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Inhibition of Virus-Induced Cell Fusion by Apolipoprotein A-I and Its Amphipathic Peptide Analogs

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Abstract Apolipoprotein A-I (apoA-I), the major protein component of serum high-density lipoproteins (HDL), was found to inhibit herpes simplex virus (HSV)-induced cell fusion at physiological (~ 1 μM) concentrations, whereas HDL did not exert any inhibitory effect. Lipid-associating, synthetic amphipathic peptides corresponding to residues 1–33 (apoA-I[1–33]) or residues 66–120 (apoA-I[66–120]) of apoA-I, also inhibited HSV-induced cell fusion, whereas a peptide corresponding to residues 8–33 of apoA-I (apoA-I[8–33]), which fails to associate with lipids, did not exert any inhibitory effect. These results suggest that lipid binding may be a prerequisite for peptide-mediated fusion inhibition. Consistent with this idea, a series of lipid-binding 22-amino-acid-residue-long synthetic amphipathic peptides that correspond to the amphipathic helical domains of apoA-I (A-I consensus series), or 18-residue-long model amphipathic peptides (18A series), were found to exert variable levels of fusion-inhibitory activity. The extent of fusion-inhibitory activity did not correlate with hydrophobic moment, hydrophobicity of the nonpolar face, helix-forming ability, or lipid affinity of the different peptides. Peptides in which the nonpolar face was not interrupted by a charged residue displayed greater fusion-inhibitory activity. Also, the presence of positively charged residues at the polar-nonpolar interface was found to correlate with higher fusion-inhibitory activity.

Key words: herpes simplex virus, high-density lipoproteins, amphipathic helixes, fusion-inhibitory peptides

Entry of enveloped virus into susceptible cells involves fusion between viral and cell membranes. Also, many viruses, including herpes simplex virus (HSV), induce cell fusion as a major cytopathic effect. Variants of HSV known as syncytial (Syn) mutants display a cytopathology characterized by extensive cell fusion [1]. The process of virus-induced cell fusion is thought to be analogous to the processes involved in the fusion of viral membranes with the cell membranes. In model membrane systems, certain agents that destabilize membrane bilayers have been shown to cause membrane fusion [2], whereas agents that stabilize the membrane bilayer have been shown to inhibit membrane fusion [3,4]. Our laboratory has been investigating structure-function relationships among the lipid-associating amphipathic helices found in apolipoproteins [5–13]. A number of amphipathic peptide analogs of apolipoprotein A-I (apoA-I) have been synthesized and characterized. Many of these peptides significantly perturb the physical and chemical properties of these bilayers. In a previous study we observed that apoA-I and its synthetic amphipathic peptide analogs inhibit HSV penetration, cell to cell spread, and virus-induced cell fusion [14]. In an attempt to determine the structural features of the amphipathic peptide motif that influence its fusion-inhibitory activity, we have investigated the fusion-inhibitory activity of a series of peptide analogs.

MATERIALS AND METHODS

Cells and Viruses

Vero cells (clone 76; American Type Culture Collection, Baltimore, MD) were grown and maintained in Dulbecco's modified minimal essential medium (DMEM) supplemented with 8% fetal calf serum. HSV-1 (MP), a syncytium-inducing variant of HSV, was obtained from Dr.

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S. Chatterjee. The viruses were propagated on Vero 76 cells.

Cell Fusion and Fusion-Inhibition Assay

Vero cells were grown as monolayers in sterile 96-well tissue culture plates. Confluent monolayers were infected with HSV-1 (MP) at a multiplicity of 10 pfu/cell. The virus was allowed to adsorb for 2 h at 37°C. The inoculum was removed and replaced with DMEM containing serial twofold dilutions of the peptides high-density lipoprotein (HDL) or apoA-I. The cells were incubated for 18 h at 37°C. The monolayers were stained with Wright-Giemsa stain and examined microscopically for areas of cell fusion. Under these experimental conditions, nearly the entire Vero cell monolayer was found to fuse into a large syncytium in virus-infected cultures. Uninfected Vero cell cultures contained predominantly mononuclear cells, and a few cells with two or three nuclei were occasionally seen. Accordingly, multinucleate cells containing five or more nuclei were regarded as areas representing cell fusion. The peptides inhibited virus-induced cell fusion in a dose-dependent manner. The lowest concentration of the agent that completely inhibited virus-induced cell fusion was determined and expressed as its minimum inhibitory concentration (ID_{100}). In certain experiments, the percentage of the monolayer involved in cell fusion was determined by morphometry. The entire monolayer was photographed under low magnification, and the area of monolayer involved in cell fusion was calculated from the photomicrographs using a Bio-Quant digitizer.

HDL and apoA-I

HDL was prepared from plasma samples from normal human volunteers using previously described techniques [8]. Apolipoproteins were isolated from HDL particles and purified by high-performance liquid chromatography (HPLC) [8]. The HDL and apoA-I preparations were dialyzed extensively against DMEM prior to use in fusion inhibition assays.

Synthesis, Purification, and Characterization of Peptides

All the peptides were synthesized by a solid-phase procedure using an automated peptide synthesizer (Advanced Chemtech Inc., Louisville, KY) [5]. Benzyl-based protecting groups were used for side-chain protection during peptide synthesis. The peptides were cleaved from the resin by hydrogen fluoride (HF). The crude peptides were first analyzed by HPLC under various solvent systems to identify optimum separation conditions. These conditions were translated to preparative systems, and the peptides were purified by preparative HPLC.

The composition of the purified peptides was verified by amino acid analysis. Most (A-I consensus and 18A series) of the peptides used in this study have been characterized before [5–13]. The characterization of peptides corresponding to native apoA-I sequences will be published elsewhere. The peptides were dissolved in distilled water by elevating the pH and neutralized after solubilization, and the required amounts were mixed with an equal volume of 2× DMEM prior to fusion-inhibition assays.

Helical Wheel Plots, Hydrophobic Moment, and Mean Hydrophobicity per Residue of the Amphipathic Peptides

The helical wheels [15] were plotted using a program developed for VAXstation 2000 that orients the helix with its nonpolar face directed toward the top of the wheel. Helical hydrophobic moments were calculated according to Eisenberg et al. [16,17]. The values for the hydrophobic moment are expressed as mean hydrophobic moment per residue. The values for the hydrophobicity of the amphipathic peptides are expressed as the mean hydrophobicity per residue on the nonpolar face and were calculated by averaging the hydrophobicity of the residues that map to nonpolar face of the amphipathic helix.

Lipid Affinity of Amphipathic Peptides

We have previously shown that apoA-I and its synthetic peptide analogs bind to dimyristoylphosphatidyl choline (DMPC) to form discoidal complexes, and the lipid affinities of the amphipathic peptides correlate inversely with the mean diameter of peptide DMPC complexes [5]. The mean diameters of the discoidal complexes were fairly uniform at lower DMPC: peptide weight ratios (1:1 to 2.5:1), whereas a considerable heterogeneity was observed in the discoidal diameters at higher DMPC/peptide weight ratios. Accordingly, we prepared DMPC-peptide complexes at a weight ratio of 2:1 by previously described procedures [5]. Briefly,

DMPC (Avanti Polar Lipids, Birmingham, AL) was evaporated under nitrogen gas and resuspended in phosphate-buffered saline (PBS; pH 7.4) at a concentration of 100 μg/ml. The peptides were suspended in PBS at a concentration of 50 μg/ml. Equal volumes of peptide and DMPC were mixed together and incubated for 1 h at room temperature. The peptide-DMPC complexes were placed on formvar-coated copper grids and stained with phosphotungstic acid (pH 7.0). The grids were examined under a Philips EM 301 electron microscope. The diameters of the discoidal peptide-DMPC complexes were determined by morphometry of the electron micrographs; the diameters of at least 100 individual discs were determined using a graduated magnifying lens, and the data were used to calculate the mean discoidal diameter.

Circular Dichroism

The α-helix content of the amphipathic peptides in buffer and in the presence of lipids was determined by circular dichroism (CD). The CD spectra were obtained using a Jasco J-500A spectropolarimeter connected to a DPN500 data processor unit. The instrument was calibrated with *d*-10-camphorsulphonic acid. The peptides were dissolved in 10 mM phosphate buffer (pH 7.4) containing 150 mM NaCl to a concentration of 50 μg/ml, and the CD spectra were obtained using a 10 mm sample cell. To obtain CD spectra of the peptides in the presence of lipids, peptide-DMPC complexes were prepared at a weight ratio of 1:1 according to the procedures described above. The complexes contained a final peptide concentration of 50 μg/ml. About eight to 16 scans were averaged and corrected for the baseline. The corrected data sets were used to estimate the α-helical contents according to the calculations described earlier [18]. The α-helical contents for some of the peptides used in this study have been reported before (18A and 18R [5], [Glu^{1,8} Leu^{11,17}]18A and [Glu^{4,9} Leu ^{11,17}]18R [11], [Met^{3}]18A [8]).

RESULTS

Inhibition of HSV-Induced Cell Fusion by HDL, ApoA-I, and Synthetic Peptide Fragments of ApoA-I

HSV-infected Vero monolayers displayed extensive cell fusion, which involved the entire monolayer by 18 h postinfection (Fig. 1, panel 2). ApoA-I did not induce cell fusion or exert any toxic effects on uninfected Vero cells (Fig. 1, panel 3). HSV-induced cell fusion was completely inhibited in cells treated with 1 μM apoA-I (Fig. 1, panel 4). In contrast to apoA-I, no inhibitory effect was observed with HDL at concentrations as high as 1 mg protein/ml (Fig. 1, panel 6). These results suggest that the inhibitory effects of apoA-I may be mediated by its lipid-associating domains and the lack of fusion inhibition by HDL particles may be due to the fact that these domains are already lipid-bound in HDL particles.

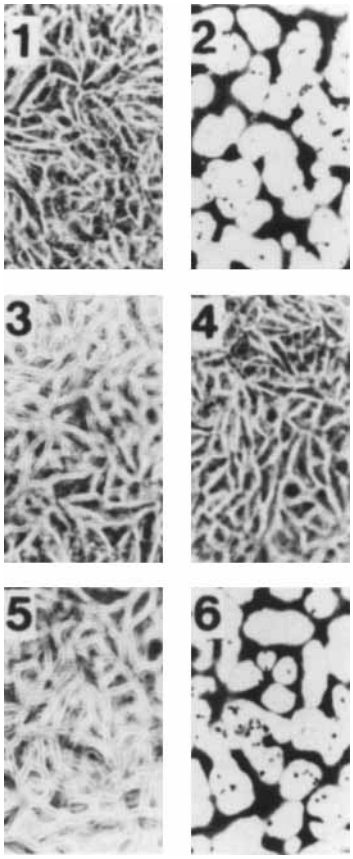


Fig. 1. Inhibition of HSV-induced cell fusion by apoA-I and HDL. Uninfected (**panels 1, 3, 5**) or HSV-infected (**panels 2, 4, 6**) Vero cells were incubated in the presence of DMEM (panels 1, 2), DMEM containing 1 μM apoA-I (panels 3, 4), or DMEM containing 1 mg/ml HDL (panels 5, 6). After 12 h, the monolayers were stained with Wright-Giemsa stain and examined microscopically for areas of cell fusion. The results show that apoA-I inhibits HSV-induced cell fusion (panel 4), whereas HDL does not (panel 6).

The amino terminus (residues 1–33) of apoA-I is highly conserved among different apolipoprotein species and was initially thought to be the functional domain of apoA-I [6]. The amino acid sequence of residues 1–33 reveals proline residues at position 3, 4, and 7 (see Fig. 3A). The

presence of a proline residue at positions other than N1 may exert a strong helix-breaking effect [19]. We therefore synthesized two peptides, one corresponding to residues 1–33 and the other corresponding to residues 8–33. The C-terminus (residues 44–198) of apoA-I has a propensity to form multiple amphipathic helical 22mer segments (see Fig. 3A). This structural feature of apoA-I is thought to be responsible for its lipid associating properties [13]. Based on studies with synthetic peptides, residues 66–120 have been implicated as the major LCAT (lecithin: cholesterol acetyltransferase)-activating domain of apoA-I [9]. Therefore, we also tested a peptide corresponding to residues 66–120 of apoA-I. Physicochemical characterization of these peptides will be published elsewhere.

The lipid-binding properties of the synthetic peptide fragments of apoA-I are shown in Figure 2 and Table I. We have previously shown that lipid affinity of the amphipathic peptides correlates inversely with the mean diameter of peptide DMPC complexes [6]. A-I [66–120] formed small complexes with DMPC, whereas, A-I [1–33] formed very large DMPC discoidal complexes with a mean diameter of 218 Å. Among the series of peptides that we have tested, A-I [1–33] has the lowest lipid affinity (see Tables I and III). A-I [8–33] did not bind to lipids and failed to form discoidal complexes with DMPC. The reasons for the lack of lipid binding by A-I [8–33] are presently not clear. Analysis of the peptide sequence arranged in a helical wheel representation indicated that the peptide can assume an amphipathic helical configuration. Furthermore, calculated values for the mean hydrophobic moment per residue (0.506) and the mean hydrophobicity per residue on the nonpolar face (0.473) are consistent with values expected for a lipid-binding amphipathic α-helix (Table I). However, algorithms to predict protein secondary structure (e.g., Chou-Fasman or Robeson-Garnier) failed to identify helix-forming domains in region 8–33 of apoA-I. The CD spectra of A-I [8–33] peptide did not reveal any α-helical content either in buffer or in the presence of lipids (data not shown). A more detailed characterization of these peptides is currently in progress.

The fusion-inhibitory properties of the apoA-I peptides are summarized in Table I. The lipid-binding peptides A-I [1–33] and A-I [66–120] inhibited HSV-induced cell fusion at concentrations of 500 and 2 μM, respectively. In contrast, A-I [8–33], which failed to bind lipids, did not inhibit virus-induced cell fusion (Table I). These results suggest that lipid binding may be a prerequisite for fusion-inhibitory activity of apoA-I and its peptide analogs. The 243-residue-long apoA-I and A-I [66–120] are fairly long sequences and contain internal proline residues that may break the helix into shorter α-helical

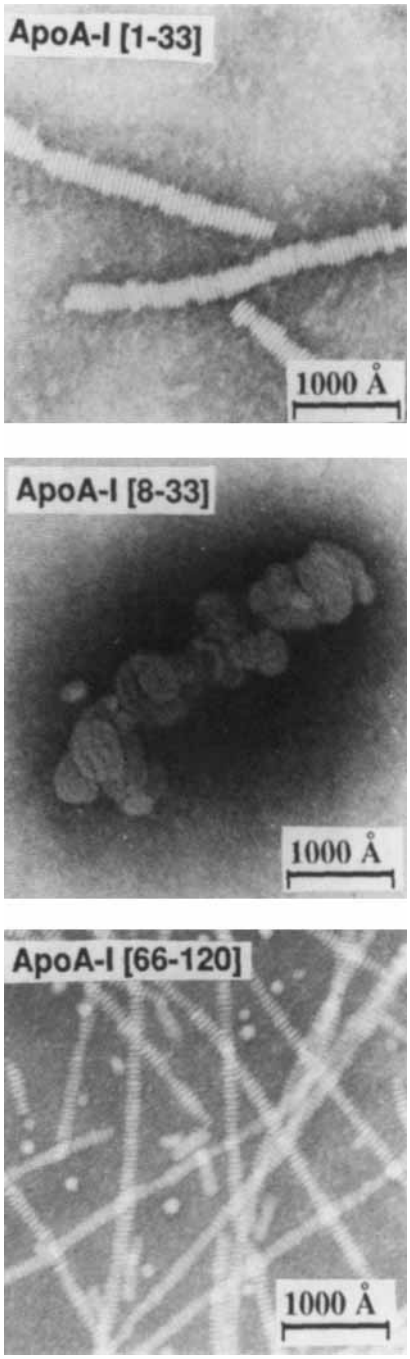


Fig. 2. Electron microscopy of peptide-DMPC (1:2 wt/wt) complexes from A-I[1–33], A-I[8–33], and A-I[66–120] ×135,000.

TABLE I. Inhibition of HSV-Induced Cell Fusion by Synthetic Peptide Fragments of ApoA-I

Peptide	Fusion inhibition (ID_{100})	Hydrophobic moment per residue	Mean hydrophobicity per residue	Mean diameters of peptide-DMPC complexes[a] (Å)
A-I[1–33]	500	0.412	0.517	218
A-I[8–33]	>500	0.506	0.473	—
A-I[66–120]	2	0.382	0.146	117

[a]Values calculated from data shown in Figure 2.

segments. In an attempt to obtain more effective inhibitors of virus-induced cell fusion and to identify features of the amphipathic helix that influence fusion-inhibitory activity, we tested a series of peptide analogs that had been synthesized before as a part of our ongoing studies to understand molecular properties of apoA-I.

Design and Synthesis of Peptide Analogs

The C-terminal domain of apoA-I, derived from the fourth exon, is composed of eight 22-amino-acid-long repeats punctuated either by proline residues or by 11mer repeats (Fig. 3A) [20,21]. Each of these 22mers is capable of forming an amphipathic α-helix. These repeat sequences are thought to have evolved by duplication of a primordial nucleotide sequence. The amino acid sequence deduced from a consensus nucleotide sequence did not form an amphipathic helix when represented as a helical wheel [9]. We therefore attempted to derive a consensus amino acid sequence from these eight structurally similar 22-amino-acid regions of apoA-I by choosing the most prevalent type of (hydrophobic, positively or negatively charged) residue at each position. As can be seen in Figure 3A, considerable polymorphism was observed at positions 11, 12, 13, 16, 19, and 22. Compared with the other residues in this sequence, polymorphism at residue 13 is rather unusual; when the consensus sequence is organized as a helical wheel, this residue is located on the nonpolar face (Fig. 3C). However, six of the eight tandem repeats have a charged residue at this position, and a seventh repeat has a polar residue at this position. We therefore synthesized a series of four peptides, designated as the A-I consensus series, in which residue 13 was chosen to represent a negative charged glutamic acid ([Glu^{13}]A-I_{con}), a positively charged arginine ([Arg^{13}]A-I_{con}),

TABLE II. Amino Acid Composition of Amphipathic Peptides

Amino acid	[Glu^{13}] A-I_{con}	[Ala^{13}] A-I_{con}	[Arg^{13}] A-I_{Con}	18A	18hA	[Met^{3}] 18A	[$Glu^{1,8}$ $Leu^{11,17}$] 18A	18R	[$Glu^{4,9}$ $Leu^{11,17}$] 18R
Ala	1 (1.3)[a]	2 (2.5)	1 (1.1)	3 (3.0)	3 (3.7)	3 (2.6)	1 (1.0)	3 (3.0)	1 (1.0)
Asp/Asn	2 (2.3)	2 (1.7)	2 (2.3)	2 (1.8)	2 (2.2)	2 (1.9)	—	2 (1.7)	—
Glu/Gln	6 (7.2)	5 (5.8)	5 (5.4)	2 (2.1)	2 (2.5)	2 (2.2)	4 (4.2)	2 (1.9)	4 (3.6)
Phe	1 (1.1)	1 (0.7)	1 (0.9)	2 (2.2)	2 (2.0)	2 (2.0)	2 (2.0)	2 (2.1)	2 (2.0)
Lys	4 (3.9)	4 (3.4)	4 (3.4)	4 (3.7)	4 (3.7)	4 (4.1)	4 (3.7)	4 (3.9)	4 (3.6)
Leu	5 (5.8)	5 (5.4)	5 (4.9)	2 (2.2)	—	1 (1.1)	4 (3.8)	2 (2.0)	4 (3.6)
Met	—	—	—	—	—	1 (0.8)	—	—	—
Pro	1 (1.3)	1 (1.3)	1 (0.9)	—	—	—	—	—	—
Arg	1 (1.2)	1 (1.2)	2 (2.1)	—	—	—	—	—	—
Val	1 (1.2)	1 (0.8)	1 (0.9)	1 (1.0)	1 (1.0)	1 (1.1)	1 (1.0)	1 (1.0)	1 (1.0)
Trp	—	—	—	1[b]	—	1[b]	1[b]	1[b]	1[b]
Tyr	—	—	—	1 (0.9)	—	1 (1.0)	1 (1.0)	1 (0.8)	1 (1.0)
β-Naphthyl-alanine	—	—	—	—	4 (4.0)	—	—	—	—
Total	22	22	22	18	18	18	18	18	18

[a]The figures show the expected numbers of each amino acid residue. The observed numbers of residues per mole peptide are shown in parenthesis.
[b]Tryptophan residues are destroyed during acid hydrolysis.

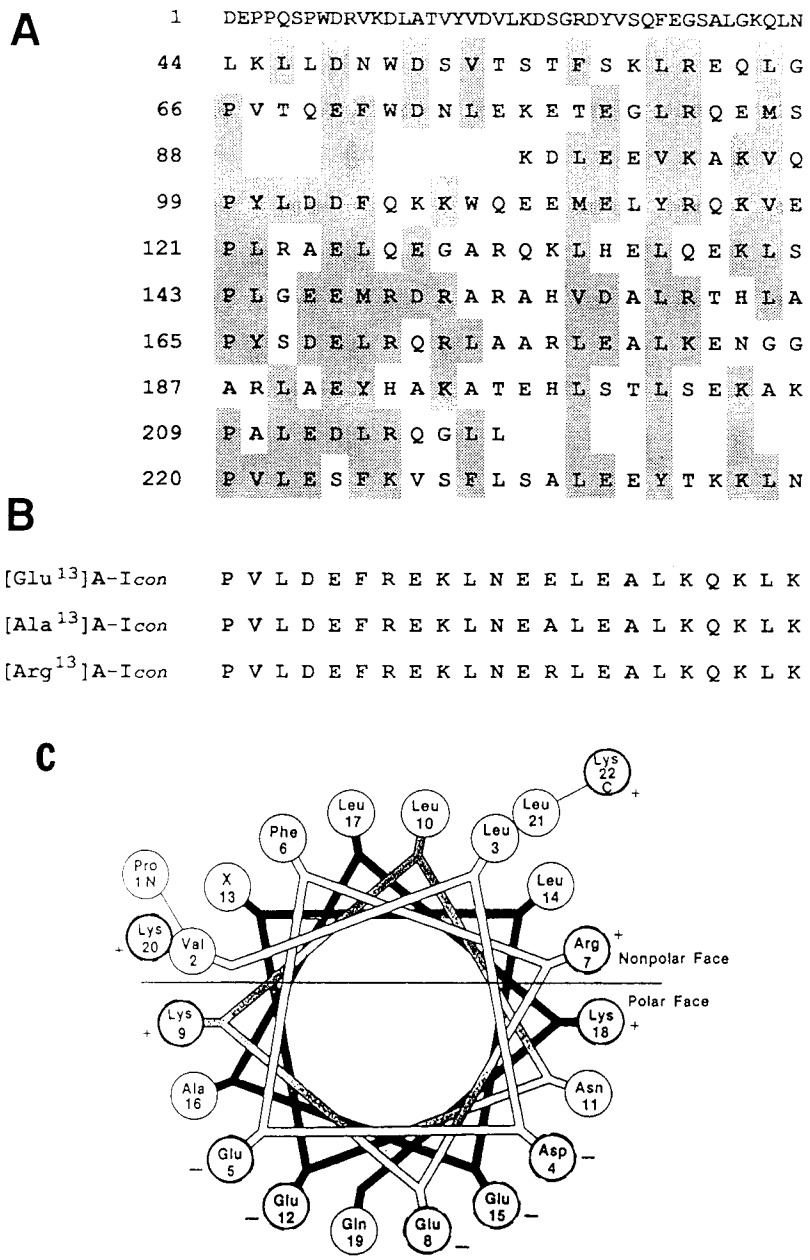
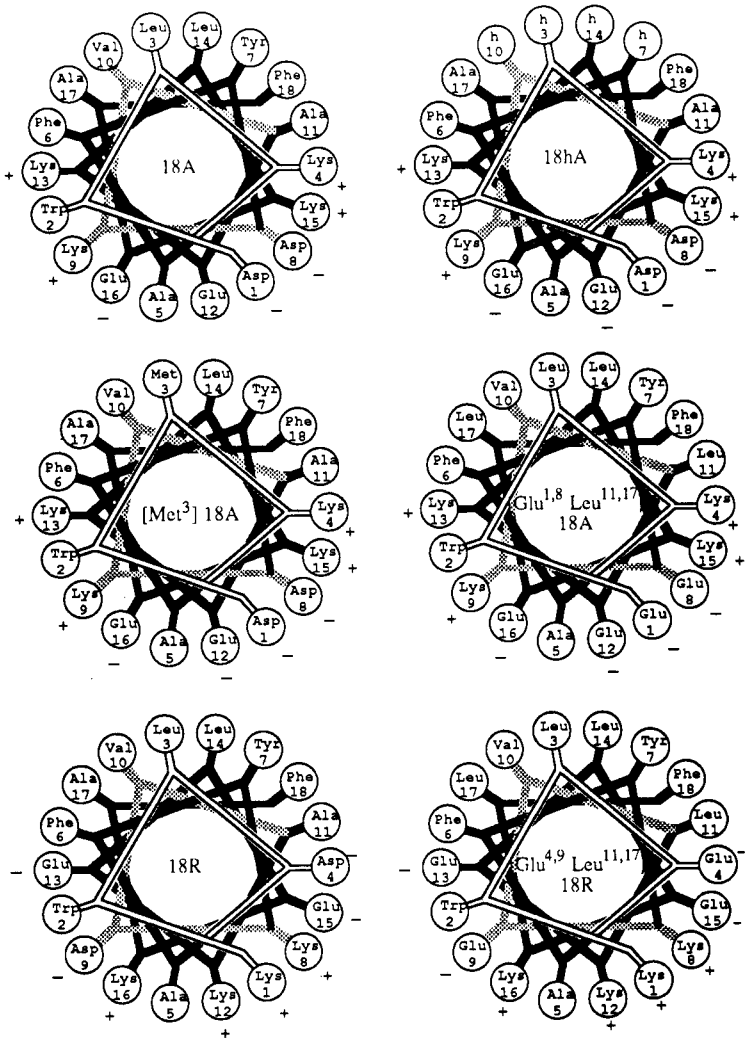


Fig. 3. Design of A-I consensus peptides. **A** shows the amino acid sequence of apolipoprotein A-1. The C-terminal 198 residues (residues 44–122) derived from exon 4 have been arranged to show the tandem amphipathic helical 22mer repeats. The most prevalent types of residue (hydrophobic, positively charged, or negatively charged) at each position are shown by the shaded box. **B** shows the sequences of the A-I consensus peptides: [Glu^{13}]A-I_{con}, [Ala^{13}]A-I_{con}, and [Arg^{13}]A-I_{con}. These peptides share an identical sequence except for the residue at position 13. **C** shows a helical wheel representation of the consensus peptide, in which residue 13 is denoted by an X.

or a nonpolar alanine ([Ala^{13}]A-I_{con}) residue. The amino acid sequences of these peptides are shown in Figure 3B.

We also synthesized a model amphipathic peptide, which retains the charge distribution profile of the apoA-I consensus amphipathic helices but does not possess any charged residues at the nonpolar face. In view of the fact that 3.6 amino acid residues are involved in one complete turn in the α-helix, an 18-residue-long peptide was synthesized to form five complete turns and was designated as 18A (Fig. 4). The positively charged



18A	DWLKAFYDKVAEKLKEAF
18hA	DW**h**KAF**h**DK**h**AEK**h**KEAF
[Met^3] 18A	DW**M**KAFYDKVAEKLKEAF
[Glu^{1,8}Leu^{11,17}] 18A	**E**WLKAFY**E**KV**L**EKLKE**L**F
18R	**K**WL**D**AFY**KD**VA**KE**L**EK**AF
[Glu^{4,9}Leu^{11,17}] 18R	**K**WL**E**AFY**KE**V**LKE**L**EKL**F

Fig. 4. Design of 18A series amphipathic peptides. The amino acid sequences of different 18A series amphipathic peptides and their helical wheel representations are shown. The residues that differ from the prototype peptide 18A are highlighted in bold letters. The letter code h has been used to denote β-napthylalanine residues in peptide 18hA.

clusters at the polar-nonpolar interface in apoA-I amphipathic helical domains were thought to provide additional favorable free energy of lipid association via the contribution of the significantly hydrophobic portion of the lysine side chain to the overall hydrophobicity of the amphipathic helix [22]. A peptide designated 18R was therefore synthesized by reversing the position of the charged residues such that resulting helix would now contain negative charges at the polar-nonpolar interface of the helix [5] (Fig. 4). The peptide analogs 18hA (in which leucine residues in 18A were replaced by β-napthylalanine) and [Met^3]18A (in which the third residue

from the N-terminus of 18A is replaced with a methionine residue) were synthesized to increase the hydrophobicity of the nonpolar face of the 18A helix. We also synthesized additional peptide analogs expected to show a greater helix-forming propensity, based on helix-probability calculations [23], and these included: [Glu^{1,8}, Leu^{11,17}]18A and [Glu^{4,9}, Leu^{11,17}] 18R [11,24].

All the peptides were synthesized by solid-phase synthesis method, cleaved from the resin, and purified by HPLC. The analytical HPLC profiles of the purified peptides are shown in Figure 5. The compositions of the peptides were determined by amino acid analysis of acid hydrolysates prepared from these peptides, and the results are shown in Table II. Based on these analyses, all the peptides were found to be > 90% pure.

Amphipathicity of Synthetic Peptides

The calculated values for mean hydrophobic moment per residue and the mean hydrophobicity per residue on the nonpolar face of the helix for the peptides are summarized in Table III. All the peptides exhibited high hydrophobic moments, with values ranging from 0.454 to 0.577. In general, the apoA-I consensus peptides displayed a slightly lower hydrophobic moment than the model 18A series peptides. The peptides varied considerably in the hydrophobicity

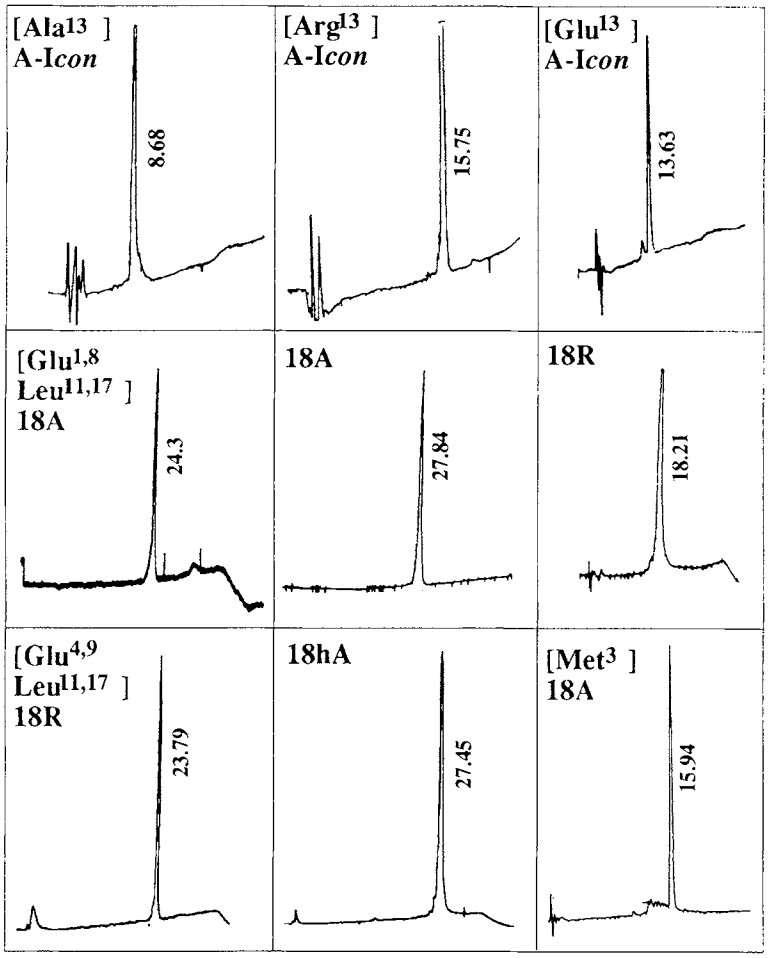


Fig. 5. Analytical HPLC profiles of amphipathic peptides; 50–100 μg of peptides was injected on HPLC columns, and the eluates were monitored by absorbance at 220 nm. [Ala^{13}]A-I$_{con}$, [Arg^{13}]A-I$_{con}$, and [Glu^{13}]A-I$_{con}$ were injected on a C-18 Vydac (25 × 0.45 cm) column and eluted with a gradient of acetonitrile containing 0.1% trifluoracetic acid (TFA) (30–60%) in 30 min at a flow rate of 1.2 ml/min. [Met^{3}]18A was injected on a C-18 Vydac (25 × 0.45 cm) column and eluted with a gradient of acetonitrile containing 0.1% TFA (25–58%) in 30 min at a flow rate of 1.2 ml/min. 18A, 18R, 18hA, [Glu^{1,8} Leu^{11,17}]18A, and [Glu^{4,9} Leu^{11,17}]18R were injected on a C-4 Vydac (25 × 0.45 cm) column and eluted with a gradient of acetonitrile containing 0.1% TFA (5–50%) in 30 min at a flow rate of 1.2 ml/min.

TABLE III. Inhibition of HSV-Induced Cell Fusion by Synthetic Amphipathic Peptide Analogs of ApoA-I

Peptide	Fusion inhibition (ID_{100})	Hydrophobic moment per residue	Mean hydrophobicity per residue	Mean diameters[a] of peptide-DMPC complexes (Å)	α-Helix[b]	
					Buffer	Lipids
[Glu^{13}]A-I_{con}	>500	0.485	0.355	202Å [9]	0.32	0.35
[Arg^{13}]A-I_{con}	500	0.454	0.262	93Å [9]	0.24	0.35
[Ala^{13}]A-I_{con}	250	0.563	0.577	88Å [9]	0.22	0.41
18A	4	0.568	0.742	90Å (Fig. 6)	0.15	0.30 [11]
18hA	4	?[c]	?[c]	95Å (Fig. 6)	nd	nd
[Met^{3}]18A	4	0.575	0.758	164Å (Fig. 6)	0.13	0.25 [8]
[$Glu^{1,8}$ $Leu^{11,17}$]18A	12	0.560	0.773	115Å (Fig. 6)	0.22	0.55 [11]
18R	100	0.572	0.742	182Å (Fig. 6)	0.00	0.01 [11]
[$Glu^{4,9}$ $Leu^{11,17}$]18R	50	0.577	0.773	147Å (Fig. 6)	0.43	0.42 [11]

[a]The mean discoidal diameters of the peptide-DMPC complexes were determined by morphometry of the electron micrographs. The values were obtained from a previous study, where indicated.
[b]The α-helical contents of the peptides were determined by circular dichroism. The values were obtained from a previous study, where indicated. nd, not determined.
[c]Exact values are not available because a specific value has not been assigned to β-napthyl alanine in the consensus GES scale.

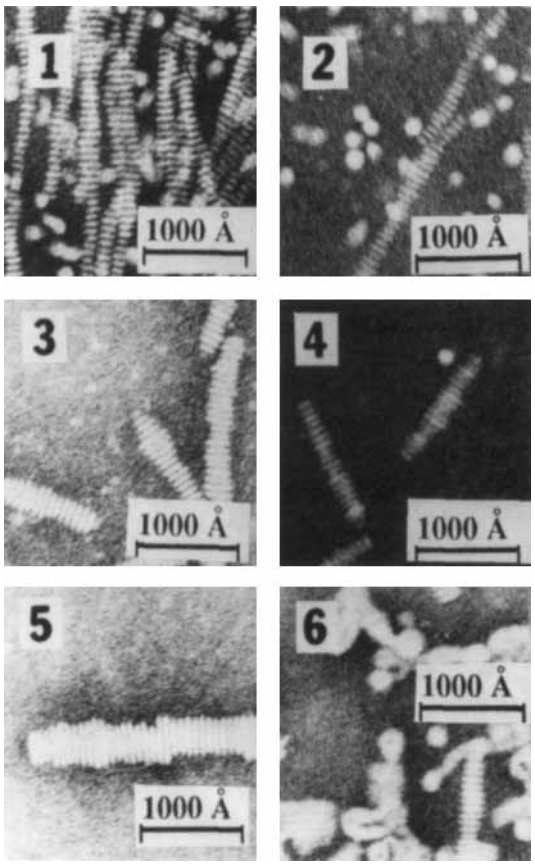


Fig. 6. Electron microscopy of peptide-DMPC (1:2 wt/wt) complexes from 18A series peptides: **1**; 18A, **2**; 18hA, **3**; [Met^{3}]18A, **4**; [$Glu^{1,8}$ $Leu^{11,17}$]18A, **5**; 18R, **6**; [$Glu^{4,9}$ $Leu^{11,17}$]18R. ×135,000.

of their nonpolar face. The A-I consensus peptides displayed lower mean hydrophobicity per residue on the nonpolar face, and particularly [Glu^{13}]A-I_{con} and [Arg^{13}]A-I_{con} which contain a charged residue in the nonpolar face, displayed very low hydrophobicity.

Lipid Affinity

The lipid affinities of the amphipathic peptides were investigated by electron microscopy of peptide-DMPC complexes. The results are summarized in Figure 6 and Table III. The peptides bound to DMPC with varying affinity, as evidenced by the differences in the mean diameters of the peptide-DMPC complexes [5]. Based on these data, the rank order of lipid affinity of these peptides can be summarized as [Ala^{13}]A-I_{con} > 18A > [Arg^{13}]A-I_{con} > 18A > [$Glu^{1,8}$ $Leu^{11,17}$]18A > [$Glu^{4,9}$ $Leu^{11,17}$]18R > [Met^{3}]18A > 18R > [Glu^{13}]A-I_{con}.

Circular Dichroism

The α-helical contents of the amphipathic peptides in buffer or in the presence of lipids were determined by CD and the results are summarized in Table III. All the peptides studied were found to adapt an α-helical conformation, although their α-helical content varied considerably. Consistent with the idea that α-helical conformations of the peptides are stabilized by lipid binding, [Ala^{13}]A-I_{con} displayed a large increase in the α-helical content in presence of lipids, whereas no such increase in helicity was

observed with the peptides [Glu^{13}]A-I_{con} or [Arg^{13}]A-I_{con}. The helicity of unbound peptides probably indicates aggregation, and the aggregated peptides were still capable of lipid binding as evidenced by their ability to bind DMPC (Table III), as has been demonstrated for several apolipoproteins, including apoA-I. The model amphipathic peptide 18A and its analogs displayed varying degrees of helicity. The helicity of [Met^3]18A in buffer and in the presence of lipids was comparable to that of 18A. The peptide [$Glu^{1,8}$ $Leu^{11,17}$]18A, designed to obtain a peptide with high helix probability, displayed a high α-helical content in the presence of lipids. In contrast to 18A peptides, the 18R peptide displayed a very low α-helical content. 18R has a Lys^+ residue at position 1, whereas 18A has an Asp^- residue at this position. It is possible that the Asp^{1-} residue in 18A is stabilized by interaction with the partial positive charge created near the N-terminus by the helix dipole, whereas the presence of a Lys^+ residue at this position in 18R may result in helix destabilization. However, [$Glu^{4,9}$ $Leu^{11,17}$]18R also has a Lys^+ residue at position 1 but displays a high helical content, both in buffer and in presence of lipids. Thus, the low helical content of 18R cannot be attributed entirely to the helix dipole effects. The helical content of [$Glu^{4,9}$ $Leu^{11,17}$]18R did not increase significantly upon lipid binding, suggesting that this peptide may undergo aggregation.

The CD studies presented here are by no means complete. In view of the fact that we have not purified the DMPC-peptide complexes, the CD spectra analyzed in this study represent an average of bound and unbound peptides. Nevertheless, an increase in the α-helical content of the peptides upon association with lipid has been interpreted by several investigators to be indicative of modifications in peptide secondary structure by lipid binding. For several peptides, this interpretation has been supported by shifts in the intrinisic tryptophan fluorescence [11]. A detailed investigation of the secondary structure analyses of the different peptides is currently in progress.

Inhibition of HSV-Induced Cell Fusion by Peptide Analogs

The inhibitory activity of amphipathic peptides on HSV-induced cell fusion in Vero cell monolayers was investigated, and the results are shown in Table III. The results are expressed as the minimum concentration (in μM) of the peptide required to show complete inhibition (ID_{100}) of virus-induced cell fusion. All the peptides, with the exception of [Glu^{13}]A-I_{con}, inhibited virus-induced cell fusion. The fusion-inhibitory activity of the peptides varied considerably, and the inhibitory concentrations ranged from 4 to 500 μM for different peptides. In general, the 22-residue-long A-I consensus peptides were less active than the model 18A series peptides. One common feature of these peptides compared with 18A peptides is that they all show lower hydrophobic moment and contain a less hydrophobic nonpolar face. Of these, [Arg^{13}]A-I_{con} and [Glu^{13}]A-$I_{con,}$ which contain a charged residue in the nonpolar face of the helix, were least active as inhibitors of virus-induced cell fusion (Table III). It is possible that the charged residues are incompatible with the hydrophobic core of the lipid bilayer and affect lipid binding properties of these peptides. Consistent with this idea, [Glu^{13}]A-I_{con} displayed a weak lipid affinity. However, [Arg^{13}]A-I_{con} displayed high lipid affinity despite the presence of a charged residue on its nonpolar face. It is possible that the long alkyl side chain of the arginine residue contributes to the overall hydrophobicity of the helix and facilitates lipid association. The peptides [Ala^{13}]A-$I_{con,}$ which had an uninterrupted nonpolar face, displayed high lipid affinity, although it was not an effective inhibitor of virus-induced cell fusion (ID_{100} = 250 μM). Compared with 18A series peptides, the A-I consensus peptides displayed a higher helical content in buffer, which did not increase appreciably in the presence of lipids. These data suggest that these peptides have a higher propensity for self-association and aggregation. The increase in the relative strength of peptide-peptide interaction may mitigate the relative strength of interaction of peptides with the complex lipid bilayers of the viral and cell membranes, although this feature was not evident in their interaction with a simple lipid (DMPC) vesicles.

The model amphipathic peptide 18A was one of the most active peptides (ID_{100} = 4 μM) in fusion inhibition assays; 18hA and [Met^3]18A, analogs of 18A with a more hydrophobic nonpolar face, were equally effective (ID_{100} = 4 μM) in fusion inhibition assays. These results suggest that the hydrophobicity of the nonpolar face of the amphipathic helix, per se, does not influence the fusion-inhibitory activity of the amphipathic peptides. [$Glu^{1,8}$ $Leu^{11,17}$]18A, an analog of 18A designed to obtain a peptide with increased heli-

cal content, was slightly less active (ID_{100} = 12 μM) than 18A. 18R and [$Glu^{4,9}$ $Leu^{11,17}$]18R, analogs of 18A, in which the positively charged residues at the polar-nonpolar interface were replaced with negatively charged residues, were significantly less active (ID_{100} = 100 μM and 50 μM, respectively) than 18A, suggesting that the presence of a positively charged residue at the helix interface strongly influences its fusion-inhibitory activity.

Reversal of Peptide-Induced Fusion Inhibition

To study the nature of the association of the amphipathic peptides with cell (or viral) membranes, we tested the effect of removal of peptides on the fusion process. Monolayers of Vero cell cultures grown in 96-well tissue culture plates were infected with HSV-1 (MP) at a multiplicity of 10 pfu/cell. The virus was allowed to adsorb for 2 h at 37°C. After adsorption, the inoculum was replaced with 100 μl of DMEM containing serial twofold dilutions of the amphipathic peptide 18A or peptide-free medium. Extensive cell fusion was observed by 10 h postinfection, which involved nearly 80% of the monolayer in cultures not treated with the peptide, whereas cell fusion was completely inhibited in cells treated with 5 μM 18A (Fig. 7). At this time point, peptide-containing media were removed from the culture, washed, and incubated with peptide-free medium. Areas of cell fusion were readily seen by 30 min to 2 h in these cultures (Fig. 7). A rapid reversal of peptide-induced fusion inhibition upon removal of peptide-containing medium indicates that the peptides are loosely associated with cell (or viral) membranes. It is possible that free and membrane-bound peptides exist in an equilibrium in peptide-treated cultures. Replacement of peptide-containing medium with peptide-free medium would then favor the release of membrane-bound peptides, leading to a reversal in peptide-mediated fusion inhibition. These results are consistent with the idea that the amphipathic peptides do not traverse the lipid bilayer but lie perpendicular to the plane of the phospholipid bilayer, with their hydrophobic face buried in the membranes. It is possible that the amphipathic peptides associate with a macromolecular component (other than the phospholipid bilayer) of the virus or the cells. In such an event, this interaction would also appear to be a rapid and reversible association.

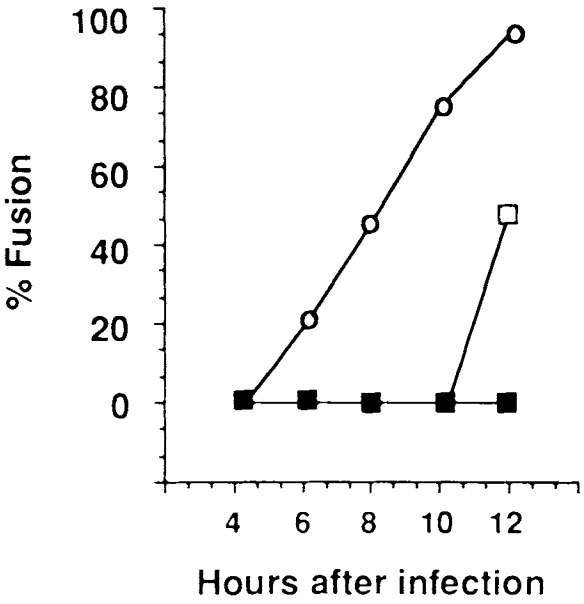


Fig. 7. Reversal of peptide-mediated fusion inhibition. Confluent monolayers of Vero cells were infected with HSV-1 and maintained in DMEM with (■) or without (○) 5 μM 18A. In one set of cultures (□), the peptide containing medium was removed at 10 h after infection, washed, and incubated further with peptide-free DMEM. At different time points, the monolayers were stained with Wright-Giemsa and examined microscopically for areas of cell fusion. Percent fusion was calculated from the photomicrographs using a Bio-Quant digitizer.

DISCUSSION

We previously observed that apoA-I and its synthetic amphipathic peptide analogs inhibit HSV penetration, cell to cell spread and virus-induced cell fusion [25]. In this study, we tested a total of 12 synthetic amphipathic peptide analogs with different physicochemical characteristics for their ability to inhibit HSV-induced cell fusion in an attempt to identify features of the amphipathic helix motif that influence its fusion-inhibitory activity.

The mean hydrophobic moment per residue of the peptides varied from 0.382 to 0.577. The mean hydrophobic moment per residue did not correlate with their inhibitory concentrations. With the exception of A-I[66–120], peptides with a mean hydrophobic moment per residue lower than 0.525 (A-I[1–33], A-I[8–33], [Arg^{13}]A-I_{con}, and [Glu^{13}]A-I_{con}) were weak inhibitors of virus-induced cell fusion ($ID_{100} \geq$ 500 μM). A-I[66–120] was an effective inhibitor of HSV-induced cell fusion despite the fact that it had the lowest hydrophobic moment. One possible explanation for this finding is that this peptide is 55 residues long and contains regions of greater hydropho-

bic moment. We therefore suggest that a mean hydrophobic moment per residue > 0.525 may be a prerequisite for fusion inhibition by short amphipathic helical peptides. Among the peptides tested, we did not observe any correlation between the mean hydrophobicity per residue on the nonpolar face of the amphipathic helix and their fusion-inhibitory concentrations. The presence of charged residues on the nonpolar face of the helix was found to affect the fusion-inhibitory activity of the peptides. Among the 22-residue-long A-I consensus peptides that vary in sequence at a single residue, peptides containing a charged residue in the nonpolar face were found to be poor inhibitors of cell fusion. Thus an uninterrupted nonpolar face appears to facilitate fusion inhibition by amphipathic helical peptides.

The nature of the charged residue at the polar-nonpolar interface was found to exert a profound influence on the fusion-inhibitory activity of the amphipathic helical peptides. Peptides containing positively charged lysine residue at the polar-nonpolar interface (e.g., 18A, 18hA, [Met^{3}]18A, and [Glu^{1,8} Leu^{11,17}]18A) were found to be more effective inhibitors of cell fusion than peptides containing a negatively charged glutamic residue at the interface (e.g., 18R and [Glu^{4,9} Leu^{11,17}]18R). The reasons for these findings are presently not clear. It is possible that the long alkyl side chains of arginine and lysine residues contribute to the overall hydrophobicity of the amphipathic helix and provide additional favorable free energy of lipid association, allowing the helix to bury deeper in the phospholipid bilayer [22]. Alternatively, the positive charges at the helix interface may facilitate interaction with the negative charges on the phospholipid bilayers, whereas negatively charged residues will hinder such interactions by charge repulsion.

A lack of fusion inhibition by the A-I[8–33] peptide, which fails to fold into α-helical structures in the presence of lipids (data not shown), suggests that the ability to adapt an α-helical conformation in the presence of lipids is a prerequisite for fusion inhibition by the amphipathic peptides. However, we could not detect a quantitative correlation between the α-helical content of the peptides and their fusion-inhibitory activities. The fusion-inhibitory activities of modified peptides (e.g., [Glu^{1,8} Leu^{11,17}]18A and [Glu^{4,9} Leu^{11,17}]18R) with an increased α-helical content were not significantly different from the fusion-inhibitory activities of their unmodified counterparts (18A and 18R). Many peptides with a high α-helical content in the presence of lipids also displayed a high α-helical content in buffer, presumably due to aggregation [11,24]. The aggregated peptides, in general, displayed a lower fusion-inhibitory activity.

Two lines of evidence suggest that the amphipathic helices exert their fusion inhibitory effect, at least in part, by their lipid binding properties: 1) A-I [8–33], which did not bind to lipids, failed to inhibit cell fusion and 2) free apoA-I effectively inhibited cell fusion, whereas HDL, which contains lipid-associated apoA-I, failed to inhibit cell fusion. [Glu^{13}]A-I$_{con}$ bound to lipids but did not inhibit cell fusion. This result suggests that lipid binding is necessary, but not sufficient, for fusion inhibition by the amphipathic helical peptides. There was no correlation between lipid affinity of the peptides as determined by the sizes of peptide-DMPC complexes and their fusion-inhibitory activities. The peptides [Arg^{13}]A-I$_{con}$ and [Ala^{13}]A-I$_{con}$ had a high lipid affinity but were found to be poor inhibitors of cell fusion. In contrast, [Met^{3}]18A, a peptide with a moderate lipid affinity, was a good inhibitor of virus induced cell fusion. The reasons for the lack of correlation between lipid affinity and fusion-inhibitory activities are presently not clear. It is possible that affinity of the peptides for DMPC does not reflect entirely the affinity of these peptides for complex biological membranes such as the viral and cellular membranes. Furthermore, we cannot rule out the possibility that the peptides interact with proteinaceous components of cells (e.g., receptors) and viruses (glycoproteins) involved in the fusion process.

We have previously shown that the inhibitory effects mediated by peptide analogs of apoA-I are exerted on a wild-type HSV-1 isolate as well [25]. In other studies, we have found that apoA-I and its peptide analogs can inhibit human immunodeficiency virus (HIV)-induced cell fusion [26,27]. Studies are currently in progress to determine whether the different peptide analogs vary in their spectra of antiviral activities. The precise mechanism by which the amphipathic peptide analogs inhibit virus-induced cell fusion remains to be established. Available evidence from two different virus systems indicates that the peptides interfere with the fusion process per se and do not affect synthesis, processing, or assembly of viral proteins [14,25–27]. The peptides may therefore interact directly with some

component of the fusion mechanism. Consistent with this idea, the amphipathic peptides also inhibited entry of the virus into cells, a process that involves fusion of viral membranes with cell membranes. Interestingly, the inhibitory effects on virus penetration were manifest when the viruses (but not the cells) were pretreated with the peptides. It is therefore likely that the peptides either interact with a macromolecule (e.g., viral fusion proteins) or alter the supramolecular structure associated with the viral membranes.

Certain carbobenzoxy di- and tripeptides have been shown to inhibit measles virus and herpes virus penetration, virus-induced cell fusion, and hemolysis [28–30]. Short hydrophobic peptides (and their carbobenzoxy derivatives), which are homologous to the hydrophobic amino-terminal domain of the viral fusion proteins, also inhibit virus infection and virus-induced cell fusion by myxo- and paramyxoviruses [31–33]. The antiviral carbobenzoxy peptides, unlike the apoA-I analogs, appear to exert their effect on the cells and not the virus. It has been suggested that these antiviral peptides may bind to a putative fusion receptor on the cell membranes. These peptides have also been shown to elevate the bilayer to hexagonal phase transition temperature in model membrane systems, and it has been suggested that this bilayer-stabilizing property of the peptides may play a role in their antiviral activity [3]. Consistent with this hypothesis, other molecules such as tromantadine and cyclosporine, which stabilize the membrane bilayer, have been shown to inhibit virus-induced cell fusion [3,4,34,35]. Preliminary results show that some of the analogs of amphipathic peptides (e.g., 18A) used in this study have an ability to elevate the bilayer to hexagonal phase transition temperature in model membrane systems (R.M. Epand et al., unpublished results); whether the amphipathic peptides exert a bilayer-stabilizing effect on the complex biological membranes remains to be investigated. It will also be of interest to determine whether these different classes of fusion-inhibitory molecules display any additive or synergistic effects.

The biological significance of the in vitro antiviral effect exerted by apoA-I (and its amphipathic peptide analogs) is presently not clear. High plasma levels of HDL have been found to be a common and perhaps hereditary factor in long-lived individuals, and the beneficial effects of high HDL levels can be observed even after correcting for prevented deaths due to coronary heart diseases, in which HDL may play a direct role [36]. Several studies have suggested a participation of lipoproteins in host defense against infectious and noninfectious tissue destructive processes [37–42], although the mechanisms involved in this process remain unclear. Available information indicates that certain amphipathic molecules such as acute-phase serum proteins can displace apoA-I from HDL [43]. It is possible that the displaced apoA-I serves to prevent the spread of certain viral infections.

Amphipathic helices have been observed in naturally occurring antimicrobial agents such as cecropins and magainins [44,45]. However, these helices are characterized by the presence of a positively charged cluster along the polar face of the amphipathic helix, which is thought to facilitate pore formation and lysis of bacterial cells after peptide oligomers insert into membranes [46]. Different species of interferons also contain a region of similar overall length, charge distribution, and hydrophobicity that can fold into amphipathic helical segments. The amphipathic helices observed in interferons contain a highly hydrophobic nonpolar face [47]. Interestingly, interferon treatment has been shown to render cell membranes rigid and inhibit HSV-induced cell fusion [48,49]. The role, if any, of the amphipathic helical segments in interferon-mediated inhibition of HSV remains to be investigated. Future studies on amphipathic helices with different charge distribution and hydrophobicity and omission analogs similar to those described for magainins [50] should facilitate understanding structure-function relationships and design of effective antiviral peptides.

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REFERENCES

1. Hoggan MD, Roizman B: Am J Hygiene 70:28–39, 1959.
2. Verkleij AJ, Leunissen-Bijvelt J, deKruijff B, Hope M, Cullis PR: Non-bilayer structures in membrane fusion.

In CFS 103 (eds): "Cell Fusion." London: Pitman Books, 1984, pp 45–59.
3. Epand RM: Biosci Rep 6:647–653, 1986.
4. Epand RM, Epand EF, Mckenzie RC: J Biol Chem 262:1526–1529, 1987.
5. Anantharamaiah GM, Jones L, Brouillette CG, Schmidt CF, Chung BH, Bhown AS, Hughes TA, Segrest. JP: J Biol Chem 260:10248–10255, 1985.
6. Anantharamaiah GM: Methods Enzymol 128:627–647, 1986.
7. Anantharamaiah GM, Gawish A, Iqbal M, Gupta KG, Brouillette CG, Segrest JP: Protides Biol Fluids 34:63–66, 1986.
8. Anantharamaiah GM, Hughes TA, Iqbal M, Gawish A, Neame PJ, Medley MF, Segrest JP: J Lipid Res 29:309–318, 1987.
9. Anantharamaiah GM, Venkatachalapathi YV, Brouillette CG, Segrest JP: Arteriosclerosis 10:95–105, 1990.
10. Chung BH, Anantharamaiah GM, Brouillette CG, Nishida T, Segrest JP: J Biol Chem 260:10256–10262, 1985.
11. Epand RM, Gawish A, Iqbal M, Gupta KB, Chen CH, Segrest JP, Anantharamaiah GM: J Biol Chem 262: 9389–9396, 1987.
12. Kanellis P, Romans AY, Johnson BJ, Kercret H, Chiovetti RJ, Allen TM, Segrest JP: J Biol Chem 255:11464–11470, 1980.
13. Segrest JP, Jackson RL, Morrisett JD, Gotto AM: FEBS Lett 38:247–253, 1974.
14. Srinivas RV, Birkedal B, Owens RJ, Anantharamaiah GM, Segrest JP, Compans RW: Virology 176:48–57, 1990.
15. Schiffer M, Edmundson AB: Biophys J 7:121–135, 1967.
16. Eisenberg D, Weiss RM, Terwilliger TC: Nature 299: 371–374, 1982.
17. Eisenberg D, Schwarz E, Komaromy M, Wall R: J Mol Biol 179:125–142, 1984.
18. Greenfield N, Fasman GD: Biochemistry 8:4108–4116, 1969.
19. Richardson JS, Richardson CD: Nature 240:1648–1652, 1988.
20. Barker WC, Dayhoff MO: Comp Biochem Physiol 57B: 309, 1977.
21. Edelstein C, Kezdy FJ, Scanu AM, Chen BW: J Lipid Res 20:143, 1979.
22. Venkatachalapathi YV, Gupta KB, DeLoof H, Segrest JP, Anantharamaiah GM: In JE Rivier, GR Marshall (eds): "Peptides, Proceedings of the Eleventh American Peptide Symposium." Leiden: ESCOM Press, 1990, pp 672–673.
23. Mattice WL, Robinson RM: Biopolymers 20:1421–1434, 1981.
24. Epand RM, Surewicz WK, Hughes DW, Mantsch H, Segrest JP, Allen TM, Anantharamaiah GM: J Biol Chem 264:4628–4635, 1989.
25. Srinivas RV, Anantharamaiah GM, Owens RJ, Compans RW, Segrest JP: Circulation 80:II-386.
26. Owens RJ, Anantharamaiah GM, Kahlon JB, Srinivas RV, Compans RW, Segrest JP: J Clin Invest 86:1142–1150,1990.
27. Owens RJ, Srinivas RV, Anantharamaiah GM, Segrest JP, Compans RW: J Cell Biochem Suppl 13B:286, 1989.
28. Miller FA, Dixon GJ, Arnett G, Dice JR, Rightsel WA, Schabel FM Jr, McLean IW Jr: Appl Microbiol 16:1489–1496, 1968.
29. Nicolaides E, DeWald H, Westland R, Lipnick M, Posler J: J Med Chem 11:74–79, 1968.
30. Norrby E: Virology 44:599–608, 1971.
31. Richardson CD, Scheid A, Choppin PW: Virology 105: 204–222, 1980.
32. Richardson CD, Choppin PW: Virology 131:518–532, 1983.
33. Choppin PW, Richardson CD, Scheid A: In Stuart-Harris C, Oxford J (eds): "Problems in Antiviral Therapy" London: Academic Press, 1983, pp 12–33.
34. Cheetham JJ, Epand RM: Biosci Rep 7:225–230, 1987.
35. Mckenzie RC, Epand RM, Johnston DC: Virology 159: 1–7, 1987.
36. Gleuck CJ, Gartside P, Fallot RW: J Lab Clin Med 88:941–957, 1976.
37. Fahie-Wilson M, Mills R, Wilson K: Clin Chim Acta 167:197–209, 1987.
38. Cabana VG, Siegel JN, Sabesin SM: J Lipid Res 30:39–49, 1989.
39. Coombs E, Shakespeare P, Batstone F: J Trauma 20: 971–975, 1980.
40. Friedland M, Herbert P: J Am Med Assoc 248:82, 1982.
41. Heldenberg D, Rubinstein A, Levtov O, Berns L, Werbin B, Tamir I: Atherosclerosis 35:433–437, 1980.
42. Alvarez C, Ramos A: Clin Chem 32:142–145, 1986.
43. Coetzee GA, Strachan AF, van der Westhuysen DR, Hoppe H, Jeenah MH, de Beer FC: J Biol Chem 261: 9644–9651, 1986.
44. Boman HG, Faye I, Holsten VP, Kockum K, Lee J, Xanthopoulos KG: Dev Comp Immunol 9:551–558, 1985.
45. Zasloff M: Proc Natl Acad Sci USA 84:5449–5453, 1987.
46. Christensen B, Fink J, Merrifield RB, Mauzerall D: Proc Natl Acad Sci USA 85:5072–5076, 1988.
47. DeGrado WF, Wasserman ZR, Chowdhry V: Nature 300:379–381, 1982.
48. Chatterjee S, Lakeman AD, Whitley RJ, Hunter E: Virus Res 1:81–87, 1984.
49. Chatterjee S, Hunter E, Whitley R: J Virol 56:419–425, 1985.
50. Cuervo JH, Rodriguez B, Houghten RA: Peptide Res 1:81–86, 1988.