Surface Tensiometry of Apolipoprotein B Domains at Lipid Interfaces Suggests a New Model for the Initial Steps in Triglyceride-rich Lipoprotein Assembly*5

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Background: Apolipoprotein B (apoB) is the essential protein component of chylomicrons and very low density lipoprotein that transports lipids in plasma.

Results: Domains of apoB interact with model membranes dependent on lipid composition and surface pressure.

Conclusion: ApoB domains interact with membranes to initiate lipid recruitment.

Significance: The apoB lipid recruitment mechanism provides a target to regulate the secretion of VLDL.

Apolipoprotein B (apoB) is the principal protein component of triacylglyceride (TAG)-rich lipoproteins, including chylomicrons and very low density lipoprotein, which is the precursor to LDL. TAG-rich lipoprotein assembly is initiated by the N-terminal βα1 superdomain of apoB, which co-translationally binds and remodels the luminal leaflet of the rough endoplasmic reticulum. The βα1 superdomain contains four domains and is predicted to interact directly with lipids. Using drop tensiometry, we examined the interfacial properties of the α-helical and C-sheet domains and several subdomains to establish a detailed structure-function relationship at the lipid/water interface. The adsorption, stress response, exchangeability, and pressure (II)-area relationship were studied at both triolein/water and triolein/1-palmitoyl, 2-oleoeylphosphatidylcholine/water interfaces that mimic physiological environments. The α-helical domain spontaneously adsorbed to a triolein/water interface and formed a viscoelastic surface. It was anchored to the surface by helix 6, and the other helices were ejected and/or remodeled on the surface as a function of surface pressure. The C-sheet instead formed an elastic film on a triolein/water interface and was irreversibly anchored to the lipid surface, which is consistent with the behavior of amphipathic β-strands. When both domains were adsorbed together on the surface, the C-sheet shielded a portion of the α-helical domain from the surface, which retained its globular structure. Overall, the unique secondary and tertiary structures of the N-terminal domains of apoB support the intrinsic capability of co-translational lipid recruitment. The evidence presented here allows the construction of a detailed model of the initiation of TAG-rich lipoprotein assembly.

Chylomicrons and very low density lipoprotein (VLDL), collectively known as triglyceride-rich lipoproteins (TRLs),6 distribute triacylglycerides (TAGs) to peripheral tissues for utilization or storage. The remaining particle remnants are smaller, more dense, and enriched in cholesterol esters. Low density lipoprotein (LDL) is the remnant particle of VLDL (1, 2), and high plasma concentrations of LDL are associated with heart attack and stroke (3).

ApoB is essential for the assembly and stability of TRLs and, in the case of LDL, its cellular uptake in humans. There are two physiological isoforms of human apoB as follows: apoB100 (4536 residues) produced in hepatocytes (2, 4) and apoB48 (the N-terminal 2152 residues or 48% of the mature apoB sequence) synthesized by enterocytes and associated with chylomicrons (5). Both isoforms of apoB are highly water-insoluble and are only found associated with lipoprotein particles in vivo (4). Because of apoB’s large size and hydrophobicity, attempts to determine apoB’s high resolution structure have been unsuccessful.

ApoB contains five superdomains, NH2-βα1-β1-α2-β2-α3-COOH (4), as depicted in Fig. 1A. The β1 and β2 domains are composed of a series of amphipathic β-strands that were proposed to bind directly to a lipoprotein surface and act as an anchor for the protein (4). Amphipathic β-strands are formed

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6 The abbreviations used are: TRL, triglyceride-rich lipoprotein; αHD, α-helical domain containing 17 helices (apoB6-13); apoB, apolipoprotein B; A-sheet, apoB87–20.5; C-sheet, apoB13–17; ER, endoplasmic reticulum; LV, lipovitellin; POPC, 1-palmitoyl, 2-oleoyl phosphatidylcholine; SUV, small unilamellar vesicles; TAG, triacylglyceride; TP, transition point; mN, millinewton.

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from a sequence of alternating hydrophobic-hydrophilic amino acids that associate to form a sheet where one side is hydrophilic and the other side is lipophilic (6). The α2 and α3 domains of apoB are predicted to form amphipathic α-helices. These helices have both a hydrophobic and hydrophilic face (7).

The N-terminal βα1 superdomain, corresponding to the first 20.5% of apoB, has both α-helical and β-sheet secondary structure and has a putative globular tertiary fold (8, 9). The βα1 domain is required for the initial assembly of TRL in enterocytes and hepatocytes (4). However, the mechanism and function of the βα1 domain during the initiation of lipoprotein assembly remain unclear. The C-sheet is composed of six anti-parallel amphipathic α-helices with an additional long loop (apoB residues 706–728 in Fig. 2) missing in the LV crystal structure between strands 4 and 5, which may contain some amphipathic α-helices but little or no amphipathic β-strands (Fig. 1C) (13). The very hydropho-
bic face of the C-sheet is predicted to be in direct contact with the lipid core (8, 9). The hydrophilic face is predicted to form salt-bridge interactions with the C-terminal half of the HD (8, 9). By itself, the C-sheet sequence clears 1,2-dimyristoyl-sn-glycero-3-phosphocholine vesicles extremely rapidly (12). The A-sheet is less homologous to LV than the rest of the domain and is only partially modeled. It is predicted to form eight amphipathic β-strands but is not soluble in solution without detergent (12). The A-sheet is predicted to interact with the β-barrel and α-helical domains and is considered the beginning of the β1 domain.

The co-translational lipidation of apoB is the initial and essential step in TRL secretion. It is postulated that as domains of apoB are translocated into the rough ER, they recruit neutral lipids and remodel the ER lipids to bud off from the membrane in a primordial particle complexed with polar and neutral lipids. Budding may occur by forming a “micro-lens” of neutral lipid in the membrane, which ultimately buds off into the lumen of the rough ER. The molecular details of how the structure of the βα1 superdomain supports the initiation of TRL assembly remain a mystery.

We postulate that the mechanism of lipid recruitment is determined by the physical properties of each domain at the lipid/water interface. We and others have shown that drop tensiometry is a powerful tool for investigating protein functions at the lipid/water interface (14–17).

Here, we report on the -area isotherm at a triolein/water interface of the HD, the C-sheet, and a construct containing both domains (αHD+C) and several other critical segments of apoB within this region. Several remodeling intermediates within the helices of the αHD at the interface are visible as the surface pressure (II) is changed. These remodeling intermediates correspond to transitions in specific helices as they are reproduced in truncated constructs containing subsets of helices. We also characterize the adsorption of our constructs to a triolein/POPC/water as it has a monolayer of phospholipid that more closely resembles the ER membrane interface. We quantitate binding by determining the exclusion pressure (II_EX), which is the pressure above which a construct cannot bind the interface (18). These insights into the structure-function relationship of the βα1 superdomain based on the interfacial prop-

![FIGURE 2. Sequence alignment of apoB17 (1st line) and lamprey lipovitellin (2nd line). Black bars above the apoB17 sequence indicate conserved sequence motifs (N1–N13), and the highlighted amino acids are residues that are conserved within the large lipoprotein transfer protein family (30). Red residues are >90% conserved; green residues are 90–65% conserved, and yellow residues are 65 to 40% conserved. Above the apoB17 sequence are arrows that indicate the beginning or end of sequences studied labeled with percentages of the 4536 amino acids of mature apoB protein. On the 3rd line are the known point mutations causing familial hypobetalipoproteinemia. The 4th line is the predicted secondary structure of those amino acids according to the homology model (8, 9), H is helix; S is sheet. Below the secondary structure prediction is the name which refers to that structural motif.]

![FIGURE 3. Helical wheel diagrams of the 17 α-helices of the αHD. Yellow amino acids are hydrophobic; blue amino acids are basic; red amino acids are acidic, gray amino acids are polar, and pink amino acids are glycines or prolines. The one-letter codes indicate the actual amino acid.]

![TABLE 1 Constructs used in this study
Residues refer to the amino acid sequence of mature human apoB lacking the signal sequence. Percentage is the approximate starting and ending percentile of each construct relative to the 4536 amino acids of mature apoB (i.e. apoB100).

| Name | Schematic | Residues | Percentage |
|------|-----------|----------|------------|
| αHD | α-Helical | C-sheet | 292-782 | ApoB16-17 |
| αHD | α-Helical | C-sheet | 292-593 | ApoB16-13 |
| H1-10 | | | 292-496 | ApoB16-10 |
| H1-6 | | | 292-396 | ApoB16-9 |
| H1-5 | | | 292-379 | ApoB16-8.5 |
| H1-4 | | | 292-365 | ApoB16-8 |
| H9-17 | | | 369-593 | ApoB9-13 |
| S1-4 | | | 611-680 | ApoB13-15 |
| C-Sheet | | | 611-782 | ApoB13-17 |

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![Graph showing linear isotherms of protein at a triolein/water interface](image)

**FIGURE 4. Protocol for reversible isotherms.** In this example experiment, αHD was adsorbed to a triolein/water interface and washed out after reaching an equilibrium II. After the washout, the drop was linearly expanded and then linearly compressed to determine the isotherm. To test the reversibility, the drop was repeatedly linearly compressed and expanded. There was 1500 s of wait time between expansions and compressions. The isotherms were determined by plotting area versus II.

**EXPERIMENTAL PROCEDURES**

**Drop Tensiometry**

All experiments were performed with an IT Concepts Oil-Drop Tensiometer (Longessaigne, France) (19). A 16-μl drop of triolein (99.7% pure, Nu-Chek Prep, Elysian, MN) was formed at the tip of a J-tube submerged in 6 ml of bulk buffer. The bulk buffer was 2 mM sodium phosphate buffer at a pH of 7.4. A set of experiments were performed.

**Linear Isotherms of Protein at a Triolein/Water Interface—ApoB-derived proteins** (see below) were added to the solution surrounding a triolein drop. The triolein drop had a surface tension of 32 mN/m, which is considered “0” pressure. The protein spontaneously adsorbed to the triolein/water interface and raised the surface pressure (Πeq), the excess unbound protein was removed from the bulk solution by flowing 220 ml of peptide-free bulk buffer through the cuvette. This “washout” effectively depleted the bulk peptide by >99.9% (16, 20). After the washout, the drop was slowly and linearly expanded at a rate of ~2.7 mm²/min, held at a large area for ~1000 s, then linearly compressed at a rate of ~2.7 mm²/min. In some experiments the linear expansions and compressions were repeated (see Fig. 4). The isotherm was determined by plotting II versus area during a compression. The expanded II/area curves shown in Figs. 6–10 were obtained using this protocol.

**Exclusion Pressure (ΠEX) of Protein at a Triolein/POPC/Water Interface—A triolein/POPC/water interface was created by adsorbing POPC (Avanti Polar Lipids) small unilamellar vesicles (SUWs) to a triolein/water interface as described by Mitsche et al. (20). SUVs were made by sonicating aqueous POPC for 1 h using a probe sonicator (Branson). After a washout to remove SUVs, the drop was compressed to the specific initial pressure (II) (Fig. 11). Then the protein was added to a bulk concentration of ~3 μg/ml. After adding the protein, the surface area was increased in ~2-mm² steps until increasing the area did not cause II to decrease due to protein adsorption. The details for estimating ΠEX from these data are explained in Fig. 11.

**RESULTS**

**II-area Isotherms of the α-Helical and C-sheet Domains at the Triolein/Water Interface—The II-A relationship of a monolayer at constant temperature, called the “isotherm,” is an important indicator of monolayer properties. For complex monolayers, the isotherm is particularly useful because it yields compressibility information and highlights phase transitions and molecular rearrangements, which cause nonidealities during a compression of the surface area. The isotherms were determined for each protein by first adsorbing the protein to a triolein/water interface in the drop tensiometer, washing out...**

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**Protein Preparation**

The following peptides were studied: αHD+C, αHD, H1–10, H1–6, H1–5 H1–4, H9–17, S1–4, and C-sheet (Table 1). The sequence for each peptide is shown in Fig. 2. There is an N-terminal methionine and a C-terminal His₆ tag on each construct. The proteins were cloned in pET24a vector (Novagen) and expressed in BL21 DE3 *Escherichia coli* cells (Novagen) using standard protocols. For expression, cells were grown at 37 °C to an optical density of 0.6–0.8 at 600 nm in Luria broth supplemented with 34 μg/ml kanamycin. Protein expression was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside, and the cells were grown for an additional 3 h. Cell pellets were lysed with 1 mg/ml lysozyme at room temperature for 30 min and then sonicated. The protein inclusion bodies were dissolved in 8 M urea after washing with 1% Triton X-100 and 1 M urea. Soluble proteins were loaded on nickel-nitrilotriacetic acid-Sepharose (Qiagen) and eluted with 250 mM imidazole in 6 M guanidine hydrochloride. Protein refolding was achieved by rapidly diluting concentrated protein stock in 6 M guanidine hydrochloride to a final protein concentration of 1 μM into a refolding buffer containing 50 mM Tris, 800 mM arginine, 10 mM reduced glutathione, 2 mM oxidized glutathione, 0.02% sodium azide at pH 8.0. The refolding mixture was incubated at 4 °C overnight and then dialyzed extensively against 10 mM Tris, 150 mM sodium chloride, pH 7.5 (TS buffer). The refolded protein was later re-concentrated on another nickel-nitrilotriacetic acid column, and eluted with 250 mM imidazole in TS buffer. Finally, the protein was desalted to 2 mM sodium phosphate, pH 7.4, using a PD-10 desalting column (GE Healthcare). The final solution had a protein concentration of 0.1–0.3 mg/ml and was stored at 4 °C for no more than a week. The protein concentration was determined using a standard Lowry method (21) and by the adsorption at 280 nm (22). Both methods were consistent within 5% of the concentration. The αHD+C, αHD, S1–4, and C-sheet constructs have previously been shown to be folded with the secondary structure expected from the lipovitellin model by circular dichroism spectroscopy (CD) (11) as have H1–10 and H9–17 (23). The H1–6, H1–5, and H1–4 constructs have the expected helical CD signal and unfold cooperatively in urea denaturation experiments monitored by CD (data not shown). For drop tensiometry experiments, a small amount of peptide stock (~100 μl) was added to 6 ml of bulk buffer resulting in a final bulk concentration of ~3 μg/ml. See Table 2 for the bulk protein concentration and equilibrium surface pressure (ΠEX) of each protein.
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FIGURE 5. Protocol used to determine the isotherms of the apoB domains. The protein was adsorbed to the surface of a triolein droplet in the Oil-Drop Tensiometer. The shape of the drop was recorded by the camera of the tensiometer and provides the surface tension (32 mN/m for pure triolein). The decrease in surface tension due to protein binding causes the drop to become less spherical and the difference between 32 mN/m and the new surface tension is the surface pressure, \( \Pi \). After equilibrating to a constant \( \Pi \), a standard was washed out by changing the buffer (bar below the data on the far left). After the washout, the surface of the drop was linearly expanded and then linearly compressed by changing the volume of the drop which is attached to a syringe full of triolein controlled by a stepper motor. The isotherms (see Figs. 6–10) were determined by plotting drop area versus \( \Pi \). These experiments were all done with a pre-washout bulk concentration of \(-3\ \mu g/ml\). This example is of \( \alpha\)-helical C-sheet. Above the time axis are schematic representations of the triolein drop, which is buoyant in the aqueous buffer, held above the syringe tip. With no protein, the drop is nearly spherical (left). Upon addition of protein (black rectangles) the decrease in surface tension causes the drop to become more elongated. As the surface is expanded, the surface concentration of protein decreases, and after compression, the surface concentration is increased (far right).

During a linear compression at a triolein/water interface, the \( \Pi \) increased for both the C-sheet and the \( \alpha\)-helical C-sheet as the area was reduced by removing volume from the drop in the tensiometer (Fig. 6). The isotherm of the C-sheet (Fig. 6A) was linear both during expansions and compressions. The isotherms of both proteins were reversibly compressible (see Figs. 7 and 8). The slope of the C-sheet isotherm was smooth without any clear or reproducible breaks or discontinuities (i.e. rapid changes in slope). This is indicative of an elastic surface that quickly remodeled upon compression of the surface. The lack of breaks or discontinuities in the isotherm suggests no major remodeling events of the protein structure or the surface in response to compressions. Instead, there was a gradual re-aligning of the strands to exert more \( \Pi \) as their surface density increased. The compression was reversible and showed a large hysteresis. There was a wait time of 1500 s between each expansion and compression. During the compression, there were distinct bumps in the isotherms that repeatedly occurred at the same \( \Pi \) and area. The black dot on the x axis indicates the area of the drop during the initial adsorption.

The excess protein, and then linearly expanding and compressing the surface by changing the volume of the drop via the motorized syringe (as described in Fig. 5). The reversibility of the isotherms was determined by repeatedly linearly expanding and compressing the surface in progressively larger intervals (see Fig. 4). During a linear compression at a triolein/water interface, the \( \Pi \) increased for both the C-sheet and the \( \alpha\)-helical C-sheet as the area was reduced by removing volume from the drop in the tensiometer (Fig. 6). The isotherm of the C-sheet (Fig. 6A) was linear both during expansions and compressions. The isotherms of both proteins were reversibly compressible (see Figs. 7 and 8). The slope of the C-sheet isotherm was smooth without any clear or reproducible breaks or discontinuities (i.e. rapid changes in slope). This is indicative of an elastic surface that quickly remodeled upon compression of the surface. The lack of breaks or discontinuities in the isotherm suggests no major remodeling events of the protein structure or the surface in response to compressions. Instead, there was a gradual re-aligning of the strands to exert more \( \Pi \) as their surface density increased. The expansion and compression isotherms of the C-sheet were roughly parallel to one another indicating little hysteresis.

### Table 2

Adsorption characteristics of proteins from the \( \beta\alpha\)1 domain of apoB of the triolein/water interface

| Protein | Predicted structure | Standard concentration | Equilibrium \( \Pi \) (\( \Pi_{\text{EQ}} \)) |
|---------|----------------------|------------------------|-------------------|
| \( \alpha\)-Helical C-sheet | \( \alpha\)-helical C-sheet | 3.2 \( \mu g/ml \) | 18.8 \( \pm 0.4 \) mN/m |
| \( \alpha\)-Helical (H1–17) | \( \alpha\)-helical (H1–17) | 2.8 \( \mu g/ml \) | 18 \( \pm 0.4 \) mN/m |
| H1–4 | H1–4 | 3.6 | 16.5 \( \pm 1.2 \) mN/m |
| H1–5 | H1–5 | 3.5 | 16.9 \( \pm 0.7 \) mN/m |
| H1–6 | H1–6 | 3.2 | 19.4 \( \pm 0.3 \) mN/m |
| H1–10 | H1–10 | 2.9 | 17.1 \( \pm 0.3 \) mN/m |
| H9–17 | H9–17 | 2.7 | 16.5 \( \pm 0.9 \) mN/m |
| C-sheet | C-sheet (S1–6) | 2.9 | 19.7 \( \pm 0.6 \) mN/m |
| S1–4 | S1–4 | 3.2 | 19.4 \( \pm 0.4 \) mN/m |

The equilibrium surface tension, \( \Pi_{\text{EQ}} \), is dependent on concentration. A standard concentration near 3 \( \mu g/ml \), the mean \( \pm \) S.D. of \( \Pi_{\text{EQ}} \) was calculated from eight independent experiments (\( n = 8 \)).

The isotherms was determined by repeatedly linearly expanding and compressing the surface by changing the volume of the drop which is attached to a syringe full of triolein controlled by a stepper motor. The isotherms (see Figs. 6–10) were determined by plotting drop area versus \( \Pi \). These experiments were all done with a pre-washout bulk concentration of \(-3\ \mu g/ml\). This example is of \( \alpha\)-helical C-sheet. Above the time axis are schematic representations of the triolein drop, which is buoyant in the aqueous buffer, held above the syringe tip. With no protein, the drop is nearly spherical (left). Upon addition of protein (black rectangles) the decrease in surface tension causes the drop to become more elongated. As the surface is expanded, the surface concentration of protein decreases, and after compression, the surface concentration is increased (far right).
Thus, the C-sheet was highly compressible and elastic, a property characteristic of other amphipathic β-strands (6, 24). The HD had a fascinating compression isotherm (Fig. 6B). Foremost, unlike the C-sheet, its isotherm is nonlinear. More interestingly, there were clear discontinuities in the isotherm with rapid increases in the slope (Fig. 9A). We term these discontinuities “transition points” (TP), and five of these TPs reproducibly occurred at the same area when the isotherm was repeated within the same or independent experiments (Fig. 8). At these transition points, the area decreased with little or no change in pressure, consistent with a very large compressibility, indicating a conformational phase transition in the protein monolayer. The average pressure of each TP is shown in Table 3. When the surface was re-expanded (Fig. 8), the TPs occurred roughly at the same area but at a lower pressure due to hysteresis.

TP 1 occurs at the highest pressure (smallest surface area), and TP 5 occurs at the lowest pressure (largest surface area). Because these transition points are observed in the exchangeable, amphipathic α-helices of the HD, but are notably absent from the nonexchangeable amphipathic β-sheets of the C-sheet (17), the obvious physical explanation for these transition points is the ejection or expulsion of individual or subsets of helices from the triolein/water interface into the aqueous phase. Thus, the HD undergoes five major structural rearrangements over this range of surface pressures.

II-area Isotherms of Portions of the α-Helical Domain—To determine which helices of HD are ejected from the drop surface by increasing II at each TP, isotherms of smaller constructs containing only part of the 17-helix HD were examined. Four additional proteins were studied as follows: H1–4, H1–6, H1–10, and H9–17 (Table 1). The isotherms of these smaller constructs also had TPs, and intriguingly, these TPs are subsets of the TPs and to determine the area where each TP occurs. The TPs are labeled 1–5 (TP 1 occurs at the smallest drop surface area and therefore at the highest II; TP 5 occurs at the largest drop area and therefore at lowest II), B, H1–4, C, H1–6; D, H1–10; E, H9–17. B–E, the experimental data are shown in blue; the derivative of the data is in black, and the TPs are labeled as in A. The black dots on the x axis indicate the initial pressure after addition of proteins before commencing compressions and expansions.

**TABLE 3**
Transition points in the isotherm of apoB helical domain constructs at the triolein/water interface

| Transition Point Number | Pressure [mN/m ± S.D.] | αHD (1–17) | H1–4 | H1–6 | H1–10 | H9–17 | Possible Transition Helices |
|-------------------------|------------------------|------------|------|------|-------|-------|---------------------------|
| 1                       | 26.1 ± 0.9             | Yes        | Yes  | Yes  | Yes   | 5-6   | H1–6                      |
| 2                       | 23.2 ± 0.3             | Yes        | Yes  | Yes  | Yes   | 1-4   | H1–6                      |
| 3                       | 21.6 ± 0.2             | Yes        | Yes  | Yes  | Yes   | 1-4   | H1–6                      |
| 4                       | 20.1 ± 0.5             | Yes        | Yes  | Yes  | Yes   | 9-10  | H1–6                      |
| 5                       | 17.2 ± 1.1             | Yes        | Yes  | Yes  | Yes   | 10-17 | H1–6                      |
| II at Original Area [mN/m] | 21.9 ±0.6 | 22.4 ± 0.9 | 21.3 ± 0.2 | 21.9 ± 0.3 | 18.4 ±0.4 | H1–6 |
| Number of Experiments  | 6                      | 4          | 4    | 6    | 3     |       | H1–6                      |

**FIGURE 9.** II-A isotherms at the triolein/water interface of helical domain constructs feature TP. A, αHD. The raw data for αHD is the same as in Fig. 6B. The derivative of the isotherm is shown below the data in gray to highlight the TP 1 occurs at the smallest drop surface area and therefore at the highest II; TP 5 occurs at the largest drop area and therefore at lowest II; B–E, the experimental data are shown in blue; the derivative of the data is in black, and the TPs are labeled as in A. The black dots on the x axis indicate the initial pressure after addition of proteins before commencing compressions and expansions.
of TPs observed for the complete αHD (Fig. 9, B–E). The II of each TP was approximately the same for all αHD proteins. However, none of the smaller proteins had all five TPs of the αHD isotherm (see Table 3 for a summary). H1–4 exhibited only two TPs corresponding the II of TPs 2 and 3 of the intact αHD. The isotherm remained smooth at the transition pressures expected for TPs 1, 4, and 5 showing the absence of these TPs. Therefore, helices 1–4 undergo transitions at TPs 2 and 3. The H1–6 isotherm has TPs 1–3 of the intact αHD. Because TP 1 was present in H1–6 but not in H1–4, TP 1 could be attributed to a change in helices 5–6, which are part of H1–6 but absent in H1–4. Similarly, H1–10 exhibited TPs 1–3 like H1–6 but also had TP 4, indicating TP 4 occurs within helices 7–10. H9–17 had TPs 4 and 5, and a sixth TP at a low II that was only seen with H9–17. Because TP 4 is found in H1–10 and H9–17, it must arise from helices 9–10 found in both constructs. Also, H9–17 undergoes TP 5, indicating that TP 5 must occur within helices 11–17, as helices 9–10 undergo TP 4.

We also examined the triolein/water isotherm of a larger construct, αHD+C, that contains the αHD and C-sheet to determine whether it underwent similar TPs. αHD+C only exhibited TPs 1–4, the four high-II TPs, but they were not as dramatic as the isolated αHD domain (see Fig. 10). The equilibrium adsorption II was between that of TP 3 and 4. At II values less than TP 4, II dropped at a linear rate, similar to the C-sheet. Therefore, αHD+C adsorbed to the surface at an equilibrium conformation where the N-terminal helices (H1–10) were likely in contact with the surface, but the C-terminal helices (H11–17) were not. When the surface was compressed, the helices remodeled at TP 1–4 as observed with αHD. This suggests that helices 11–17 did not bind to the interface. Instead, they fold against the more polar face of the C-sheet whose hydrophobic face is in direct contact with the triolein of the drop. This conformation is quite similar to that proposed for αHD+C based on its compression isotherm at an air/water interface measured with a Langmuir trough (16).

Protein Exclusion Pressure (Π_{EX}) at a Triolein/POPC/Water Interface—Physiological TAG/water interfaces are mostly covered with an amphipathic monolayer composed primarily of phospholipids. Therefore, a triolein/POPC/water interface is a better model to study the initial adsorption of the αHD or C-sheet to a lipid surface like the ER luminal membrane. The affinity of a protein for a triolein/POPC/water interface can be quantified by determining its exclusion pressure (Π_{EX}). Π_{EX} is defined as the surface pressure of a phospholipid monolayer where protein cannot penetrate the surface (18, 25, 26). When the initial pressure of the interface, Π_{I}, is greater than the exclusion pressure (i.e., Π_{I} > Π_{EX}), the protein is unable to insert into the interface. With Π_{I} < Π_{EX}, the protein can insert.

In our experiments, Π_{EX} was determined by adding the peptide to the bulk buffer surrounding a POPC-covered triolein drop with a high Π_{I} (>15 mN/m). The triolein/POPC/water interface was made by adsorbing POPC SUVs to a triolein/water interface followed by a washout (20). After the washout of POPC, the surface was compressed to increase Π_{I}. After the protein was added to the surrounding solution, the drop was slowly expanded in a stepwise fashion (with the protein in the bulk) until increasing the area did not decrease Π_{I} (see Fig. 11). In the presence of protein, the Π_{I} would be increased if the initial pressure, Π_{I}, was less than the exclusion pressure, Π_{EX}, of the protein (Π_{I} < Π_{EX}). If Π_{I} > Π_{EX}, there was no change in Π_{I} as the peptide cannot penetrate the surface (for example see the arrow in Fig. 11A). The surface was then expanded in a stepwise fashion (each step was less than 1 μl (~2 mm²)). The change in Π_{I} from adsorbing protein (ΔΠ_{I, protein}) was equal to the equilibrium II after an expansion, minus the Π_{I} of the POPC at the interface without any added protein that was determined in another experiment (black line, Fig. 11B). We can then determine Π_{EX} of the protein by plotting the ΔΠ_{I, protein} at each expansion, versus the known II of the POPC at that expanded area (that is the Π_{I}) and extrapolating to a ΔΠ_{I, protein} of 0 (Fig. 11C).

The relationship between ΔΠ_{I, protein} and Π_{I} was linear for all proteins (see Figs. 11 and 12). The Π_{EX} of αHD and the constructs with portions of the αHD ranged from 21–23 mN/m (see Table 4 and Fig. 12). The C-sheet had a Π_{EX} = 18.7 mN/m, significantly lower than Π_{EX} of the αHD. A construct containing only the first four of the six strands (S1–4) of the C-sheet had a Π_{EX}, identical to the full C-sheet construct. The αHD+C construct, with both the C-sheet and αHD domains, had a Π_{EX} = 22.2 mN/m, which is characteristic of the αHD alone. This suggests that the αHD+C construct initiates binding to triolein/POPC/water interface with the α-helices of the αHD, especially at high Π_{I}.

The difference between the αHD and C-sheet domains is more pronounced when binding at the triolein/POPC/water interface (Π_{EX}) is compared with that at the triolein/water interface (Π_{EQ}) (see Table 4). The αHD proteins’ Π_{EX} at the triolein/POPC/water interface is 4–5 mN/m greater than Π_{EQ} at the triolein/water interface. Therefore, the αHD has a higher affinity for a triolein/POPC/water interface than a triolein/water interface so phospholipids increase the binding affinity of the helices. In contrast, for the C-sheet proteins Π_{EX} ≈ Π_{EQ}, which indicates the C-sheet has very little affinity for interfacial phospholipids. The C-sheet only adsorbed to the triolein/POPC/water interface at low surface pressures of POPC where there are probably patches of continuous triolein available to form a binding site. Thus, the C-sheet has an equivalent inter-
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FIGURE 11. Exclusion pressure determination of αHD at the triolein/POPC/water interface. A, triolein/POPC/water interface was formed by adsorbing POPC small unilamellar vesicles to a triolein drop, washing out the excess POPC, and then compressing the surface to ~22 mN/m (28). Protein was then added to the bulk solution (black arrow), but no change in pressure (black line) was observed as the high surface concentration of POPC (IPOPC, where I is surface concentration) prevented αHD from binding. After ~2000 s of equilibration time, the drop was expanded in a stepwise manner to determine the adsorption of protein at the lowered pressure and resulting lowered IPOPC. Blue dots represent individual areas, and blue squares indicate equilibrium pressures reached by binding of protein to the interface. These values are plotted against each other in B. B, II of a triolein/POPC/protein/water interface was determined at different IPOPC. Before the protein was added, the number of POPC molecules on the surface and therefore the initial pressure I0 was calculated. II is the II of the triolein/POPC/water interface in the absence of protein (solid curve) (18). The blue diamonds correspond to the pressure attained by the protein adsorbing to the surface (blue squares in A) plotted against the increase in drop area (blue circles in A). The difference between the II of a triolein/POPC/protein/water interface and a pure triolein/POPC/water interface is defined as ΔIIprotein (double-headed arrow). C, exclusion pressure (IIEX). The ΔIIprotein for each experimental point in B is plotted against II0, which is the pressure exerted by POPC in the absence of protein at equivalent drop areas (solid curve in B). The IIEX is equal to the x-intercept of the ΔIIprotein versus II0 line.

facial affinity for both interfaces, indicating the C-sheet preferentially binds triolein at both interfaces.

DISCUSSION

The process of lipid recruitment by the βα1 domain of apoB requires structural rearrangements of the protein to first recognize TAGs exposed in the ER membrane, bind to the membrane, recruit additional lipids, and finally create a binding site for the β1 superdomain of apoB when it is translocated. Given the disruptive nature of hydrophobic lipids during protein folding, the βα1 must orchestrate this highly coordinated process to avoid protein misfolding or improper particle assembly. Importantly, the translation rate of the nascent apoB (minutes) is much slower than the expected folding rate of the domains encoded in the βα1 superdomain (<< seconds) so these individual domains are likely to fold as they exit the translocon, well before they are assembled into the final primordial particle.

The βα1 domain contains both α-helical and β-sheet structure. Amphipathic α-helices and amphipathic β-strands have different properties at a lipid interface (6, 24). The helices exchange on and off a hydrophobic interface and behave viscoelastically. The helices remodel in response to changes in II and can be ejected from the surface in a sequential and II-dependent fashion (27). The large αHD displays discrete remodeling/ejection events as seen by the five transition points in its compression isotherm at the triolein/water interface (Fig. 9). Furthermore, these TPs are conserved in the smaller constructs allowing us to localize the helices that are remodeled/ejected. Each TP most likely indicates a surface pressure-dependent protein rearrangement.

It is instructive to follow the rearrangement of the helices of the αHD construct at the triolein/water interface during a compression from a high to low surface area (Fig. 13). At high area, the surface pressure is low (II < 17 mN/m) and both the N- and C-terminal helices spread on the surface. As the protein is compressed, the C-terminal helices (H9–17) are first ejected from the surface (TP 5 and 4). At a moderate surface pressure (II = 22–25 mN/m), helices 1–4 remodel and are ejected in two steps (TP 3 and TP 2). At the highest surface pressure (above TP 1), the core helices (H5–8) remodel, but the αHD remains anchored to the surface by helix 6. The very hydrophobic helix 6 remains embedded in the interface to anchor the peptide at the surface even at the highest surface pressure. This anchoring prevents all constructs containing H6 from desorbing from the surface and allows a rapid response to changes in area.

In our experiments, the αHD adsorbed to the triolein/water interface from the bulk solution to an equilibrium pressure between TP 2 and TP 3. Therefore, it adsorbed in a compact conformation, where the C-terminal nine helices were excluded from the surface and only some of the N-terminal eight helices were on the surface. Compression caused the N-terminal four helices to be ejected from the surface, whereas expansion led to binding by the C-terminal helices H10–17. Thus, the conformationally flexible interfacial structure of the αHD allows the βα1 superdomain to remodel as the composition and surface pressure of bound protein changes during initial lipo-protein assembly.
FIGURE 12. Exclusion pressure data. Raw data used to measure $\Pi_{EX}$ of the series of proteins at a triolein/POPC/water interface is reported in Table 4. $\Pi_{EX}$ is equal to the linear regression of $\Delta \Pi_{protein}$ and $\Pi_{I}$ (see Fig. 11). The correlations were linear and had an $R^2$ greater than 0.9.
Amphipathic \( \beta \)-strands of the C-sheet have a higher affinity for a triolein/water interface than amphipathic \( \alpha \)-helices and exert more surface pressure both when adsorbed and expanded (6, 24). The C-sheet binds irreversibly to a triolein/water interface and cannot be pushed off the droplet surface by compression. Based on their exclusion pressures (\( \Pi_{EX} \)) at the triolein/POPC/water interface (Table 4), they do not appear to interact with phospholipid and prefer the more hydrophobic triolein.

The \( \alpha \)-helical domain proteins and C-sheet domains have different lipid binding activities (see Table 4). The C-sheet exerted more II on a triolein/water interface. However, the \( \alpha \)HD exerted more II on a triolein/POPC/water interface and penetrated a higher initial surface pressure (\( \Pi_I \)) than the C-sheet. For the C-sheet to bind a triolein/POPC/water interface, the \( \Pi_I \) must be lower than C-sheet triolein/water equilibrium surface pressure (\( \Pi_{EQ} \)). Thus, the monolayer of POPC shields the C-sheet from binding to the hydrophobic TAG core. Conversely, POPC enhances the binding of the \( \alpha \)HD. The \( \alpha \)HD was able to bind a triolein/POPC/water interface with a \( \Pi_I > \Pi_{EQ} \) for the triolein/water interface. Therefore, the \( \alpha \)HD has a higher affinity for a mixed triolein/POPC interface than a pure triolein interface.

\( \alpha \)HD + C, which includes both domains, also adsorbed to a triolein/water interface in a compact structure (Fig. 10). In the adsorbed conformation, the C-terminal half of the \( \alpha \)HD (H11–17) had a globular conformation and was shielded from the interface by the C-sheet. The N-terminal helices (H1–10) were partially unfolded and spread at the interface (16). When the monolayer of adsorbed \( \alpha \)HD + C was compressed, the N-terminal helices remodeled to adapt to the smaller area per molecule. When the monolayer was expanded, the C-sheet remodeled while still shielding H11–17 from the lipid.

The unique two-layered helical structure of the \( \alpha \)HD provides remarkable adaptability in both lipid binding and domain folding. Putting these findings into context with other studies of the \( \beta \)E1 superdomain, such as the LV structure of the \( \beta \)E1 superdomain (8–11, 13, 28), in vitro lipid binding assays (12, 14, 17, 23, 29), the conserved sequence motifs (9, 30), and \( \alpha \)B C-terminal truncation secretion (31–33), we have developed a more detailed mechanism of initial co-translational apoB lipid binding (see Fig. 14), using the TPs we report here as a road map. Note that we have measured the binding of isolated domains, not intact apoB, and that our system uses model interfaces and not the ER membrane or ER-resident lipid droplets, nor does our system contain microsomal triglyceride transfer protein or any of the other chaperones that are essential for VLDL secretion. Thus, our mechanism only addresses the interaction of apoB domains with lipids and not the critical role of microsomal triglyceride transfer protein, chaperones, or any other proteins that are essential for VLDL secretion.

The ER membrane likely resembles a highly compressed POPC monolayer with a high II. There is a high concentration of PC, but other molecules like TAG and proteins are dispersed in the membrane, which lower the II and create potential binding sites for apoB. The \( \alpha \)HD can bind to these small binding

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**TABLE 4**

| Peptide (no. of data points for \( \Pi_{EX} \)) | Structure | Exclusion pressure (\( \Pi_{EX} \)) (mN/m ± S.E. triolein/POPC/water) | \( \Pi_{EQ} \) (mN/m ± S.D. POPC/water) |
|-----------------------------------------------|-----------|-------------------------------------------------|---------------------------------|
| \( \alpha \)HD + C (45) | Both domains | 22.2 ± 0.2 | 18.8 ± 0.4 |
| **\( \alpha \)-Helical domain proteins** | | | |
| \( \alpha \)HD (40) | H1–17 | 22.3 ± 0.3 | 18.0 ± 0.4 |
| H1–4 (29) | H1–4 | 21.4 ± 0.3 | 16.5 ± 1.2 |
| H1–6 (29) | H1–6 | 23.6 ± 0.5 | 19.4 ± 0.3 |
| H1–10 (32) | H1–10 | 20.9 ± 0.4 | 17.1 ± 0.3 |
| H9–17 (29) | H9–17 | 21.2 ± 0.5 | 16.5 ± 0.9 |
| **C-sheet proteins** | | | |
| C-sheet (47) | Strands 1–6 | 18.7 ± 0.3 | 19.7 ± 0.6 |
| S1–4 (47) | S1–4 | 18.3 ± 0.1 | 19.4 ± 0.4 |

**FIGURE 13. Potential structures of the \( \alpha \)HD at the triolein/water interface at each transition point.** The helices of \( \alpha \)HD are numbered 1–17. Yellow indicates the hydrophobic triolein. Surface area increases from left to right. Conversely, surface pressure (II) decreases from left to right. The five transition points are labeled, and the surface pressure they occur at is given above the arrow indicating the reversibility of these transitions.
sites, but the C-sheet cannot because of the high concentration of PC. The β-barrel domain and first four helices of the αHD do not have a high lipid affinity, so they are translocated into the lumen of the rough ER without binding lipid. As they are translocated, chaperones and microsomal triglyceride transfer proteins (not shown in the figure) are recruited to the site to facilitate the folding of the more hydrophobic portions of apoB. When the hydrophobic H6 is expressed and translocated, the protein binds the luminal leaflet of the rough ER and perhaps nucleates a TAG-enriched membrane patch (Fig. 14A and supplemental video 1). When the αHD binds the membrane, H1–4 partially unfolds to bind the membrane. As additional helices are translocated (H7–17), they also partially unfold to bind the membrane surface (Fig. 14B). As the αHD binds the surface, TAGs diffuse to the binding site and remain shielded from the water by the protein. This results in the passive accumulation of neutral lipids at the binding site of apoB as it is expressed. When the C-sheet is expressed and translocated, it first displaces H11–17 and then recruits a patch of TAG that binds to its hydrophobic face (Fig. 14C). The architecture of the ER-membrane does not dramatically remodel until the A-sheet has been translocated. Translocation of the A-sheet displaces H1–4 from the surface allowing it to bind to TAG and increase II (Fig. 14D). In this extended membrane-bound conformation, the βα1 domain will have created a high surface pressure in the ER membrane. To alleviate the high surface pressure, the protein remodels the ER membrane to form a TAG-rich “bud” such that the amphipathic β-strands can bind favorably to the TAG-enriched membrane (Fig. 14E), but the overall surface pressure is reduced. The loops of the A-sheet can now interact with the conserved β-barrel domain forming a tertiary structure similar to that of lipovitellin with the lipid bud filling the lipovitellin lipid pocket. Once a bud is formed, the β1 superdomain will be capable of binding to the neutral lipids exposed at the membrane surface. Detachment of the bud as a nascent lipoprotein is required to complete the early stage of TRL assembly.

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