP, Inc. (Elysian, MN). POPC, dissolved in chloroform at 25.0 mg/ml, was purchased from Avanti Polar Lipids (Alabaster, AL) and stored at −20°C. Purity of both lipids was checked using high-dose thin-layer chromatography. POPC was dried under nitrogen and re-suspended in standard buffer at 1.0 or 2.5 mg/ml. POPC in buffer was sonicated for 60 min with a pulsed duty cycle of 30% to form small unilamellar vesicles (SUV) of d ~22 nm (42, 43).

**Interfacial tension (γ) measurement**

An I. T. Concept (Longessaigne, France) Tracker oil-drop tensiometer was used to measure γ (45, 46). All tensiometry experiments were conducted at 25.0 ± 0.2°C in a thermostated system and repeated at least twice. Triolein drops of 16 μl were formed in 6.0 ml of gently stirred standard buffer, containing 0–750 μg/ml of POPC. After POPC was adsorbed but before addition of peptide, the buffer was exchanged with 150–250 ml of POPC-free buffer, as previously described (43). By removing >99.9% of the original buffer, all suspended POPC vesicles were removed in this procedure. After TO/W interfaces had stabilized at an initial γ of γi = 32.0 mN/m or POPC/TO/W interfaces reached γi = 23.0–25.5 mN/m, varied amounts of peptide stocks were added to the aqueous phase to obtain different protein concentrations (1.3–5.0 × 10−6 M) (48), and Pro substitutions in the apolar helical face reduce self-association (48), such that the apoC-I variants are expected to be largely monomeric in standard buffer at the concentrations used.

Peptide adsorbed to both types of interfaces, and γ was monitored continuously as it fell to an equilibrium value (γeq) (Fig. 2). Surface pressure (Π) was obtained from γ of a TO/W interface (γTO = 32.0 mN/m) minus γ of the interface with POPC and/or peptide (Π = γTO − γ). Adsorption curves were approximated by sigmoidal functions:

\[
\gamma(t) = \gamma_{eq} + \left( \gamma_i - \gamma_{eq} \right) \left\{ 1 + \exp \left( \frac{t-t_{1/2}}{w} \right) \right\}
\]

Here, \( t_{1/2} \) is the midpoint corresponding to 50% decrease in γ due to peptide adsorption, and \( w \) is the transition width.

**Rapid compression and expansion of the interfaces**

After peptide adsorption lowered γ of either interface to a γeq, the volume of the TO drop (16 μl initially) was decreased by a sequence of rapid compressions ranging 2–8 μl (Fig. 3). These decreases in volume decreased surface area, resulting in abrupt decreases in γ to certain values, γ, (where Πi = γTO − γ). The TO drop was held at reduced volumes for more than 5 min. If the compressed interface underwent relaxation via desorption of peptide, γ rose toward γeq observed as a desorption curve. If γ did not change, no net desorption or absorption occurred. At 5–10 min after each compression, the interface was expanded by increasing drop volume back to 16 μl. As surface area increased upon expansion, γ abruptly increased. If aqueous peptide

**Table 1. Helical content and hydrophobic moment of apoC-I variants**

| Protein | In Solution | On DMPC | <μH> (kcal/mol residue) |
|---------|-------------|---------|------------------------|
| G15A    | 48          | 75      | 0.30                   |
| WT      | 31          | 65      | 0.30                   |
| G15P    | 20          | 44      | 0.29                   |
| R23P    | 0-5         | 40      | 0.30                   |
| M38P    | 0-5         | 45      | 0.28                   |

Helical content and mean hydrophobic moment (<µH>) of apoC-I variants. Protein α-helical content for each apoC-I form was determined by CD from the measured value of [θ222] as described previously (37) with 5% accuracy. Results are from Refs. 37–39, but we verified the aqueous helical content of each apoC-I form at identical protein concentrations (0.02 μg/ml). Hydrophobic moments (µH) for the two predicted α-helices within each peptide were calculated as previously described (58) using the hydrophobicity scale of Ref. 59. The average of these hydrophobic moments represents the mean hydrophobic moment (<µH>) for each peptide.
adhered to the newly formed extra surface, $\gamma$ fell toward the initial $\gamma_{eq}$ observed as a readesorption curve. A similar sequence of rapid compressions and reexpansions was conducted after the bulk buffer was exchanged with 150 ml of peptide-free buffer.

**Values of $\Pi_{MAX}$**

At a given interface, $\Pi_{MAX}$ is the maximum pressure a protein can withstand before all or part of it is ejected from the interface. For each compression, the change in tension ($\Delta \gamma$) prior to reexpansion due to peptide desorption was plotted against $\Pi$. A linear regression of $\Delta \gamma$ versus $\Pi$ gave $\Pi_{MAX}$ as the x-intercept where $\Delta \gamma = 0$ mN/m (Fig. 4), such that peptide desorbs on compression to $\Pi > \Pi_{MAX}$ (49, 50).

**Ramping protocol and corresponding $\Pi$-$A$ isotherms**

After POPC adsorption to a TO drop lowered $\gamma$ to 23.0–25.5 mN/m, bulk buffer was exchanged with 150–250 ml of POPC-free buffer. TO drop volume was increased or decreased at a rate of 0.02 $\mu$l/s, decreasing or increasing $\Pi$ to an initial pressure $\Pi_i$ (43). $\Pi_i$ was used to find POPC surface concentration ($\Gamma_{POPC}$) in $\mu$m of POPC/$m^2$ of TO drop surface area and as a percentage of TO drop coverage by POPC, as described previously (43). Protein was added to the buffer and adsorption lowered $\gamma$ to a $\gamma_{eq}$, increasing $\Pi$ by $\Delta \Pi$. Buffer was exchanged with 150 ml of peptide-free buffer. Drop volume was increased at a rate of 0.02 $\mu$l/s to 25.0–28.0 $\mu$l. After $\gamma$ equilibrated for >200 s, volume was decreased at 0.02 $\mu$l/s with the lower limit varying, based on the requirement that $\gamma$ remain above 5.0 mN/m. Pressure-area ($\Pi$-$A$) isotherms were calculated from the $\gamma$ and surface area (A) profiles of each compression and expansion as previously described (43).

**Values of $\Pi_{ENV}$**

For compressions to $\gamma < \gamma_{eq}$, $\Pi$-$A$ isotherms exhibit an envelope point, which marks a change in slope. The envelope point (*)&

![Diagram A](image1.png)

**Fig. 3.** Examples of desorption and readesorption curves of G15A at TO/W (A) and POPC/TO/W (B) interfaces. (A) G15A was added to the aqueous phase at 1.3 $\times$ 10^{-7} M. After G15A adsorption to a 16 $\mu$l TO drop lowered $\gamma$ to $\gamma_{eq}$ = 19.5 mN/m, the drop was compressed and subsequently reexpanded in increments of $\pm 2$ $\mu$l, $\pm 4$ $\mu$l, $\pm 6$ $\mu$l, and $\pm 8$ $\mu$l. G15A was removed from the aqueous phase by a 150 ml buffer exchange (shown by the bar). The drop was compressed and reexpanded in increments of $\pm 4$ $\mu$l, $\pm 6$ $\mu$l, $\pm 8$ $\mu$l, and $\pm 10$ $\mu$l. (B) A 16 $\mu$l TO drop was formed and POPC adsorption lowered $\gamma$ to 25.2 mN/m. POPC was removed from the aqueous phase by a 250 ml buffer exchange (shown by the first bar). G15A was added to the aqueous phase at 1.3 $\times$ 10^{-7} M, and adsorption lowered $\gamma$ to $\gamma_{eq}$ = 9.5 mN/m. The TO drop was compressed and reexpanded in increments of $\pm 2$ $\mu$l, $\pm 4$ $\mu$l (×2), $\pm 5$ $\mu$l, and $\pm 6$ $\mu$l. G15A was removed from solution by a 150 ml buffer exchange (shown by the second bar). The drop was compressed and reexpanded in increments of $\pm 2$ $\mu$l, $\pm 6$ $\mu$l, $\pm 8$ $\mu$l, and $\pm 10$ $\mu$l.

![Diagram B](image2.png)
is the surface area ($\Lambda_{\text{ENV}}$) and $\Pi$ ($\Pi_{\text{ENV}}$) at which protein begins to be ejected from a POPC/TO/W interface. For a protein at a given interface, envelope pressure ($\Pi_{\text{eq}}$), and $\Pi_{\text{MAX}}$, both represent the maximum $\Pi$ up to which the protein is retained at that interface. However, as $\Pi_{\text{ENV}}$ is directly observed from a single experiment, the protocol to find $\Pi_{\text{ENV}}$ is better suited for determining the dependence of peptide retention pressure on $\Gamma_{\text{POPC}}$ (calculated from $\Pi$).

**Values of $\Pi_{\text{EX}}$**

Exclusion pressure ($\Pi_{\text{EX}}$) for a peptide is the $\Pi$ at which the peptide is excluded from POPC/TO/W interfaces (i.e., it cannot penetrate into the interface to raise surface pressure) (43, 51). Repetition of the ramping protocol over various $\Pi$ gave different $\Delta\Pi$ values for each $\Pi$, $\Delta\Pi$ values were plotted against $\Pi$. A linear extrapolation of the plot gave $\Pi_{\text{EX}}$ as the $x$-intercept where $\Delta\Pi = 0$ mN/m, such that peptide is excluded from the interfaces at $\Pi > \Pi_{\text{EX}}$ (49).

**RESULTS**

**Modification of TO/W and POPC/TO/W interfaces depends on the $\alpha$-helical content of apoC-I variants**

Apolipoproteins bind to TO/W and POPC/TO/W interfaces and decrease $\gamma$ to equilibrium values, $\gamma_{\text{eq}}$. This corresponds to $\Pi$ increases of $\Delta\Pi = \Pi_{\text{eq}} - \Pi$. We aimed to compare $\Delta\Pi$ of apoC-I variants at apolar TO/W and at more polar POPC/TO/W interfaces to assess how secondary structure in apoC-I affects binding to and modification of these interfaces. As peptides with greater $\alpha$-helical propensity form more extensive interactions with lipid (52), we hypothesized that greater $\alpha$-helical propensity favors peptide adsorption to lipid surfaces, leading to larger $\Delta\Pi$.

Fig. 2 shows a typical set of interfacial tension-time curves for peptides at TO/W (Fig. 2A) and POPC/TO/W (Fig. 2B) interfaces. In each case, peptide was added to the aqueous phase at $2.5 \times 10^{-7}$ M. The initial $\gamma$ of the TO/W interface was $32.0 \pm 0.5$ mN/m and that of the POPC/TO/W interface was $25.0 \pm 0.5$ mN/m ($\Gamma_{\text{POPC}} = 37.0 \pm 1.0\%$).

Adsorption of apoC-I variants to both interfaces showed a sigmoidal time course. The midpoint $t_{1/2}$ and transition width, $w$, were calculated as described in Materials and Methods and are shown for G15A in Fig. 2A. This sigmoidal behavior indicates that, once peptide adsorbs to lipid interfaces at adequate concentrations to significantly change $\gamma$, $\gamma$ decreases more rapidly as more peptide adsorbs (positive cooperativity) until the binding reaches an equilibrium. At $\gamma_{\text{eq}}$, lipid-bound peptide is in equilibrium with bulk peptide.

At a TO/W interface (Fig. 2A), all five peptides showed similar adsorption kinetics ($t_{1/2}$ varied from 266 to 400 s, and $w$ from 39 to 72 s). The ability of apoC-I to remodel the interface by changing $\Pi$ followed the rank order of G15A > WT > G15P = R23P = M38P. This order was observed for adsorption of peptide at multiple aqueous concentrations to a TO/W interface (Table 2). All peptides have a pI of 7.9 and are slightly basic in buffer of pH 7.4, except for R23P (pI = 6.2), which is acidic. To see whether net charge affected apoC-I binding to a TO/W interface, identical protocol as in Fig. 2A was repeated for WT and R23P in buffer of pH 6.2. Adsorption kinetics did not change for either peptide between buffers (data not shown). This result suggests that electrostatic peptide-peptide interactions do not alter the kinetics of peptide adsorption to an apolar lipid interface.

At a POPC/TO/W interface (Fig. 2B), most peptides showed similar adsorption kinetics ($t_{1/2}$ varied from 172 to 208 s, and $w$ from 42 to 72 s), except for R23P, ($t_{1/2} = 430 \pm 90$ s, $w = 120 \pm 30$ s). This indicates that cooperative binding to POPC/TO/W interfaces is significantly weaker for R23P than for the other peptides. The rank order for $\Delta\Pi$ was G15A > WT > G15P = M38P > R23P over multiple aqueous concentrations (Table 2). This indicates that R23P remodels POPC/TO/W interfaces by increasing surface pressure to a much lower extent than G15P or M38P, despite the similar helical content of the three peptides (Table 1).

Previous studies by Krebs and Phillips showed that the ability of various apolipoproteins to modify the surface
pressure of an air/water interface did not correlate with the mean hydrophobic moment ($\mu_H$) or helical content (F) of the proteins (53, 54). However, larger II changes correlated strongly with larger values of the product of the two variables ($\mu_H F$). Our results showed that, at both types of interface, II results did not correlate with $\mu_H$, as all peptides have very similar $\mu_H$ (Table 1). However, differences in II at both types of interfaces correlated strongly with differences in lipid-free or phospholipid (DMPC)-bound helical content (F) and with differences in the product $\mu_H F$ (as $\mu_H$ effectively serves as a scale factor) (supplementary Fig. I). These results suggest that peptides with greater α-helical propensity modify the surface pressure of lipoprotein surfaces to a greater extent.

At TO/W and POPC/TO/W interfaces, $\Pi_{\text{MAX}}$ correlates strongly with the phospholipid-bound α-helical content of apoC-I variants

The first aim of rapid compression and reexpansion protocol was to show that each apoC-I form was exchangeable at TO/W and POPC/TO/W interfaces, confirming the validity of our experimental approach. The second aim was to compare peptide retention at these interfaces. Physiologic processes, such as the catabolism of lipoproteins, rapidly change lipoprotein size and II. The ability of apolipoproteins to be retained as exchangeable. Hydrophobic lipid interfaces induce folding of amphipathic α-helices, we hypothesized that qualitative differences in the DMPC-bound helical content of the peptides would correlate with ability to be retained on lipid/water interfaces.

Fig. 3 shows γ and area changes during the rapid compression and reexpansion of G15A/TO/W (Fig. 3A) and G15A/POPC/TO/W (Fig. 3B) interfaces with and without peptide in the aqueous phase (i.e., before and after buffer exchange). After G15A adsorption lowered γ to $\gamma_{\text{eq}} = 13.5$ (Fig. 3A) or 9.5 mN/m (Fig. 3B), the 16 μl TO drop underwent a series of compressions and reexpansions, which corresponded to changes in volume of 2–8 μl and changes in area of 7–34%. At both interfaces, decreases in γ increased with the magnitude of compression, such that $\Pi_{\text{eq}}$ increased with larger compressions. For most compressions, γ rose toward $\gamma_{\text{eq}}$ at TO/W (Fig. 3A) and POPC/TO/W (Fig. 3B) interfaces when the compressed volume was held for more than 5 min. This rise in γ meant that some peptide desorbed from the surface after rapid compressions.

When the TO drop was reexpanded to 16 μl, γ increased above $\gamma_{\text{eq}}$, indicative of a lower amount of surface-active material present at the interface. Interfacial tension fell to $\gamma_{\text{eq}}$ at TO/W and POPC/TO/W interfaces as peptide in solution readsorbed to the newly exposed surface area. If only one of the protein α-helices desorbed while the other was still anchored to these lipid surfaces, peptide adsorption after each reexpansion would be fast and the readsorption slopes steeper than that of initial adsorption. However, the slopes of all readsorption curves were identical to those of initial adsorption curves at both interfaces (Fig. 3A, B, left), indicating that G15A was fully exchangeable.

To further test the reversible binding of G15A to both interfaces, rapid compression and expansion protocol was repeated after a 150 ml buffer exchange, which removed aqueous peptide (shown by bars in Fig. 3). After each reexpansion, γ remained elevated at both interfaces (Fig. 3A, B, right). If only one α-helix desorbed on compression, it would readsorb on reexpansion and lower γ to $\gamma_{\text{eq}}$, even without peptide in the aqueous phase. Similar protocol showed the exchangeability of WT (49), G15P, R23P, and M38P (data not shown). We conclude that the binding of all apoC-I variants to TO/W and POPC/TO/W interfaces is reversible.

To compare the retention of apoC-I mutants at both interfaces, $\Pi_{\text{MAX}}$ was obtained for each at TO/W and POPC/TO/W ($\Gamma_{\text{POPC}} = 37.0 ± 1.0\%$) interfaces (Fig. 4). The $\Pi_{\text{MAX}}$ data for G15P and M38P at a TO/W interface are not shown, as they superimpose with R23P data. The rank orders for $\Pi_{\text{MAX}}$ were G15A > WT > G15P = R23P = M38P at a TO/W interface, and G15A > WT > G15P > M38P > R23P at a POPC/TO/W interface (Fig. 4 and Table 3). We conclude that greater lipid-bound helical content increases the extent of apoC-I interactions with lipid, resulting in retention up to higher pressures.

### Table 2. Adsorption-induced II modifications of TO/W and POPC/TO/W interfaces by apoC-I variants

| Interface   | [Peptide] (M) | G15A | WT | G15P | R23P | M38P |
|------------|--------------|------|----|------|------|------|
| TO/W       | $1.3 \times 10^{-2}$ | 18.1 ± 0.4 | 17.2 ± 0.2 | 15.7 ± 0.6 | 16.0 ± 0.2 | 16.3 ± 0.2 |
|            | $2.5 \times 10^{-2}$ | 18.8 ± 0.2 | 17.9 ± 0.5 | 16.5 ± 0.3 | 16.5 ± 0.3 | 16.3 ± 0.2 |
|            | $5.0 \times 10^{-2}$ | 18.6 ± 0.2 | 18.0 ± 0.3 | 17.0 ± 0.3 | 16.9 ± 0.3 | 16.5 ± 0.3 |
| Average (ΔII) |              | 18.5 ± 0.5 | 17.7 ± 0.4 | 16.4 ± 0.5 | 16.5 ± 0.4 | 16.4 ± 0.3 |
| POPC/TO/W  | $1.3 \times 10^{-2}$ | 15.9 ± 0.3 | 14.8 ± 0.2 | 13.4 ± 0.4 | 11.7 ± 0.1 | 13.2 ± 0.1 |
|            | $2.5 \times 10^{-2}$ | 16.4 ± 0.3 | 15.4 ± 0.5 | 13.8 ± 0.5 | 12.3 ± 0.2 | 13.4 ± 0.4 |
|            | $5.0 \times 10^{-2}$ | - | 15.4 ± 0.2 | 13.6 ± 0.3 | 12.9 ± 0.3 | 13.8 ± 0.4 |
| Average (ΔII) |              | 16.2 ± 0.4 | 15.2 ± 0.5 | 13.6 ± 0.5 | 12.3 ± 0.4 | 13.3 ± 0.4 |

Adsortion-induced II modifications (ΔII) of TO/W and POPC/TO/W interfaces by apoC-I variants. Each ΔII is the average value from n = 3–7 experiments. <ΔII> is the average ΔII from all experiments at all concentrations for a peptide at a given interface (n = 9–12). Uncertainty for <ΔII> and ΔII values is the standard deviation. At POPC/TO/W interfaces, $\Pi_{\text{eq}} = 7.0 ± 0.5$ mN/m ($\Gamma_{\text{POPC}} = 37.0 ± 1.0\%$) prior to peptide adsorption. Results from Fig. 2 are in bold. [Peptide], peptide concentration in the aqueous phase; ΔII, the change in surface pressure due to adsorption of the given peptide.
For most peptides, the slopes of the linear regressions used to find $\Pi_{\text{MAX}}$ fell within 0.6–0.8. However, the regression used to find $\Pi_{\text{MAX}}$ of WT apoC-I at a POPC/TO/W interface had a slope of 0.4. We speculate that the $\alpha$-helices of apoC-I could exhibit a cooperativity in desorbing from POPC/TO/W interfaces that is not preserved with single amino acid substitutions in either of the protein $\alpha$-helices.

$\Pi_{\text{ENV}}$ correlates strongly with the $\alpha$-helix content of phospholipid-bound apoC-I variants

We aimed to find envelope pressures ($\Pi_{\text{ENV}}$) for apoC-I variants at interfaces of varied POPC surface concentration ($\Gamma_{\text{POPC}}$) to compare differences in peptide retention over a range of lipid/water interfaces. Like $\Pi_{\text{MAX}}$, $\Pi_{\text{ENV}}$ is the surface pressure at which protein begins to be ejected from lipid/water interfaces as pressure increases. The difference between $\Pi_{\text{MAX}}$ and $\Pi_{\text{ENV}}$ for a peptide at a given interface is the protocol used to find them. The dependence of retention pressure (referring to both $\Pi_{\text{MAX}}$ and $\Pi_{\text{ENV}}$) on $\Gamma_{\text{POPC}}$ is more easily derived from the protocol to find $\Pi_{\text{ENV}}$ (43).

**Figure 5** shows compression $\Pi$-$A$ isotherms for apoC-I variants adsorbed to a POPC/TO/W interface of $\Gamma_{\text{POPC}} = 36.5 \pm 0.5\%$. These were generated from the $\gamma$ and surface area profiles when, following peptide adsorption and a 150 ml buffer exchange, the POPC/TO drop was expanded and compressed at ±$0.02 \mu$l/s. In Fig. 5, the envelope point (*) is the surface area ($A_{\text{ENV}}$) and pressure ($\Pi_{\text{ENV}}$) at which peptide begins to be ejected from the POPC/TO/W interface on compression (direction indicated by arrow). The envelope point marks a change in isotherm slope. For all peptides, $A_{\text{ENV}} = 31.8–32.8 \text{ mm}^2$, indicating that each peptide occupied the same surface area after adsorption. $\Pi_{\text{ENV}}$ values from Fig. 5 were equivalent to $\Pi_{\text{MAX}}$ values at a similar interface (Table 3), following the rank order G15A $>$ WT $>$ G15P $>$ M38P $>$ R23P.

**Figure 6** and Table 3 show that this $\Pi_{\text{ENV}}$ rank order was observed for several POPC/TO/W interfaces, independent of $\Gamma_{\text{POPC}}$. For all peptides, $A_{\text{ENV}}$ was similar at each $\Gamma_{\text{POPC}}$ (data not shown), such that each peptide occupied the same area on adsorption. Linear regressions of the data for all peptides, except R23P, had similar slopes ($m = 0.11–0.14$) and extrapolated to $\Pi_{\text{ENV}}$ at a TO/W interface (Fig. 6). This indicates that R23P has different desorption kinetics at TO/W and POPC/TO/W interfaces. At POPC/TO/W interfaces, retention pressures of G15P were much higher than those of M38P, despite the similar phospholipid-bound helical content of the peptides (Table 3). This indicates that the N- and C-terminal helices of apoC-I may have different lipid-binding affinities.

**Differences in peptide retention pressures ($\Pi_{\text{ENV}}$, or the average of $\Pi_{\text{MAX}}$ and $\Pi_{\text{ENV}}$) at all of the lipid/water interfaces in Table 3 correlated strongly with differences in the phospholipid-bound helical content (F) and $<\mu_{H}> \times F$ of the peptides (supplementary Fig. II). This suggests that greater lipid-bound helical content increases the extent of protein-lipid interaction, resulting in apoC-I retention up to higher lipoprotein surface pressures.**

$\Pi_{\text{EX}}$ depends on apoC-I $\alpha$-helical content

We aimed to determine the effect of apoC-I point mutations on exclusion pressure ($\Pi_{\text{EX}}$). $\Pi_{\text{EX}}$ for a peptide is the $\Pi$ of POPC/TO/W interfaces at which peptide cannot penetrate and bind to (i.e., is excluded from) the interface.
Physiologically, proteins with higher $\Pi_{\text{EX}}$ are better able to bind to and regulate the metabolism of lipoproteins with high local surface pressures, such as nascent HDL or catabolic products of TAG-rich lipoproteins (51). We hypothesized that greater $\alpha$-helical propensity would favor apoC-I binding to lipid interfaces, reflected in larger $\Pi_{\text{EX}}$.

For each peptide, repetition of the ramping protocol over several values of $\Pi_i$ (or $\Gamma_{\text{POPC}}$) gave a complementary set of $\Delta\Pi$ values. Linear regression of the $\Delta\Pi$-$\Pi_i$ data for each peptide had a slope of $m = 0.56-0.62$ and gave $\Pi_{\text{EX}}$ as the x-intercept where $\Delta\Pi = 0$ mN/m (Fig. 7). The rank order for $\Pi_{\text{EX}}$ followed G15A > WT > M38P = G15P > R23P. We conclude that Pro substitutions in the middle of either $\alpha$-helix of apoC-I decrease overall $\alpha$-helical content and, as a result, decrease the ability of apoC-I to bind to high-pressure lipoprotein surfaces. Replacing Gly with Ala in the middle of the N-terminal $\alpha$-helix increases $\alpha$-helical content and the ability of apoC-I to bind to high-pressure lipid surfaces.

**DISCUSSION**

In this study, we used oil-drop tensiometry to determine the effect of single-residue substitutions on the affinity of apoC-I for lipoprotein-mimicking TO/W and POPC/TO/W interfaces. Because apoC-I is the smallest human apolipoprotein (57 residues) and lacks substantial tertiary structure, single Ala or Pro substitutions in the middle of its helices significantly alter its $\alpha$-helical content in solution and on phospholipid (Table 1).

The four apoC-I point mutants used in this study (Table 1) exhibited significant differences in their ability to bind to and modify TO/W and POPC/TO/W interfaces, as compared with WT apoC-I. Adsorption of apoC-I variants to TO/W (Fig. 2A) and POPC/TO/W (Fig. 2B) interfaces increased surface pressure by $\Delta\Pi$. The magnitude of $\Delta\Pi$, at both types of interface, followed the rank order of G15A > WT > G15P = M38P > R23P (Fig. 2 and Table 2). Plots of $\Delta\Pi$, at both types of interface, as a function of the lipid-free or DMPC-bound helical content of the peptides showed a significant correlation between the two variables (supplementary Fig. 1). Peptide length and mean hydrophobic moment ($\langle \mu_{H}\rangle$), which were very similar for all peptides (Table 1), did not contribute to this correlation. Together, these results suggest that higher helical propensity leads to more extensive protein-lipid interactions (52), resulting in a greater ability to bind to and increase the surface pressure of any lipoprotein surface (Fig. 8).

Consistent with this model, Pro substitutions in the middle of $\alpha$-helices of 20-residue apolipoprotein peptides disrupted helical content and peptide-phospholipid association (28). In addition, all apoC-I variants had similar $A_{\text{ENV}}$ values at various POPC/TO/W interfaces. This suggests that differences in $\Delta\Pi$ are due to differences in the helical properties of peptides, rather than differences in surface area coverage on adsorption.

Differences in exclusion pressure ($\Pi_{\text{EX}}$), the surface pressure at which peptide cannot penetrate and bind to POPC/TO/W interfaces, also indicated differences in the ability of apoC-I to bind to lipid/water interfaces. $\Pi_{\text{EX}}$ followed the rank order of G15A = WT > G15P = M38P > R23P (Fig. 7). These $\Pi_{\text{EX}}$ results suggest that exchangeable apolipoproteins with lipid-binding regions of greater helical propensities can bind to lipoproteins with higher surface concentrations of proteins and lipids (Fig. 8). As a result, these proteins are better able to regulate the metabolism of lipoproteins with higher local surface pressures (due to higher concentrations of surface-active molecules), such as HDL or the catabolic products of TAG-rich lipoproteins.
addition, the negative net charge of R23P at pH 7.4 could disrupt the positive cooperativity in peptide-peptide interactions that promote apoC-I binding to more polar lipid surfaces. Reduced binding cooperativity of R23P at POPC/TO/W interfaces, compared with the other apoC-I variants, was evident in sigmoidal curves with slower adsorption kinetics (Fig. 2B).

In this study, we also determined the effect of single-residue substitutions on the ability of apoC-I to be retained on TO/W and POPC/TO/W interfaces as surface pressure increased. From rapid or gradual compression of both types of interface, the retention pressures ($\Gamma_{\text{MAX}}$ or $\Gamma_{\text{ENV}}$, depending on the protocol used) at which each apoC-I variant began to desorb were determined. Retention pressure values at TO/W and POPC/TO/W interfaces correlated strongly with the phospholipid-bound, $\alpha$-helical content of the peptides (supplementary Fig. II). The rank order for $\Gamma_{\text{MAX}}$ followed G15A > WT > G15P = M38P ≥ R23P at TO/W and POPC/TO/W ($\Gamma_{\text{POPC}=37.0\%}$) interfaces (Fig. 4 and Table 3).

Despite the similar helical propensity of the peptides, R23P binding to more polar POPC/TO/W interfaces greatly differed from that of G15P and M38P. At an interface of $\Gamma_{\text{POPC}} = 37.0\%$, $<\Delta\Pi>$ for R23P was 2 mN/m lower than that of G15P or M38P (Table 2 and supplementary Fig. I). In addition, the $\Pi_{\text{EX}}$ of R23P was 2.4–3.3 mN/m lower than that of G15P and M38P (Fig. 7). Together, these results suggest that M38P and G15P are better able to penetrate and bind to modify the $\Pi$ of POPC/TO/W interfaces than R23P.

As R23P, G15P, and M38P remodel apolar TO/W interfaces to a similar extent, we speculate that differences in $\Pi_{\text{EX}}$ and $\Pi_{\text{ENV}}$ at more polar POPC/TO/W interfaces are due to the negative charge of R23P at pH 7.4. Arg23 could stabilize protein-phospholipid interactions at the POPC/TO/W interface, as its alkyl side chains interact with apolar lipid moieties and ionic interactions form between its positive charge and the phosphates of phospholipid head groups (15, 16). Loss of Arg23 in R23P would disrupt these interactions, but the negative charge of R23P could also lead to repulsive ionic interactions between R23P and the phosphates of POPC head groups, interfering with the ability of R23P to bind to POPC/TO/W interfaces. In addition, the negative net charge of R23P at pH 7.4 could disrupt the positive cooperativity in peptide-peptide interactions that promote apoC-I binding to more polar lipid surfaces. Reduced binding cooperativity of R23P at POPC/TO/W interfaces, compared with the other apoC-I variants, was evident in sigmoidal curves with slower adsorption kinetics (Fig. 2B).

In this study, we also determined the effect of single-residue substitutions on the ability of apoC-I to be retained on TO/W and POPC/TO/W interfaces as surface pressure increased. From rapid or gradual compression of both types of interface, the retention pressures ($\Pi_{\text{MAX}}$ or $\Pi_{\text{ENV}}$, depending on the protocol used) at which each apoC-I variant began to desorb were determined. Retention pressure values at TO/W and POPC/TO/W interfaces correlated strongly with the phospholipid-bound, $\alpha$-helical content of the peptides (supplementary Fig. II). The rank order for $\Pi_{\text{MAX}}$ followed G15A > WT > G15P = M38P ≥ R23P at TO/W and POPC/TO/W ($\Gamma_{\text{POPC}=37.0\%}$) interfaces (Fig. 4 and Table 3).

From the protocol to find $\Pi_{\text{ENV}}$, the rank order of G15A > WT > G15P > M38P ≥ R23P was observed for apoC-I retention pressure at lipid/water interfaces of multiple POPC
surface concentrations ($\Gamma_{\text{POPC}}$) (Fig. 6 and Table 3). The lower $\Pi_{\text{ENV}}$ values of R23P than those of G15P and M38P at POPC/TO/W, but not TO/W, interfaces correspond to its reduced ability to bind to more polar lipid interfaces, such that it is ejected at lower surface pressures.

In addition, the $\Pi_{\text{ENV}}$ of M38P was 1.5–2.2 mN/m lower than that of G15P for $\Gamma_{\text{POPC}} > 0\%$ (Table 3, Fig. 6, and supplementary Fig. II). Since Pro in either position disrupts the phospholipid-bound helical content of apoC-I in the C-terminal $\alpha$-helix of apoC-I. Near-UV CD showed that M38P, but not R23P, G15P, or G15A substitution, significantly altered the packing of this aromatic cluster (37). These results are consistent with the theory that the C-terminal $\alpha$-helix, due to its higher hydrophobicity, is the apoC-I $\alpha$-helix that serves as the high-affinity lipid anchor (5, 18). C-terminal anchoring would allow the N-terminal $\alpha$-helix of apoC-I to desorb from the lipoprotein surface on small increases in surface pressure (Fig. 8) or to interact with metabolic enzymes.

Combining $\Pi_{\text{MAX}}$ and $\Pi_{\text{ENV}}$ data, plots of retention pressure versus the phospholipid-bound helical content of the apoC-I variants at multiple TO/W and POPC/TO/W interfaces showed a significant correlation between the two variables (supplementary Fig. II). This indicates that the ability of an exchangeable apolipoprotein to be retained on lipoproteins and regulate metabolism correlates strongly with the lipid-bound helical content of the protein’s lipid-binding region. Greater lipid-bound helical content allows for more extensive peptide-lipid interaction (52), such that peptide is retained up to higher surface pressures (Fig. 8).

In summary, we showed that changes in the $\alpha$-helical content or charge of apoC-I, due to point mutations in the middle of either apoC-I $\alpha$-helix, strongly influence the ability of apoC-I to bind to, modify, and be retained on lipoprotein-like interfaces. These results have several physiological implications. For one, increases in surface pressure occur in lipoprotein catabolism and change the conformation of bound apolipoproteins or force them off the surface. For example, if lipoprotein lipase on TAG-rich lipoproteins hydrolyzes a small amount of TAG, lipoprotein volume shrinks and local surface pressure increases (exemplified by volume changes in Fig. 8). Our results indicate that the helical propensity and charge of the amphipathic $\alpha$-helices of apolipoproteins contribute to their ability to be retained at the surface of TAG-rich lipoproteins during metabolism and to regulate lipoprotein fate.

No phenotypic point mutations of apoC-I have been identified in humans. However, several point mutations in the amphipathic $\alpha$-helices of apoC-II (W26R, L72P) and apoC-III (A23T, Q23K, K58E) alter protein expression, lipid surface-binding, or function (20, 55–57). Since apoC-II and C-III are structurally similar to apoC-I, we speculate that altered helical content or charge in apoC-II and apoC-III point mutations may alter protein-lipid, protein-protein, or protein-enzyme interactions, thereby influencing the ability of these proteins to penetrate and bind to, be retained on, and regulate the fate of TAG-rich lipoproteins.

In addition, depending on their plasma concentrations, exchangeable apolipoproteins could absorb to the lipoprotein surface and increase surface pressure to such a degree that the lower-affinity apolipoproteins are displaced (19, 51). The two lipid-binding, class A, amphipathic $\alpha$-helices of apoC-II have similar hydrophobicity and length as those of apoC-I, but they differ in helical propensity and number of charged residues, such that apoC-I has a higher predicted lipoprotein affinity than apoC-II (11, 20). Notably, apoC-II is an activator of lipoprotein lipase and an important regulator of lipoprotein metabolism (11, 20). We speculate that apoC-I could bind TAG-rich lipoproteins and increase local surface pressure above the retention pressure of apoC-II, thereby displacing apoC-II from the surface and inhibiting the catabolism of these lipoproteins by lipoprotein lipase.

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