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Hydrophobic Amino Acids in the Hinge Region of the 5A Apolipoprotein Mimetic Peptide are Essential for Promoting Cholesterol Efflux by the ABCA1 Transporter

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ABSTRACT

The bihelical apolipoprotein mimetic peptide 5A effluxes cholesterol from cells and reduces inflammation and atherosclerosis in animal models. We investigated how hydrophobic residues in the hinge region between the two helices are important in the structure and function of this peptide. By simulated annealing analysis and molecular dynamics modeling, two hydrophobic amino acids, F-18 and W-21, in the hinge region were predicted to be relatively surface-exposed and to interact with the aqueous solvent. Using a series of 5A peptide analogs in which F-18 or W-21 was changed to either F, W, A, or E, only peptides with hydrophobic amino acids in these two positions were able to readily bind and solubilize phospholipid vesicles. Compared with active peptides containing F or W, peptides containing E in either of these two positions were more than 10-fold less effective in effluxing cholesterol by the ABCA1 transporter. Intravenous injection of 5A in C57BL/6 mice increased plasma-free cholesterol (5A: 89.9 $\pm$ 13.6 mg/dl; control: 38.7 $\pm$ 4.3 mg/dl (mean $\pm$ S.D.); $P < 0.05$) and triglycerides (5A: 887.0 $\pm$ 172.0 mg/dl; control: 108.9 $\pm$ 9.9 mg/dl; $P < 0.05$), whereas the EE peptide containing E in both positions had no effect. Finally, 5A increased cholesterol efflux approximately 2.5-fold in vivo from radiolabeled macrophages, whereas the EE peptide was inactive. These results provide a rationale for future design of therapeutic apolipoprotein mimetic peptides and provide new insights into the interaction of hydrophobic residues on apolipoproteins with phospholipids in the lipid microdomain created by the ABCA1 transporter during the cholesterol efflux process.

Introduction

Apolipoprotein mimetic peptides are short amphipathic peptides that share many of the same biologic properties as full-length apolipoproteins and are being investigated as possible therapeutic agents for cardiovascular disease (Sethi et al., 2007). One such peptide is the 5A peptide, which is a bihelical amphipathic peptide that contains the 18A sequence in the first helix and a modified 18A sequence containing five Ala substitutions in the second helix (Sethi et al., 2008). Pairing of the high lipid affinity helix, 18A, with the lower affinity lipid helix containing the five Ala substitutions has been shown to improve the specificity for this peptide for promoting cholesterol efflux by the ABCA1 transporter (Remaley et al., 2003; Sethi et al., 2008). When used to treat apoE knockout mice, the 5A peptide was shown to promote cholesterol efflux in vivo, to increase overall reverse cholesterol transport, and to reduce the development of atherosclerosis (Amar et al., 2010). In addition, the 5A has been shown in several animal models to have anti-inflammatory properties (Tabet et al., 2010; Yao et al., 2011).

ApoA-I, the main structural protein in high-density lipoprotein (HDL), is commonly used in the design of apolipoprotein mimetic peptides and contains a tandem array of amphipathic helices (Segrest et al., 1994). The majority of these helices are joined together with a proline, which is thought to facilitate the curvature of apoA-I around the disc-like structure of nascent HDL (Segrest et al., 1994; Li et al., 2012). In the lipid-free state, apoA-I is predicted to form a tight helical bundle, burying most of its hydrophobic residues (Mei and Atkinson, 2011). The last helix of apoA-I, however, which is one of the most hydrophobic and is critical in the ability of apoA-I to efflux cholesterol by the ABCA1 transporter, is predicted to be largely excluded from the helical bundle and to be relatively surface-exposed (Vedhachalam et al., 2007; Mei and Atkinson,

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ABBREVIATIONS: AUC, area under the curve; biotinylated PE, (biotinyl)-1,2-dipalmitoyl phosphatidylethanolamine; CBQCA, 3-(4-carboxybenzoyl)-quinoline-2-carboxaldehyde; DMPC, dimyristoyl phosphatidyl choline; FPLC, fast-protein liquid chromatography; HDL, high-density lipoprotein; PBS, phosphate-buffered saline; POPC, 1-palmitoyl, 2-oleoyl phosphatidylcholine; Rhod-PE, (N-lissamine rhodamine B sulfonyl)-1,2 dioleoyl phosphatidylethanolamine; SA, simulated annealing; SUV, small unilamellar vesicle.

2011). It has been proposed that, in the cholesterol efflux process, this region of apoA-I first makes contact with the lipid microdomains created by the ABCA1 transporter (Kono et al., 2008). Once bound to this lipid microdomian, the helical bundle motif of apoA-I then unfolds, allowing the rest of the amphipathic helices to bind to lipids, which eventually results in the removal of lipids from cells by a detergent-like extraction process (Lund-Katz and Phillips, 2010).

In contrast to single-helical amphipathic apolipoprotien mimetic peptides, bihelical peptides, such as the 5A peptide, have an increased affinity for lipoproteins (Wool et al., 2009). It has been proposed that their longer length may better enable them to bind lipoproteins, particularly HDL (Wool et al., 2009). Like apoA-I, the 5A peptide also has proline between its two amphipathic helices, which has been proposed to allow the two helices to fold back upon each other and minimize the exposure of the hydrophobic face of the helices to the aqueous solvent (Sethi et al., 2008). How this peptide and related apolipoprotein mimetic peptides initially interact with the ABCA1 transporter in the cholesterol efflux process is not fully understood. In this study, we developed a structural model for the lipid-free form of the 5A peptide, which showed that most of the hydrophobic residues were positioned in the interfacial region between the two helices. Two hydrophobic amino acids, namely, F-18 and W-21, which are located in the hinge region of the peptide between the two helices, were predicted to be relatively surface-exposed. The importance of hydrophobic residues in the hinge region for phospholipid binding, cholesterol efflux by the ABCA1 transporter, and modulating lipoprotein levels in mice was determined by testing a series of 5A peptide analogs containing a variety of different types of amino acids in positions 18 and 21 of the 5A peptide.

Materials and Methods

Peptide Synthesis. Peptides were synthesized by a solid-phase procedure, using Fluorenylmethyloxycarbonyl amino acids, on a Biosearch 9600 peptide synthesizer (Milligen, Bedford, MA). Peptides were purified to more than 95% purity by reverse-phase high-pressure liquid chromatography on an Aquapore RP-300 column (Perkin Elmer, Waltham, MA).

Peptide Structure Modeling. The starting structure for the 5A peptide, which was later used for modeling studies, was based on secondary structure analysis. Residues from positions 1–18, containing the 18A helix (DWLKAFYDKVAEKLKEAF), and regions 20–37, containing the modified 18A helix with five Ala substitutions (DWAKAAYDKAAEKAKEAA), were predicted to form α-helices. The linkage region between them, i.e., proline-19, was predicted to reside in an extended conformational turn. Secondary structure elements in Table 1 were defined according to Kabsch and Sander (1983). A simulated annealing (SA) procedure for identifying low-energy conformations of the 5A peptide and its analogs, as well as an analysis of their conformational mobility, was performed after two consecutive stages of annealing. In the first step, a rough geometry optimization for each molecule was subject to SA in a vacuum, which included 50 ps of uniform heating from 0 to 350 K, 50 ps of molecular dynamics at a constant temperature 350 K, and gradual cooling to 0 K within 75 ps. This procedure was applied to the initial starting structures of each peptide a total of five times to select the best-energy conformation, which was then used in further computations. In the second step, a more accurate SA run was performed in the implicit solvent (water), with heating (300 K), molecular dynamics, and cooling time temperature being 200, 1000, and 75 ps, respectively. For each molecule, the final SA step was implemented five times. The Langevin thermostat with collision frequency 10 ps^{-1} was used for temperature coupling in all SA calculations, and a simple leap-frog integrator with 1.0 femto seconds time step was used to propagate the dynamics. The Hawkins-Cramer-Truhlar (Hawkins et al., 1995; Hawkins et al., 1996) pairwise generalized Born model, with parameters described by Tsui and Case (2000–2001), was used to specify the parameters of implicit solvent (water). The finite nonbonded cut-off distance was used only in the second stage of the SA simulations and was set at 12 Å. The SA calculations were performed in the program Sander from the Amber 11 package with the Amber ff10 force field (Duan et al., 2003).

Circular Dichroism (CD) Spectroscopy. Peptides (0.1 mg/ml) in sodium phosphate buffer (pH 7.4) were loaded into a quartz cuvette (d = 0.2-cm path length), and CD spectra from 185 to 240 nm were recorded on a Jasco J715 (Jasco, Easton, MD) spectropolarimeter at 24°C. Data were normalized by calculating the mean residue ellipticity (θ).

Phospholipid Binding Assay. Small unilamellar vesicles (SUV) binding assay was performed, as previously described (Davidson et al., 2006), with the following modifications. Peptides at the indicated concentrations were incubated for 30 minutes at room temperature with SUV vesicles [25–32 nm in diameter, containing 99% (w/w) 1-palmitoyl, 2-oleoyl phosphatidylcholine (POPC), 0.5% (biotinyl)-1,2-dipalmitoyl phosphatidylethanolamine (biotinylated PE), and 0.5% (N-lissamine rhodamine B sulfonyl)-1,2 dioleoyl phosphatidylethanolamine (Rhod-PE);

TABLE 1
The 5A peptide main-chain dihedrals and secondary structure elements
T = hydrogen bonded turn, S = bend for indicated peptide region, along with ϕ angles (dihedral angle for backbone atoms C'-N-C^{α}-C') and ψ angles (dihedral angle for backbone atoms N-C^{α}-C'-N) are shown.

#	Residue	Str[a]	ϕ	ψ
1	D		—	120.1
2	W	H	−61	−36.6
3	L	H	−66.9	−17.4
4	K	H	−97.8	−46.3
5	A	H	−81	−40.8
6	F	T	−116.2	−30.4
7	Y	H	−62.1	−29.4
8	D	H	−50.2	−37.8
9	K	H	−74.8	−18.2
10	V	H	−82.9	−45.5
11	A	H	−62.1	−33.9
12	E	G	−54.7	−30
13	K	G	−68	−13.4
14	L	G	−56.4	−28.1
15	K	G	−62.3	−33.8
16	E	T	−83.7	−43
17	A	S	−79.2	37.4
18	F		−63.9	−49.3
19	P	S	−77.6	152.1
20	D	H	−58.6	−36.9
21	W	H	−67.5	−21
22	A	H	−90.9	−42.9
23	K	H	−59.2	−39.1
24	A	H	−61.3	−38.6
25	A	H	−78.4	−43.3
26	Y	H	−59.6	−47.4
27	D	H	−61.4	−43.7
28	K	H	−61.7	−37.4
29	A	H	−72.1	−47.1
30	A	H	−64.1	−35.9
31	E	H	−59.2	−40.7
32	K	H	−81.8	−37.1
33	A	H	−52.7	−42.6
34	K	T	−68.7	−5.4
35	E	T	−70.5	−28.9
36	A		−88.3	157.1
37	A		−161.8	—

[a] Predicted secondary structure elements [H = α-helix, G = 3-helix (3/10 helix),

0.43 mg of phospholipids per 1 ml of phosphate-buffered saline (PBS)]. SUV-bound peptides were rapidly separated from free peptides in a 96-well microplate filter (MultiScreen filter plates from Millipore Corporation, Billerica, MA) using Pierce streptavidin UltraLink resin (Thermo Scientific, Rockford, IL), which binds to the biotin group on the biotinylated PE in the SUV. SUV-bound peptides and lipids were eluted with 1% sodium cholate and measured in a Victor^3 fluorescence microplate reader (Perkin Elmer, Waltham, MA). Fluorescence of Rhod-PE was used to estimate the amount of phospholipid bound to the SUV, and the amount of peptide bound to the SUV was measured with the 3-(4-carboxybenzoyl)-quinoline-2-carboxaldehyde (CBQCA) fluorescence protein assay (Molecular Probes; Invitrogen, Carlsbad, CA).

Dimyristoyl Phosphatidyl Choline Vesicle Solubilization Assay. Dimyristoyl phosphatidyl choline (DMPC) vesicles (100 ng/ml) were prepared by resuspension of dried DMPC with PBS and vortexing for 5 minutes. Changes in light scattering upon addition of peptides (final concentration of 33 ng/ml) were monitored at 24°C every minute for 1 hour at 660 nm, with shaking (fast setting) in a Victor3 microplate reader (Perkin Elmer, Waltham, MA). The effect of the peptides was compared with a negative control solution containing only PBS and expressed as the percentage of area under the curve (AUC).

Cholesterol Efflux Assay. Cholesterol efflux assay was performed as previously described (Remaley et al., 2003). Briefly, Baby Hamster Kidney (BHK) mock and BHK stably transfected cells expressing a mifepristone inducible human ABCA1 cDNA were labeled for 18 hours with 1 μCi/ml of ^{3}H-cholesterol in alpha modification of Eagle's medium plus 10% fetal calf serum. Cholesterol flux was measured after the addition of media containing the indicated cholesterol acceptor plus 10 nM mifepristone in alpha modification of Eagle's medium, with 0.2% fatty acid–free bovine serum albumin. After 18 hours, media were collected and filtered through a Unifilter 25μm polypropylene filter (Whatman Inc., Florham Park, NJ) and cells were lysed in 0.4 ml of 0.1% SDS and 0.1 M NaOH. Radioactive counts of cholesterol in media and cell fractions were measured by liquid scintillation counting, and results are expressed as the percentage of total counts effluxed into the media per the indicated time interval.

Analysis of Plasma Lipids and Lipoproteins. Lipids were measured enzymatically (Wako Chemicals USA, Inc., Richmond, VA) on a ChemWell 2910 analyzer (Awareness Technology, Inc., Palm City, FL). Distribution of lipids on lipoproteins was analyzed after separation by fast-protein liquid chromatography (FPLC), using two Superose 6 HR 10/30 columns (GE Healthcare Biosciences, Pittsburgh, PA) in tandem (Amar et al., 1998).

Animal Studies. Female C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) were injected retro-orbitally with 10 mg/ml (60 mg/kg) of peptides dissolved in saline or with saline only as a control. After 1 hour, heparinized-plasma samples were collected, pooled, and separated by FPLC and analyzed for lipids. For the in vivo cholesterol efflux study, J774 mouse macrophages were incubated in RPMI 1640 media with 50 μg of acetyl low-density lipoprotein preloaded, with trace amounts of ^{14}C-cholesterol ester for 24 hours, as previously described (Wang et al., 2007). Trypsinized, labeled J744 cells were injected intraperitoneally (i.p.) into mice. After 1 hour, plasma was drawn for the "zero" time point and mice were injected i.p. with 2 mg (60 mg/kg) of lipid-free peptides or saline. Plasma was drawn at 3, 6, 24, 48, and 72 hours and analyzed by liquid scintillation counting. All animal studies were approved by the Animal Care and Use Committee (National Heart, Lung, and Blood Institute protocols H-0050R1 and H-0018R1).

Results

Structural Model of Lipid-Free 5A Peptide. As described in the *Materials and Methods* section, a structural model of lipid-free 5A peptide from simulated annealing analysis is shown in Fig. 1. The two lipid-binding regions (residues 1–18 and 20–37) were predicted based on secondary structural analysis to form two amphipathic α-helices (Table 1). By molecular dynamic modeling, the amphipathic α-helices were relatively rigid (Fig. 1B), most likely because of stabilization by intrahelical hydrophobic interactions and a possible interhelical hydrogen bond (Fig. 1A, red arrow) between valine-10 on the first helix and tyrosine-26 on the second helix. Proline-19 (psi = 152.1) and to a lesser degree alanine-17 (psi = 37.4) were predicted to disrupt the overall α-helical structure of the peptide and to create an extended conformational turn in the hinge region between the two helices (Table 1). The hydrophobic faces of the two helices were predicted to face inward toward each other, thus shielding the majority of the hydrophobic residues. However, two hydrophobic residues, Phe-18 (F-18) and Try-21 (W-21), positioned in the hinge region (Fig. 1B: black arrows), were predicted to be facing outward toward the aqueous environment and to be relatively mobile. Based on this structural model, we hypothesized that the two hydrophobic residues in the hinge region of the 5A peptide (F-18 and W-21) may be critical in the initial interaction of the peptide with lipids similar to what has been proposed for the surface-exposed hydrophobic residues in the last amphipathic helix of apoA-I (Kono et al., 2008). To test this hypothesis, seven different analogs of the 5A peptide were synthesized in which either F-18 or W-21 or both were changed to either Ala (A) or Glu (E) (Table 2). Ala was chosen because it is uncharged but is considerably less hydrophobic than Phe and Trp. Glu was chosen to test the impact of a charge group in the hinge region. Each new peptide is given a two-letter name based on the amino acids in positions 19 and 21. The CD spectra of each peptide was tested, and all of the peptides had a similar α-helical content (Table 2), which indicates that amino acid changes in these two positions in the hinge region did not greatly affect the ability of the rest of the peptide to form α-helices.

Effect of 5A Analog Peptides on DMPC Vesicle Solubilization. In Fig. 2, the ability of the 5A analog peptides to decrease the turbidity of phospholipid vesicles made with DMPC was examined. It has been previously established that this assay measures the ability of amphipathic peptides to both initially bind and then solubilize vesicles into smaller structures that have a reduced ability to scatter light (Tall et al., 1978). Replacing F-18 or W-21 with another hydrophobic amino acid (W and F, respectively) or with the slightly less hydrophobic amino acid A did not significantly affect the ability of these peptides to solubilize DMPC vesicles. In contrast, replacing either hydrophobic residue in the hinge region with E, a negatively charged amino acid, almost completely blocked the ability of the peptide to solubilize DMPC vesicles.

Effect of 5A Analog Peptides on SUV Binding. In Fig. 3, peptide binding to phospholipids was assessed in an SUV binding assay. In contrast to ApoA-1, which could not solubilize SUV made with POPC (Davidson et al., 2006), the 5A (FW) peptide and some of its analogs did so at higher concentrations, so we were only able to test the peptides at relatively low concentrations below saturation. The AUC from the binding plot was used as a metric for assessing the binding of the peptides to the SUV. Overall, the results were similar to the DMPC vesicle solubilization study. The presence of

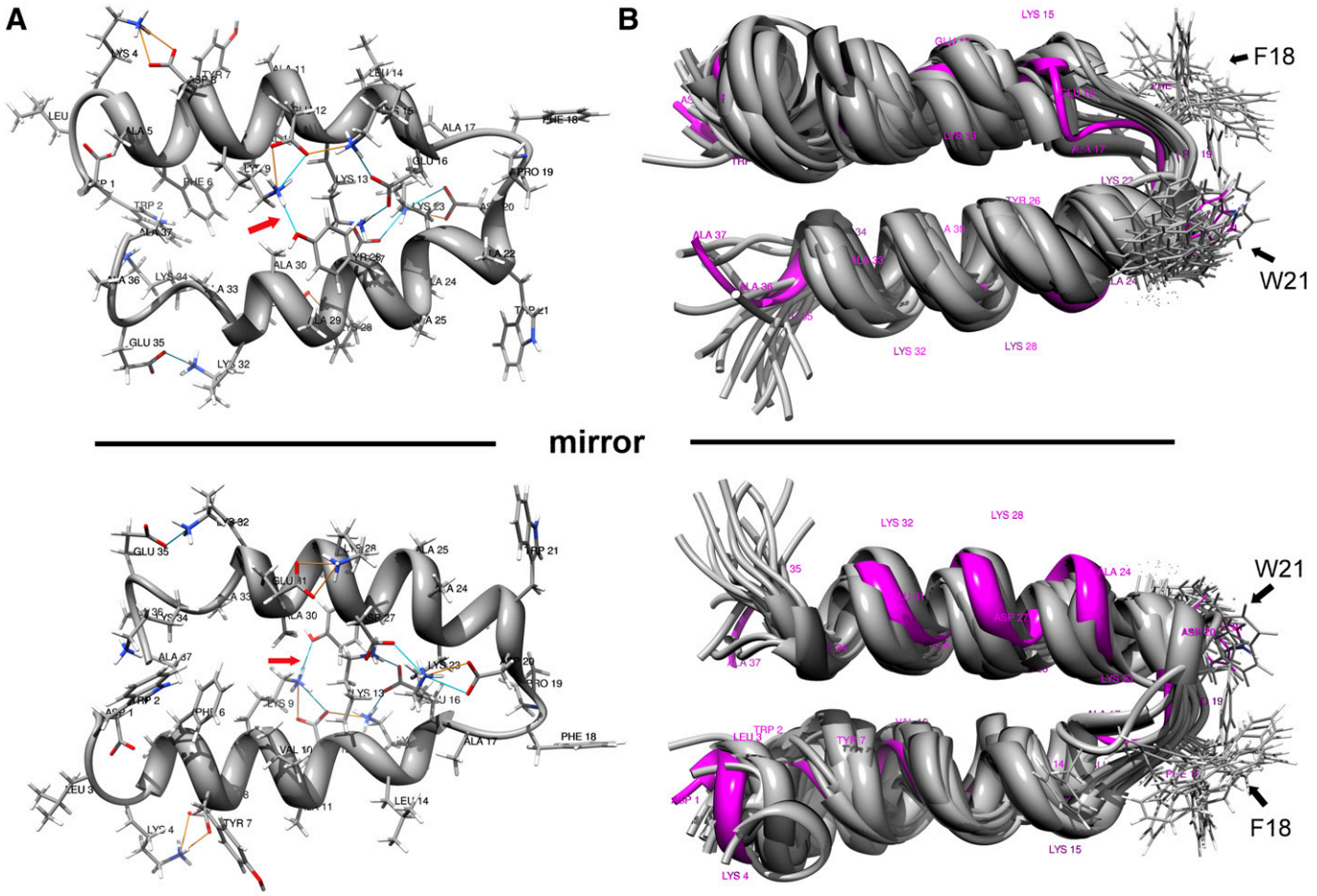

Fig. 1. Structural model of the 5A peptide. (A) Hydrogen bonds and salt bridges. Blue lines represent proper bonds, orange lines represent relaxed bonds. The red arrow indicates position of interhelical hydrogen bond between valine-10 on the first helix and tyrosine-26 on the second helix. (B) Molecular dynamic calculations. Structures of the 15-ns molecular dynamic trajectories of the 5A peptide superimposed on the initial static conformation of the molecule, colored in magenta (average Root-mean-square deviation value is 3.00 Å with a standard deviation of 0.53 Å).

hydrophobic residues in positions 18 and 21 in the hinge region was critical for SUV binding. Replacement of F-18 or W-21 with E disrupted SUV binding, although peptides with a single E substitution were not as affected in their binding to SUVs made with POPC as they were in the DMPC vesicle solubilization study (Fig. 2).

Effect of 5A Analog Peptides on Cholesterol Efflux by the ABCA1 Transporter. Next, the ability of the 5A analog peptides to promote cholesterol efflux by the ABCA1 transporter was tested (Fig. 4). It has been proposed that, like apoA-I, 5A and related amphipathic peptides can be inserted into the lipid microdomain created by ABCA1 and then efflux or remove cholesterol and phospholipids in a detergent-like extraction process similar to how they solubilize DMPC vesicles (Remaley et al., 2003; Sethi et al., 2008). The amount of cholesterol efflux was quantified by calculating the AUC from dose-response curves. The pattern for cholesterol efflux by the 5A analog peptides was similar to the results obtained by the SUV binding study (Fig. 3). The WW and FF peptides, with either W or F in both positions 18 and 21, were slightly better than the original 5A (FW) peptide in cholesterol efflux, as was found in the SUV binding experiment. Peptides containing an

TABLE 2
Peptide sequence of 5A analogs and CD analysis
Bolded and underlined residues differ from 5A sequence.

Name	Position	Sequence 18 21	Molecular Weight	Helicity
				%
FW (5A)		DWLKAFYDKVAEKLKEAF-P-DWAKAAYDKAAEKAKEAA	4217.80	22.0
FF		DWLKAFYDKVAEKLKEAF-P-D**F**AKAAYDKAAEKAKEAA	4178.77	16.1
WW		DWLKAFYDKVAEKLKEA**W**-P-DWAKAAYDKAAEKAKEAA	4256.83	19.6
FA		DWLKAFYDKVAEKLKEAF-P-D**A**AKAAYDKAAEKAKEAA	4102.67	16.8
AW		DWLKAFYDKVAEKLKEA**A**-P-DWAKAAYDKAAEKAKEAA	4141.70	16.7
EW		DWLKAFYDKVAEKLKEA**E**-P-DWAKAAYDKAAEKAKEAA	4199.74	18.3
FE		DWLKAFYDKVAEKLKEAF-P-D**E**AKAAYDKAAEKAKEAA	4160.71	17.1
EE		DWLKAFYDKVAEKLKEA**E**-P-D**E**AKAAYDKAAEKAKEAA	4142.65	17.0

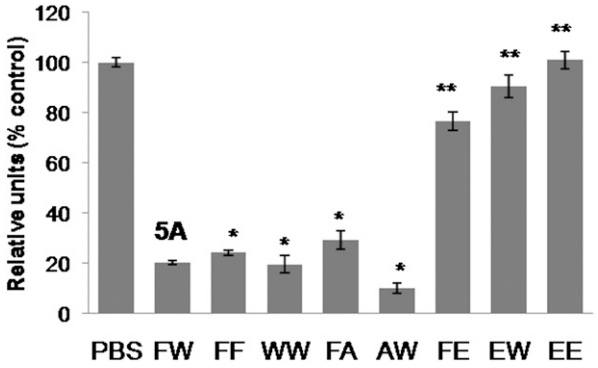

Fig. 2. Effect of peptides on DMPC vesicle solubilization. The 5A (FW) peptide analogs (33 ng/ml) dissolved in PBS or just PBS were mixed with 100 ng/ml DMPC (1,2-dimyristoyl-sn-glycero-3-phosphocholine) and incubated for 1 hour. Turbidity was monitored by measuring the absorption at 660 nm every minute. Areas under the curve were calculated and the results were presented as percent of PBS. Results are expressed as the mean ± SD ($N = 3$). *No significant difference ($P > 0.05$) and **significant difference ($P < 0.05$) compared with 5A peptide.

A residue in either hinge position showed intermediate ability to efflux cholesterol, whereas peptides containing E at either position were almost completely inactive in cholesterol efflux. A relatively high correlation coefficient (R^2) of approximately 0.93 was observed between the ability of the 5A peptide analogs to bind SUV and to efflux cholesterol by the ABCA1 transporter (Fig. 4C). None of the peptides were able to promote significant amounts of cholesterol efflux from control BHK cells not expressing the ABCA1 transporter (data not shown).

Effect of 5A Analog Peptides on Lipoproteins in Mice. Apolipoprotein mimetic peptides, including 5A, are known to have an acute effect in altering plasma levels of lipoproteins, although the 5A peptide was only previously tested in mice after first complexation with phospholipids (Amar et al., 2010). Because of the inability of the EE peptide to bind to phospholipids (Fig. 4), we compared the free EE and 5A peptide without any complexed lipids in their ability to alter lipid and lipoprotein levels in mice. A structural model comparing the EE peptide with 5A (FW) is shown in Fig. 5. Based on this model, the two E substitutions in the hinge

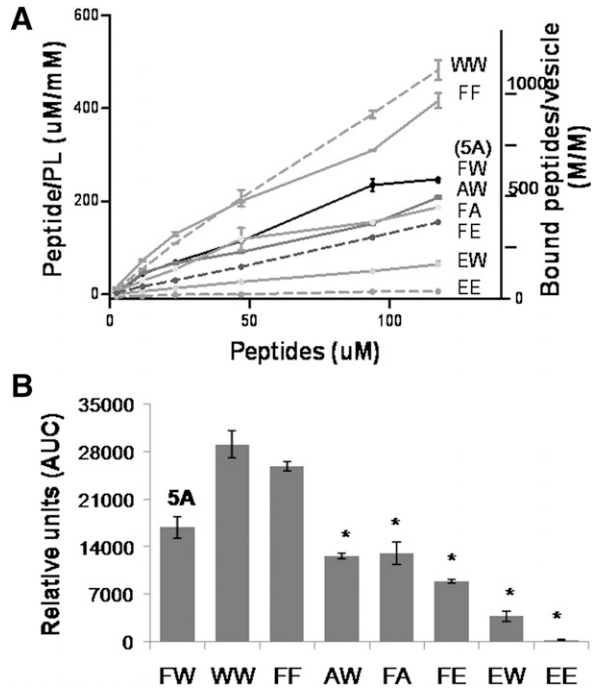

Fig. 3. Effect of peptide sequence alteration on SUV binding. (A) Peptides at the indicated concentrations were incubated for 30 minutes at room temperature with SUV vesicles made with POPC and the amount of bound peptides was measured. The right axis shows the number of bound molecules of peptide per vesicle, assuming that each vesicle contains approximately 2300 phospholipid molecules. (B) Peptide binding was quantified from AUC calculation from dose-response curves. Results are expressed as the mean ± SD ($N = 3$). *Significant difference ($P < 0.05$) compared with 5A peptide.

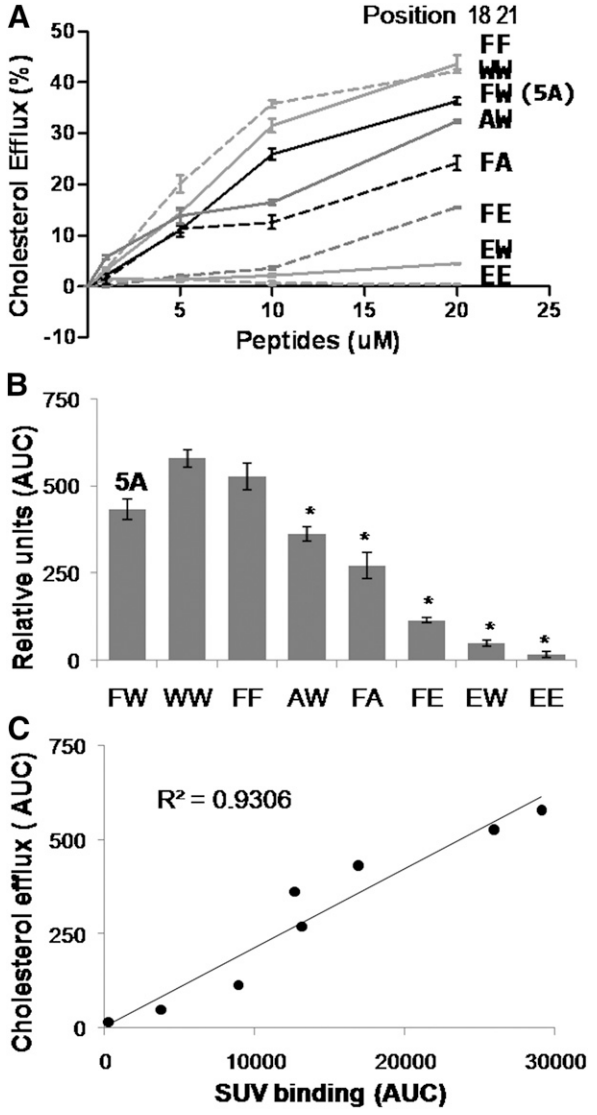

Fig. 4. Effect of peptides on cholesterol efflux. (A) BHK stably transfected cells with human ABCA1 were labeled for 18 hours with 1 μCi/ml of ^{3}H-cholesterol. The peptides were added to the media, and after 18 hours, media were collected and filtered. Radioactive counts in media and cell fractions were measured and results are expressed as the percentage of total counts. (B) AUC calculation from dose-response curves for cholesterol efflux. (C) Correlation between the ability of peptides to bind SUV made with POPC and to efflux cholesterol by ABCA1. Results are expressed as the mean ± SD ($N = 3$). *Significant difference ($P < 0.05$) compared with the FW (5A) peptide.

region of the EE peptide were predicted to result in a more open conformation in the hinge region compared with 5A, causing the two helices to be less closely apposed to each other.

The free 5A (FW) and EE peptides (60 mg/kg) were intravenously injected into mice, and plasma lipoprotein changes in mice were monitored after 1 hour. Treatment with free 5A peptide did not significantly change total cholesterol levels but did increase the percentage of free cholesterol approximately 2-fold ($P < 0.05$), whereas the EE peptide showed no difference compared with the control saline-treated mice. As has been previously described for other amphipathic peptides (Carballo-Jane et al., 2010), a relatively large increase was observed in serum triglycerides after treatment with 5A, but the EE peptide showed no such effect and was similar to the control mice. When plasma from mice was analyzed by FPLC analysis, the free 5A peptide was found to increase cholesterol on low-density lipoprotein and very low-density lipoprotein fractions and to increase triglycerides on very low-density lipoprotein (Fig. 6). No changes in cholesterol or triglyceride distribution were observed after treatment with the EE peptide. In Fig. 7, we compared the ability of the lipid-free EE or 5A peptide in promoting cholesterol flux in vivo. J744 macrophages radiolabeled with ^{14}C-cholesteryl ester were injected into the peritoneum of mice, and the efflux of cholesterol from the cells and its entry into the plasma compartment was monitored after i.p. injection of either saline, EE, or 5A peptides (60 mg/kg). Compared with the saline control, only 5A (not the EE peptide) was able to increase the mobilization of cholesterol from the radiolabeled macrophages (Fig. 7), but 5A did not show any increase in hepatic content of the radiotracer or in fecal cholesterol excretion compared with the EE peptide (data not shown).

Discussion

Apolipoprotein mimetic peptides are currently being investigated as alternatives to full-length apoA-I for acute HDL therapy, which has been proposed as a way to rapidly stabilize patients with acute coronary syndrome and reduce the likelihood of developing a future cardiovascular event (Sethi et al., 2007; Remaley et al., 2008). To date, one apolipoprotein mimetic peptide, D4F, and its stereoisomer, L4F, have been tested in early-stage clinical trials (Bloedon et al., 2008; Watson et al., 2011). The 5A apolipoprotein mimetic peptide, like several other related peptides, has been designed to promote cholesterol efflux by the ABCA1 transporter (Sethi et al., 2008), which is thought to be a key antiatherogenic property of apoA-I (Sethi et al., 2007; Bielicki et al., 2010). The presence of an amphipathic helix is known to be necessary for these peptides to promote cholesterol efflux (Remaley et al., 2003), but the necessity of other structural motifs for the cholesterol efflux process is not well known. The main finding from this study is that, in case of bihelical amphipathic peptides like 5A, the hydrophobicity of residues in the hinge region appears to be critical in their ability to efflux cholesterol by the ABCA1 transporter.

Computer modeling of the structure of 5A predicted that two hydrophobic amino acids, namely, F-18 and W-21, in the hinge region are relatively surface-exposed and readily interact with the aqueous solvent (Fig. 1). Based on the Wimley and White hydrophobicity ranking system (Wimley and White, 1996), F and W are predicted to be two of the most hydrophobic amino acids and to have the highest lipid affinity, whereas E is very polar and has one of the lowest affinities for interacting with lipid membranes. These results are consistent with site-directed mutagenesis studies involving substitutions of these residues in proteins that bind to phospholipids (Wimley and White, 1996). Because the exposure of F-18 and W-21 in the 5A peptide to the aqueous solvent would not be thermodynamically favored, we hypothesized that these two residues may be important in the initial interaction of the peptide with lipids. A similar model has been proposed for how the last helix of apoA-I, which has one of the highest hydrophobic moments of all the helices of apoA-I but is the most surface-exposed, is also involved in the initial interaction of apoA-I with phospholipid membranes (Kono et al., 2008; Lund-Katz and Phillips, 2010; Rothblat and Phillips, 2010).

Fig. 5. Comparison of structures for EE and 5A peptides. Superimposition of best-energy structure of the EE peptide (cyan) onto the 5A peptide (magenta) is shown.

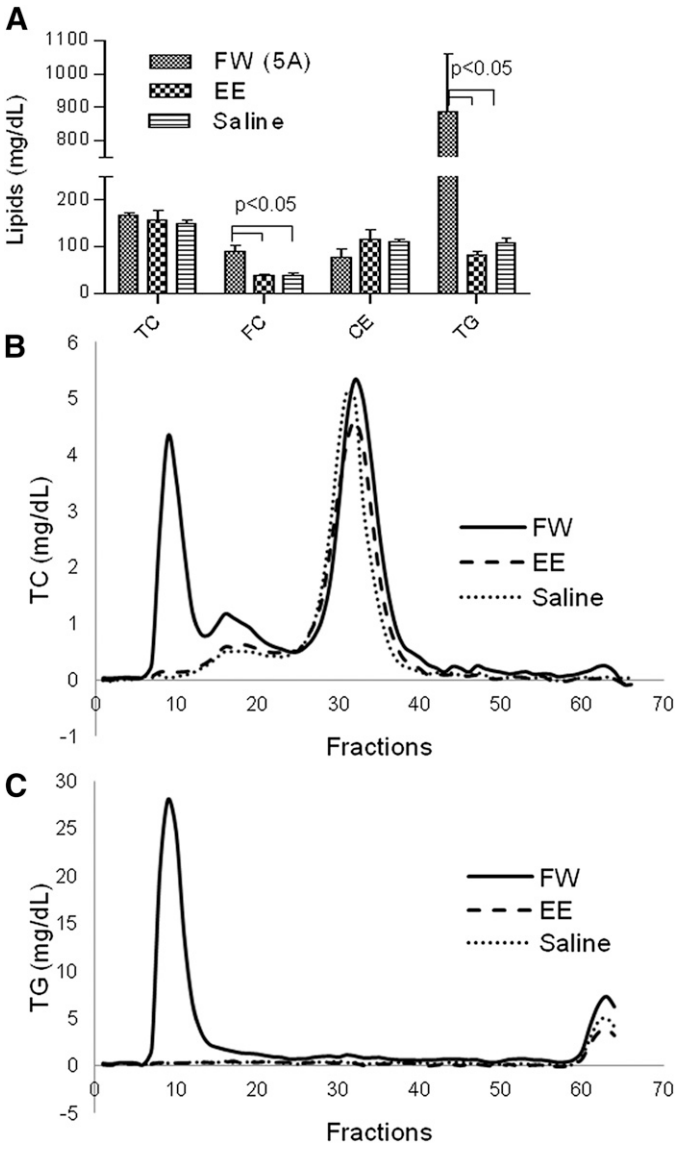

Fig. 6. Effect of peptides on plasma lipids and lipoproteins. (A) Total cholesterol (TC), free cholesterol (FC), cholesteryl esters (CE), and triglycerides (TG) were measured from plasma from C57BL/6 mice treated with either 5A (FW), EE, or saline. Peptides (60 mg/kg) or saline was injected retro-orbitally in mice ($N = 3$) and plasma was analyzed after 1 hour. Total cholesterol (B) or triglycerides (C) were measured from major lipoprotein fractions after separation of plasma by FPLC analysis. Results are expressed as the mean ± SD ($N = 3$) compared with saline control ($P < 0.05$).

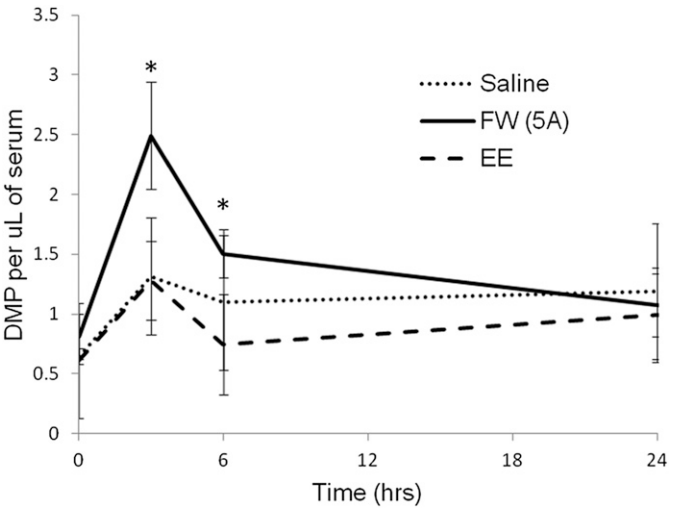

Fig. 7. Effect of peptides on in vivo cholesterol efflux. C57BL/6 mice were injected i.p. with J744 macrophages radiolabeled with cholesteryl esters and then treated with either 5A or EE peptides (60 mg/kg) or saline. Radioactive counts in plasma were monitored at the indicated time points. Results are expressed as the mean ± SD ($N = 3$) compared with saline control ($P < 0.05$).

Based on studies involving a series of 5A analogs in which F-18 and W-21 were changed to either F, W, A, or E (Table 2), we provide support for this model on how the 5A peptide interacts with lipids via hydrophobic residues in the hinge region. First, we show that hydrophobic amino acids in positions 18 and 21 are necessary for the solubilization of phospholipid vesicles made with DMPC (Fig. 2). Using SUV made with POPC, we then demonstrate that only peptides with hydrophobic amino acids in these two positions show high affinity for binding to phospholipids (Fig. 3). When the same 5A peptide analogs were tested for effluxing cholesterol from cells expressing the ABCA1 transporter, we observed a striking correlation between the ability of the peptides to bind to SUV and to efflux cholesterol by the ABCA1 transporter (Fig. 4). Peptides with polar residues in these two positions in the hinge region were unable to efficiently bind to SUV and to efflux cholesterol by the ABCA1 transporter. Peptides containing the most hydrophobic residues in the hinge region, W or F, were the best for these two processes. Because of their relatively large size, F and W residues in the hinge region may potentially change the angle between the two helices, which could also affect the function of the peptide. The predicted angle between the two helices, however, for peptides containing alanine in either position in the hinge region (AW or FA peptide) was unchanged compared with the FW peptide (data not shown).

The results from these studies on the 5A peptide analogs are consistent with our current understanding of how the ABCA1 transporter promotes cholesterol efflux (Oram and Heinecke, 2005; Lund-Katz and Phillips, 2010). The exact phospholipid substrate or ligand for ABCA1 is not known, but it has been proposed to promote the flipping of phospholipids from the inner to outer membrane leaflet (Williamson et al., 2007). The presence of additional phospholipids in the outer membrane layer from ABCA1 results in a buckling or an evagination of the plasma membrane (Vaughan and Oram, 2003), which then creates phospholipid packing defects. It is the presence of these packing defects in phospholipids that is believed to then promote the initial insertion of hydrophobic regions of apolipoproteins into the lipid microdomain created by ABCA1. Lipid-free apoA-I, once bound to the lipid microdomain created by ABCA1, then unfolds, allowing the rest of the amphipathic helices to bind to the lipid, which ultimately leads to the release of phospholipids and cholesterol from the cell membrane and the formation of discoidal HDL (Lund-Katz and Phillips, 2010; Rothblat and Phillips, 2010). Presumably, the 5A peptide promotes cholesterol efflux in a similar way, although with a different stoichiometry (Sethi et al., 2008). The complex formed between 5A and phospholipids has a greater molar ratio of peptide to phospholipid compared with discoidal HDL made with apoA-I, although a similar ratio when the amount of the peptide or protein is

expressed in terms of mass (Sethi et al., 2008). Hydrophobic residues in the hinge region of the active 5A peptide analogs are likely necessary for the cholesterol efflux process, because they promote the initial interaction of the peptide with the phospholipid packing defects in the lipid microdomain created by the ABCA1 transporter. Although the inactive 5A analogs containing the polar residue E in positions 18 and 21 can still form amphipathic helices to a similar degree as 5A and the other active peptides (Table 2), they were unable to bind to phospholipid vesicles or efflux cholesterol by the ABCA1 transporter.

Results from the in vivo treatment of mice with the lipid-free 5A and EE peptides are consistent with the in vitro lipid binding and cholesterol efflux data. The EE peptide appeared to be relatively inert in its ability to modulate plasma lipids and lipoproteins, unlike 5A, which has already been shown to alter plasma lipids in mice but was previously only tested as a complex with phospholipids (Amar et al., 2010). Unlike the EE peptide, lipid-free 5A increased cholesterol, particularly on apoB-containing lipoproteins (Fig. 6). It also caused a marked increase in triglycerides, which, like other apolipoprotein mimetic peptides, may be due to its ability to inhibit lipoprotein lipase at high doses (Carballo-Jane et al., 2010). Previously, when complexed with phospholipids, the 5A peptide was shown to primarily increase cholesterol only on HDL (Amar et al., 2010), which suggests that the association of phospholipids with apolipoprotein mimetic peptides can alter their biologic properties. Finally, the lipid-free 5A peptide, similar to what has been reported when complexed with phospholipids (Amar et al., 2010), was able to increase cholesterol efflux in vivo from macrophages (Fig. 7), whereas the EE peptide was inactive, as it was for the in vitro cholesterol efflux assay (Fig. 4). Unlike when the 5A was reconstituted with the phospholipid (Amar et al., 2010), the free 5A peptide did not increase fecal cholesterol excretion, suggesting that the cholesterol mobilized by the free peptide was recycled and redistributed among the peripheral tissues.

In summary, using a series of 5A analog peptides, we show the importance of hydrophobic amino acids in the hinge region of bihelical amphipathic peptides for lipid binding and cholesterol efflux by the ABCA1 transporter. Results from this study will aid in the rationale design of future therapeutic amphipathic peptides and provide new insights into the interaction of amphipathic peptides with the ABCA1 transporter in the cholesterol efflux process.

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Authorship Contributions

Participated in research design: Sviridov, Andrianov, Remaley, Turner.

Conducted experiments: Sviridov, Andrianov, Anishchenko, Stonik, Amar.

Performed data analysis: Sviridov, Andrianov, Anishchenko, Stonik.

Wrote or contributed to the writing of the manuscript: Remaley, Sviridov.

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