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Determinants of Membrane Activity from Mutational Analysis of the HIV Fusion Peptide

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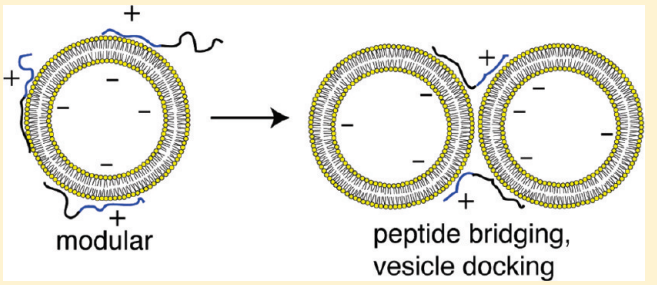
Supporting Information

ABSTRACT: We have synthesized a small library of 38 variants of the 23-residue fusion peptide domain found at the N-terminus of gp41 glycoprotein of HIV. This hydrophobic, glycine-rich sequence is critical for viral infectivity and is thought to be central in the membrane fusion of viral envelope with the host membrane. There has been extensive discussion regarding the origin of fusogenicity in this viral fusion sequence. Our library of fusion peptide variants was designed to address the biophysical importance of secondary structure, peptide flexibility, glycine content, and placement. We assayed each peptide for its ability to induce lipid mixing and membrane permeablization in synthetic vesicles. We find that the viral fusion peptide may be greatly simplified while retaining fusogenic function and minimizing membrane-permeablizing function; to the best of our knowledge, this is the first attempt to optimize fusogenic function of the HIV fusion peptide through sequence variation. Our data show that many flexible, linear, minimally hydrophobic peptides may achieve the biophysical function of fusion; glycine does not appear to be essential. These findings will be useful in the design of synthetic fusogens for cellular delivery.



We set out to dissect the origins of biophysical membrane activity in the HIV fusion peptide sequence. HIV membrane fusion[1,2] is mediated by the surface glycoprotein gp41, which is exposed following receptor binding.[3−7] A series of elegant experiments revealed a "spring-loaded" mechanism in which gp41 undergoes a dramatic conformational transformation that launches its previously buried N-terminal fusion peptide domain into the host membrane.[8−10] This 23-residue peptide is critical for viral infectivity[11−14] and itself can induce membrane fusion.[15−17] Conceptually, hydration repulsion barriers[18] must be overcome to allow membranes to dock; maturation of docking into fusion requires stabilization of either positive or negative membrane curvature,[19−22] which lowers the energy barrier to formation of a lipid membrane "fusion stalk",[18,23] a key intermediate in fusion. Consistent with this mechanism, the HIV fusion peptide has been reported to decrease the energy required to bend synthetic membranes,[24] though it is not clear how this could be generalized to unrelated fusion peptides.[25−27] The influenza virus[28−30] fusion peptide shares little sequence homology the HIV fusion sequence other than high glycine content,[31] though this is not a universal property among viral fusion peptides.[25] Fusogenic secondary structures have been proposed that are α-helical, β-sheet, or alternately helical and β-sheet; there is also considerable evidence that suggests that fusion peptides are unstructured in their functional form.[32−40] Given the diversity of primary structures in viral fusion peptides, it seems unlikely that they may all coalesce into the same functional secondary structure; an alternative explanation is that fusogenic function might be imparted at the level of general physical properties without particular sequence requirements. Such a concept has already been advanced for antimicrobial peptides,[25,41−47] and Langosch and co-workers have demonstrated that de novo hydrophobic peptides[40] based on transmembrane domains may induce membrane fusion. Wimley and co-workers demonstrated that potent membrane-permeablizing peptides selected from a combinatorial library had common compositional features centered on interfacial activity[48,49]—similar to the selection of lytic peptides from a phage library, reported by Hirosue and Weber.[50] It seemed likely that fusogenicity in native fusion peptides would also have broad determinants.

One major goal of this study was to understand what is generally required to fuse lipid membranes using a viral fusion peptide. To parse biophysical and biological function, we synthesized a library of peptides within the framework of the HIV fusion peptide and evaluated their membrane activity with regard to lipid mixing (membrane fusion) and contents leakage (lysis or permeablization). To the best of our knowledge, this is the first report of synthesis and biophysical evaluation of viral fusion peptide variants in an attempt to "reverse-engineer" fusogenic function and minimize membrane permeablization. Indeed, the HIV fusion peptide exhibits significant membrane-permeabilizing activity that appears highly sensitive to sequence variation; this has not been previously reported. Functional importance has been attached to particular glycine residues

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within the HIV fusion peptide as mutation abolishes infectivity;[51] however, loss of infectivity could have many origins aside from a loss of membrane activity. Intrigued by the high glycine content of both the HIV and Influenza fusion peptides, we questioned the functional importance of glycine pattern by "shuffling" the six glycine residues into 11 non-native patterns within the 23-residue HIV fusion peptide sequence. Though there was a significant effect on fusion yield, the system remained membrane-active, leading to the notion that there were many possible peptide fusogens within the framework of the HIV fusion peptide composition. Further synthesis and evaluation indicated that glycine is not strictly required at all, as long as peptide hydrophobicity and flexibility are maintained within a functional window. Peptides that promote vesicle docking (membrane apposition) are the most efficient fusogens, though the precise mechanism by which peptide docked vesicles fuse remains unknown; curvature alteration is one possibility that we are investigating further. We find a general connection between secondary structure and function in that peptides that are capable of helical folding at the membrane interface exhibit greater activity, perhaps simply by virtue of stronger surface binding. While others have reported both helical and sheet forms of viral fusion peptides, consideration of the enhanced interfacial binding exhibited by folded structures could unify these seemingly contradictory observations.[33,35−37,52−55] Our findings indicate that there exists an optimal peptide hydrophobicity which balances strong interfacial partitioning with peptide surface presentation and self-aggregation. This is equivalent to a bridging model for fusion, in which the fusogenic peptide simultaneously binds two surfaces to induce apposition and fusion. When membrane activity is considered this way, it is possible to account for the observations of fusogenic helical and sheet secondary structures. A fusogenic peptide must be able to bind a membrane and also present itself for interaction with other membranes. Therefore, weakly binding peptides will be inactive, as will peptides that bind so tightly that there is a high kinetic barrier to interaction with another membrane. Structures that facilitate bridging or partial anchoring should favor fusion. Using this model, we have established basic operational guidelines for preparation of fusogenic peptide sequences while minimizing membrane lytic side reactions. These design principles enable exploration of de novo designed membrane active peptides capable of mediating transmembrane passage.[56] We believe synthetic fusogens of this type will facilitate investigation on the mechanism of peptide-induced fusion, and further, will be useful in delivery applications.[56−63]

EXPERIMENTAL SECTION

General. Rink LS resin, Fmoc-amino acids were obtained from AAPTEC (Louisville, KY). Phospholipids were purchased from Avanti Polar Lipids. Fluorescent lipids were purchased from Invitrogen (Rh-DHPE, NBD-PE) and Biotium (DiI, DiO). Buffers were obtained from Sigma-Aldrich and prepared as follows: HBS (50 mM HEPES, 150 mM NaCl, pH = 7.0), PBS (50 mM phosphate, 150 mM NaCl, pH = 7.0), sucrose buffer (0.1 M sucrose, 25 mM NaH_2PO_4, 25 mM Na_2HPO_4, 25 mM NaCl, pH = 7.2), and glucose buffer (0.1 M glucose, 25 mM NaH_2PO_4, 25 mM Na_2HPO_4, 25 mM NaCl, pH = 7.2).

Peptide Synthesis. The peptides were synthesized by standard Fmoc solid-phase peptide synthesis methods on an AAPTEC Apex 396 synthesizer. Peptides were purified by C_{18} reversed-phase HPLC and an acetonitrile/water/TFA gradient (solvent A: 99% H_2O, 1% CH_3CN, 0.1% TFA; solvent B: 10% H_2O, 90% CH_3CN, 0.01% TFA). Stock solutions of peptides were prepared in deionized H_2O, and concentration was determined using tryptophan absorbance. Interfacial partitioning free energies[64] were calculated using the program Membrane Protein Explorer (MPEX 3.2) by Snider and Jayasinghe, White Laboratory, University of California, Irvine.

Lipid Mixing and Contents Release Assays. A FRET-dilution lipid mixing assay was used.[65] Anionic vesicles were prepared using 80/20 ePC/POPG and mixed with fluorescently labeled anionic vesicles ePC/POPG/NBD-PE/Rh-DHPE (76:20:2:2) in a 9:1 ratio of unlabeled to labeled vesicles that were sized to ~120 nm diameter by extrusion.[66,67] Fusogenic activity was calculated using the expression: % fusion = $[(F_t - F_0)/(F_{100} - F_0)] \times 100$. F_{100} or 100% lipid mixing was defined as NBD fluorescence in vesicles with 0.2 mol % each of NBD and Rh and total lipid concentration of 150 μM. Fusion yields are the average of three measurements. For vesicle-leakage measurements, a known calcein release assay was used.[68] Lipid mixing and release experiments were monitored using a Perkin-Elmer LS-50B luminescence spectrometer or a Molecular Devices SpectraMax M5/M5^e plate reader. All fusion and leakage measurements were carried out in triplicate at 37 °C in HBS at a 0.75 μM peptide and 150 μM lipid concentration (L/P = 200), unless otherwise specified. Percent leakage was calculated usng the formula: % leakage = $[(F_t - F_0)/(F_{100} - F_0)] \times 100$, with 100% leakage defined as the fluorescence observed upon lysis with Triton X.

GUV Preparation and Microscopy. Giant unilamellar vesicles (GUV) were prepared by the hydration method using sucrose buffer,[69] with one GUV population was labeled with DiO and another with DiI. Equimolar concentration of DiI and DiO giant liposomes were mixed with glucose buffer ($[\text{total lipid}]_{\text{final}}$ = 150 μM), placed in a CoverWell perfusion chamber gasket (Invitrogen) and equilibrated at 37 °C for at least 3 min. Giant liposomes were treated with peptide (L/P = 50 or 100) and monitored by confocal microscopy (Zeiss LSM10). DiO and DiI were observed with standard fluorescein and rhodamine filters, respectively, and images were taken using a C-Apochromat 63×/1.2W corr objective at 0, 5, 10, 20, and 30 min and processed with Zeiss LSM software.

RESULTS AND DISCUSSION

General Framework of Fusion Peptide Mutant Library. Weliky, Tamm, and co-workers have studied HIVfp-induced lipid mixing and examined the membrane-bound peptide structure by solid-state NMR at low ionic strength (5 mM HEPES).[33,70] A C-terminal hexalysine/tryptophan sequence (K_6W) was used to facilitate purification, handling, and concentration determination of the hydrophobic peptide. The cationic tag enables electrostatic peptide binding to anionic membranes; zwitterionic membranes did not appreciably bind peptide. This modification is necessary to examine the biophysical basis of peptide-triggered membrane fusion. We similarly attached a C-terminal cationic sequence to HIVfp in order to study fusion and contents release of fluid phase anionic membranes. However, it was not possible to assay membrane permeation in low ionic strength buffer using standard dye release experiments due to high osmotic pressure across the membrane. Higher salt conditions yielded significantly higher fusion yield than at low salt, consistent with increased screening of electrostatic repulsion between the anionic surfaces

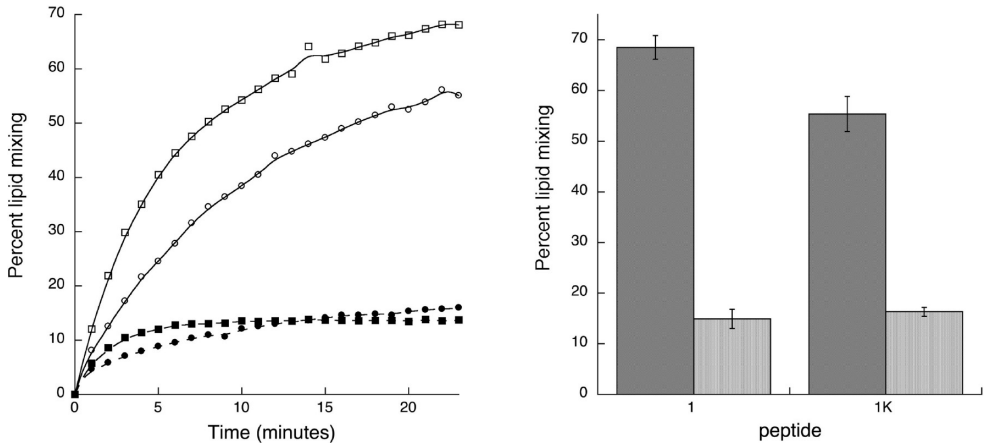

Figure 1. (left) Change in lipid mixing as a function of time for vesicle reaction with peptide **1** in standard (□) ionic strength HBS (50 mM HEPES, 150 mM NaCl, pH 7.0) and low (■) ionic strength (5 mM HEPES) and peptide **1K** in standard (○) and low (●) ionic strength buffer. (right) Percent lipid mixing with error bars derived from triplicate measurements, with standard HBS in dark gray and low ionic strength in light gray.

at higher ionic strength (Figure 1). We chose to perform our studies under more standard buffered conditions (50 mM HEPES buffer, 150 mM NaCl, pH 7.0) which decreased electrostatic effects and permitted investigation of peptide-induced membrane permeation as well as fusion. To the best of our knowledge, the lytic activity of the HIV fusion peptide has not been previously studied. It was necessary to decrease peptide/lipid ratios from 1:100 (as reported previously) to 1:200 in order to avoid vesicle precipitation at higher ionic strength. Further, we replaced the K_6W tag with an arginine sequence (R_6W), prompted by reports of enhanced membrane activity associated with arginine-containing peptides.[71−75] The arginine-tagged peptides were indeed more fusogenic than the lysine tagged variants, but increased lytic activity was also observed in some peptides. Whether this derives from specific peptide-membrane interactions or the more favorable interfacial partitioning of arginine relative to lysine is unclear.[64,76] To maximize the dynamic range of observable behavior, we performed our studies at higher ionic strength (HBS) using the R_6W tag. With this primary framework, we synthesized a library of variants in which the 23-residue fusion peptide region was altered to probe a specific question while keeping the electrostatic anchor the same. Thus, we examined membrane activity as a function of glycine placement, sequence composition, overall hydrophobicity, chain flexibility, and charge placement.

Glycine Shuffled HIV Fusion Peptide Mutants. Both HIV and influenza fusion peptides are glycine-rich, with six glycines in the 23-residue span of HIVfp.[25] Much importance has been assigned to the glycine residues, with reports of complete loss of viral infectivity upon mutation to alanine.[51] We were curious whether modulation of glycine positions within the sequence would have an equally significant biophysical effect. Eleven "glycine-shuffled" peptides (**2**−**12**) were synthesized wherein the positions of the glycines were moved throughout the 23-residue fusion peptide span, while keeping the order of non-glycine residues and overall amino acid composition constant. Though this only represents a small fraction of the numerical possibilities of such a library, the selected sequences were chosen to represent major types of glycine shuffled peptides. Noting that the native HIVfp sequence displays a near-uniform distribution of glycine throughout the sequence, we synthesized peptides with regular distribution as well as glycine-clustered sequences (Table 1). Our expectation was that glycine-clustered peptides should exhibit diminished fusogenic activity as a result of more structured hydrophobic domains that are uninterrupted by glycine. Lipid mixing and contents release experiments revealed a more complicated relationship. While the most fusogenic peptides, HIV-fp-R_6W (**1**) and variant **2**, have evenly distributed glycines, clusters of glycine residues were not strongly correlated with diminished lipid-mixing activity. However, glycine shuffling did result in a significant variation in fusion yield, from 33% to 71%, indicating that the peptide fusogenicity is not determined by amino acid composition alone. The glycine shuffled peptides induced vesicle contents release yields varying from 12% to 74% as a result of subtle sequence variation. When release is plotted against lipid mixing yield, permeation appears roughly correlated with lipid mixing; weakly lytic sequences are weakly fusogenic, and strongly lytic sequences are strongly fusogenic. However, strongly fusogenic sequences may be also be weakly lytic (Figure 2). Moreover, seemingly minor sequence variations can result in dramatic decreases (−45%) in permeation yield, as exhibited by peptides **1** and **2**, suggesting that there are multiple avenues to quash lytic activity. Conversely, peptide variants with very different glycine patterns (e.g., **2**, **4**) may have similar high lipid mixing and low permeation activity. Taken together, this limited data set suggests that the HIV-fp, as anchored by the R_6W sequence, may be predisposed to catalyze lipid mixing rather than lysis; we explore this notion further, as described below.

Modular Peptide Structure and Membrane Activity. Despite the apparently capricious balance of fusogenic and lytic activity in the glycine shuffled peptide library, exceptional lytic behavior in one glycine-shuffled variant hinted at a clearer relationship between sequence and function. Peptide **3** exhibited significant lipid mixing activity (66%) but also unusually high lytic activity (74%). This lytic activity was at least ∼17% higher than any other peptide, and on inspection of the sequence, we noted that, unlike the other 11 peptides (Table 1), this peptide featured a glycine immediately N-terminal to the R_6W anchor. We speculated that arginine natively found at position 22 in HIV-fp served as a functional part of the electrostatic anchor, and glycine insertion cleaved a 7-cation anchor (RSR_6W) into two cationic anchors composed of one (R) and six (R_6W) charges. This would change the mode of anchoring to imbed a longer part of the peptide in the membrane by electrostatic binding, which

Table 1. Fusion and Lysis Yields of Synthetic Fusion Peptide Variants[a]

#	Native fusion peptide sequence	fusion %	lysis %
1	AVGIGALFLGFLGAAGSTMGARS-R_6W	69	57
1K	AVGIGALFLGFLGAAGSTMGARS-K_6W	55	12
	Glycine Shuffled		
2	GAVIGALGFLFGLAAGSTMGARS-R_6W	71	15
3	AVIGALFGLFLGAASGTMAGRSG-R_6W	66	74
4	AVIALFLGGGFLAASGGGTMARS-R_6W	63	12
5	AVIGGALFLGGGFLGAASTMARS-R_6W	55	25
6	AVIGALFGLFGGLAASGTMGARS-R_6W	55	55
7	AVIGGGALFLGGFLAASGTMARS-R_6W	50	21
8	AVIGGALFLGGFLGAASGTMARS-R_6W	46	25
9	AVIAGGLFLFGGLAASGGTMARS-R_6W	43	33
10	AVIGGALFLGFLGAASGGTMARS-R_6W	39	19
11	AVIALFLGGGGGGFLAASTMARS-R_6W	36	35
12	AVIGGALFLGGFLAASGGTMARS-R_6W	33	16
	Electrostatic anchoring effects		
13	AVGRIGARLFLRGFLRGAARGSTRMGARSW	9	81
14	ALVRGGARLVLRGGVRLAARAGGRVLARSW	5	91
15	RSRRRRRRW	13	2
	Limited hydrophobe set		
16	AAVGGVAAVGGVAAAVGGVAARS-R_6W	2	0
17	AAVGGAAALGGLAAALGGLAARS-R_6W	24	61
18	AAVGGLAALGGLAAALGGLAARS-R_6W	60	31

#	Limited hydrophobe set (cont'd)	fusion %	lysis %
19	VLVGGLAALGGLAAALGGLAARS-R_6W	69	22
20	AALGGALLLGGLAAALGGLLARS-R_6W	74	22
21	ALLGGALLLGGLLAALGGLLARS-R_6W	71	8
22	ALLGGLLLLGGLLLALGGLLARS-R_6W	n.d	n.d
23	LLLGGLLLLGGLLLLLGGLLLRS-R_6W	n.d.	n.d.
24	ALVGGALVLGGVLAAAGGVLARS-R_6W	63	15
25	ALGVGALVLGVLGAAGAVLGARS-R_6W	61	15
	Open and cyclized (cystine) peptides	(open/cystine)	
26	ALVGGALCLGGCLAAAGGVLARS-R_6W	45/16	38/35
27	ALCGGALCLGGVLAAAGGVLARS-R_6W	53/33	47/35
28	ALCGGALVLGGCLAAAGGVLARS-R_6W	65/15	42/33
29	ALVGGALVLGGCLAAAGGCLARS-R_6W	54/14	37/34
30	ALCGGALVLGGVLAAAGGCLARS-R_6W	69/32	41/42
	Glycine content variants		
31	AVAAAAAVAVAAAVAAAAAVAARS-R_6W	73	2
32	LGLGLGLGLGLGLGLGLGLGLRS-R_6W	61	7
33	VGVGVGVGVGVGVGVGVGVGVRS-R_6W	23	8
34	AGAGAGAGAGAGAGAGAGAGARS-R_6W	1	0
35	AAAAAAAAAAAAAAAAAAAAARS-R_6W	16	0
	Double electrostatic anchor		
36	R_6-AVGIGALFLGFLGAAGSTMGARS-R_6W	60	13
37	R_6-ALVGGALVLGGVLAAAGGVLARS-R_6W	78	29
38	R_6-AVAAAAAVAVAAAVAAAAAVAARS-R_6W	69	19

[a] All fusion and lysis (release) measurements were performed in triplicate with errors indicated in the Supporting Information. Peptides **22** and **23** were insoluble and activity was not determined (n.d.). Peptide **36** was studied a peptide/lipid ratio of 1:300 while all other measurements were made at a peptide/lipid ratio of 1:200.

somehow shifts activity toward lysis. To address this possibility, we synthesized an HIV-fp variant, peptide **13**, in which the six arginines used for anchoring were evenly distributed throughout the sequence. Gratifyingly, we found that variant **13** was extremely lytic (81%) and weakly fusogenic (8.5%); such a shift of function from fusion to lysis was not previously observed for any of the other glycine-shuffled peptides (Figure 3). It appears that the framework dictates membrane activity: a modular peptide design that is divided into a hydrophobic block and cationic block directs function toward fusion, while a peptide with cationic residues integrated with hydrophobic residues directs function toward lysis/permeation. When fusion vs lysis yield is plotted for peptides **1**−**34** (Table 1), one clearly sees that fusion is favored over lysis for most peptides (Figure 3). Peptides with lytic behavior favored (top left of graph) have distributed cationic charge in their sequences rather than modular hydrophobic/cationic structure. Block structure allows peptide-membrane anchoring by either the cationic module, the hydrophobic module, or both. Such a peptide could bridge or "dock" two vesicles into apposition, an essential first step in fusion, by anchoring one vesicle electrostatically and the other vesicle hydrophobically (Figure 4). Conversely, when electrostatic binding is interdigitated with hydrophobic insertion, the entire peptide is imbedded in the membrane upon binding and it is less likely to dissociate partially to form bridging structures. Consistent with this idea, all fusogenic modular peptides, induce vesicle precipitation at peptide:lipid ratios greater than 1:200 while lytic peptides did not cause visible precipitation even at a peptide:lipid ratio of ∼1:20 (Supporting Information). Also, consistent with the bridging model, the native HIV fusion sequence **1** had minimal fusogenic activity against neutral fluid-phase vesicles (5%), indicating that electrostatic binding is essential. Diminished but significantly higher lipid mixing yields (26%) were observed when negatively charged vesicles were reacted with neutral fluid-phase vesicles

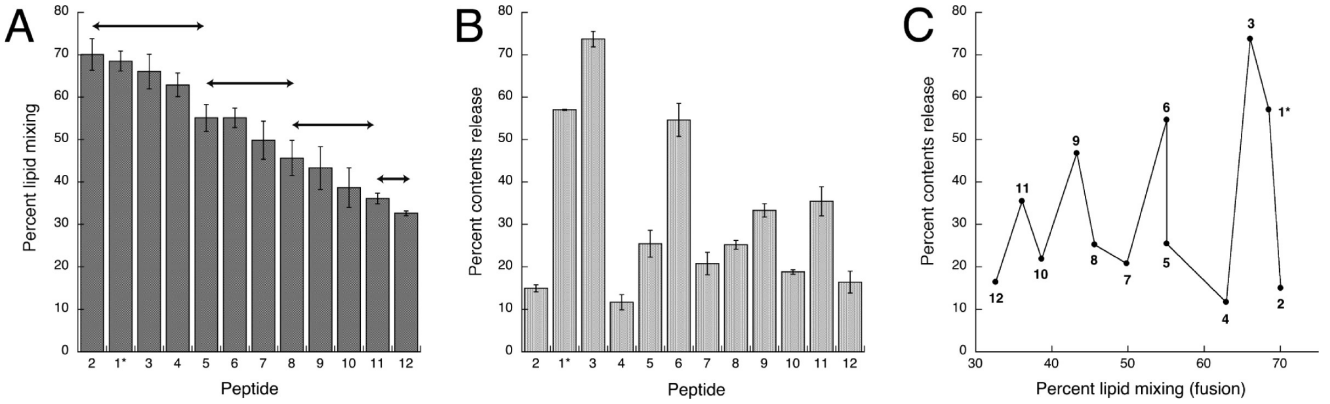

Figure 2. Percent lipid mixing (A) and percent contents release of encapsulated calcein (B) induced by glycine-shuffled peptides **1**−**12**. (C) Dependence of lytic activity on fusogenic activity of each peptide, **1**−**12**. All measurements performed in triplicate and the native HIV fusion sequence is indicated as **1***. Statistically significant differences (p value < 0.026) in percent lipid mixing are indicated with double-headed arrows in (A).

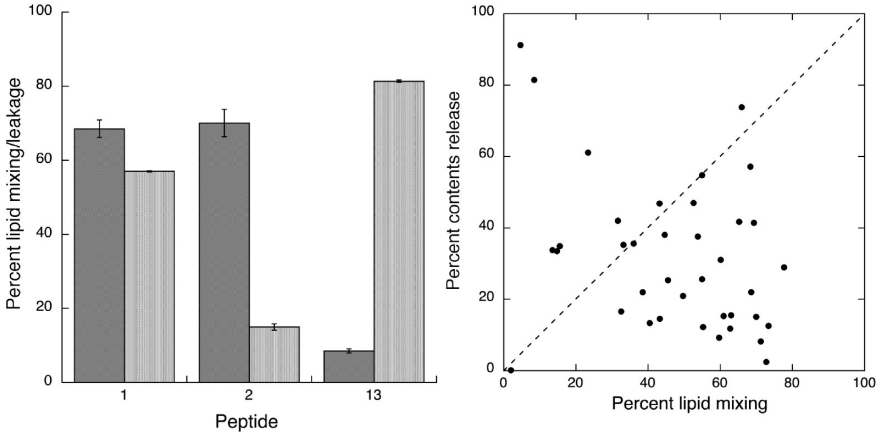

Figure 3. (left) Fusion (dark gray) and lysis (light gray) yields for peptides **1**, **2**, and **13**. (right) Fusion vs lysis plot of peptides **1**−**34** in Table 1, with dotted line indicating slope = 1.

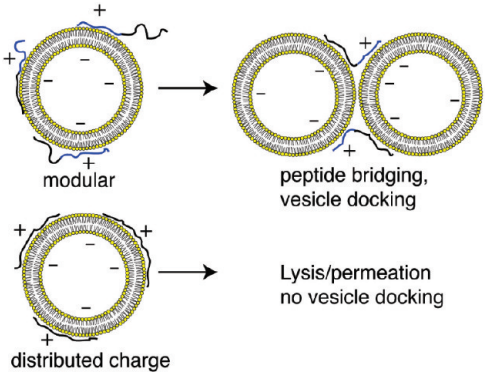

Figure 4. Hypothesized mechanism for enhanced fusion of anionic vesicles exhibited by modular hydrophobic−cationic peptide structure relative to a peptide with distributed cationic residues.

in a 1:9 ratio, indicating the ability of the hydrophobic viral sequence to capture neutral membrane fusion partners when displayed on a negatively charged membrane (Supporting Information).

De Novo Designed Fusogenic Peptides. If the bridging model for fusogenicity is correct, any modular peptide with an appropriately hydrophobic sequence could catalyze membrane fusion when presented with an electrostatic anchor. We synthesized a small set of peptides to test this hypothesis, using the calculated free energy of partitioning to the lipid−water interface as a guide. The native HIV fusion peptide composition was calculated to have a favorable interfacial partitioning ΔG_{ip} of −4.46 kcal/mol using the interfacial Wimley−White scale.[64] We chose the least active glycine variant we found among the glycine-shuffled library (**12**) and altered the hydrophobic content, with the expectation that activity could be improved by increasing hydrophobicity. Indeed, peptides in which only A, L, and V were used in non-glycine positions exhibited similar or increased fusogenic activity to the native HIVfp, with decreased lytic activity. The glycine pattern in peptide **12** that led to weak fusogenic activity (33% yield) can induce a wide range of behavior when the simpler hydrophobic amino acid set was used, leading to fusion yields ranging from 24 to 74% (Figure 5). Thus, both glycine pattern and overall hydrophobic content are important determinants of activity. The set of peptides revealed a window of partitioning free energy that permits membrane activity: when ΔG_{ip} is in the range −1.64 to −6.0 kcal/mol, both lytic and fusogenic activity are observed (Figure 5). When partitioning free energy is more positive than −1.6 kcal/mol, membrane binding is insufficient; when peptides are too

#	Limited hydrophobe set	G_{ip} (kcal/mol)
1	AVGIGALFLGFLGAAGSTMGARS-R_6W	-4.46
16	AAVGGVAAVGGVAAAVGGVAARS-R_6W	+0.78
17	AAVGGAAALGGLAAALGGLAARS-R_6W	-1.64
18	AAVGGLAALGGLAAALGGLAARS-R_6W	-2.37
19	VLVGGLAALGGLAAALGGLAARS-R_6W	-3.20
20	AALGGALLLGGLAAALGGLLARS-R_6W	-4.46
21	ALLGGALLLGGLLAALGGLLARS-R_6W	-5.92
22	ALLGGLLLLGGLLLALGGLLARS-R_6W	-7.38
23	LLLGGLLLLGGLLLLLGGLLLRS-R_6W	-9.57

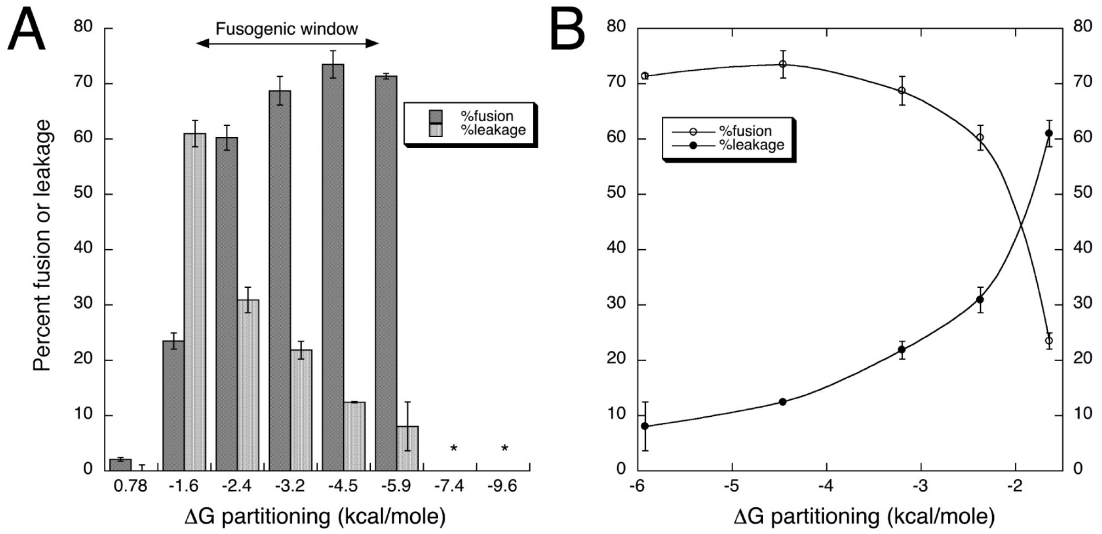

Figure 5. (top) Table of fusion peptide variants with non-native amino acid composition and their respective calculated free energies of partioning to the lipid−water interface from bulk water (ΔG_{ip}), excluding the C-terminal R_6W tag. (A) Peptide-induced fusion and leakage yields as a function of ΔG_{ip}, where the asterisk indicates insoluble peptide. (B) Line plots of fusion and leakage indicating the window of membrane activity.

hydrophobic ($\Delta G_{ip} < -6$ kcal/mol), it is possible that peptide self-aggregation competes with membrane binding, thus diminishing activity. These results are perhaps not unexpected given that transmembrane anchor variants explored by Langosch exhibit fusogenic activity. However, our de novo fusogenic sequences have only a single C-terminal electrostatic anchor, whereas Langosch's system has an anchor at either terminus. Furthermore, our fusion experiments were carried out with lipids that are not known to promote membrane fusion, whereas prior studies employed up to 20% phosphatidylethanolamine lipid, which is known to predispose a membrane toward fusion.[77] We are currently investigating the membrane activity of our peptides against membranes of different composition.

Peptide Flexibility, Linearity, and Membrane Activity. The ability of de novo sequences to induce fusion with similar efficiency as the native HIV-fp sequence (**1**) indicates the general fusogenicity of the modular peptide framework. Clearly though, there are more detailed determinants that allow membrane activity to be skewed toward lysis or fusion within this framework. Though it is not possible to extract a more precise relationship between membrane activity and sequence from the data, we felt we could address broader questions that remained. For instance, the role of the glycine pattern may be tied to both secondary structure and overall peptide flexibility, consistent with a peptide-bridging mechanism for fusion. To address the role of conformational plasticity, we chose variant **24** (which has hydrophobe content limited to A, L, and V) and replaced valine pairs with cysteine to allow for intramolecular cyclization through oxidative cystine formation. Cysteine is sterically and hydrophobically similar to valine,[78] and we expected similar membrane activity. Peptides were allowed to air-oxidize to cystines under dilute conditions in basic buffer, and the cyclization/oxidation was confirmed by HPLC/MS and Ellman's test for free thiols; the mass of disulfide dimers was never observed over a range of ionization conditions (Supporting Information). In every variant, the reduced (linear) peptide exhibited significantly higher fusogenic activity than the oxidized (cyclic) peptide, though lytic activity was less sensitive to cysteine redox (Figure 6). The linear dicysteine peptides induced fusion to a similar extent as variant **24** on which they were based but were at least twice as lytic for reasons we do not understand. Addition of a disulfide reductant such as TCEP to membrane-bound cystine peptide elevated

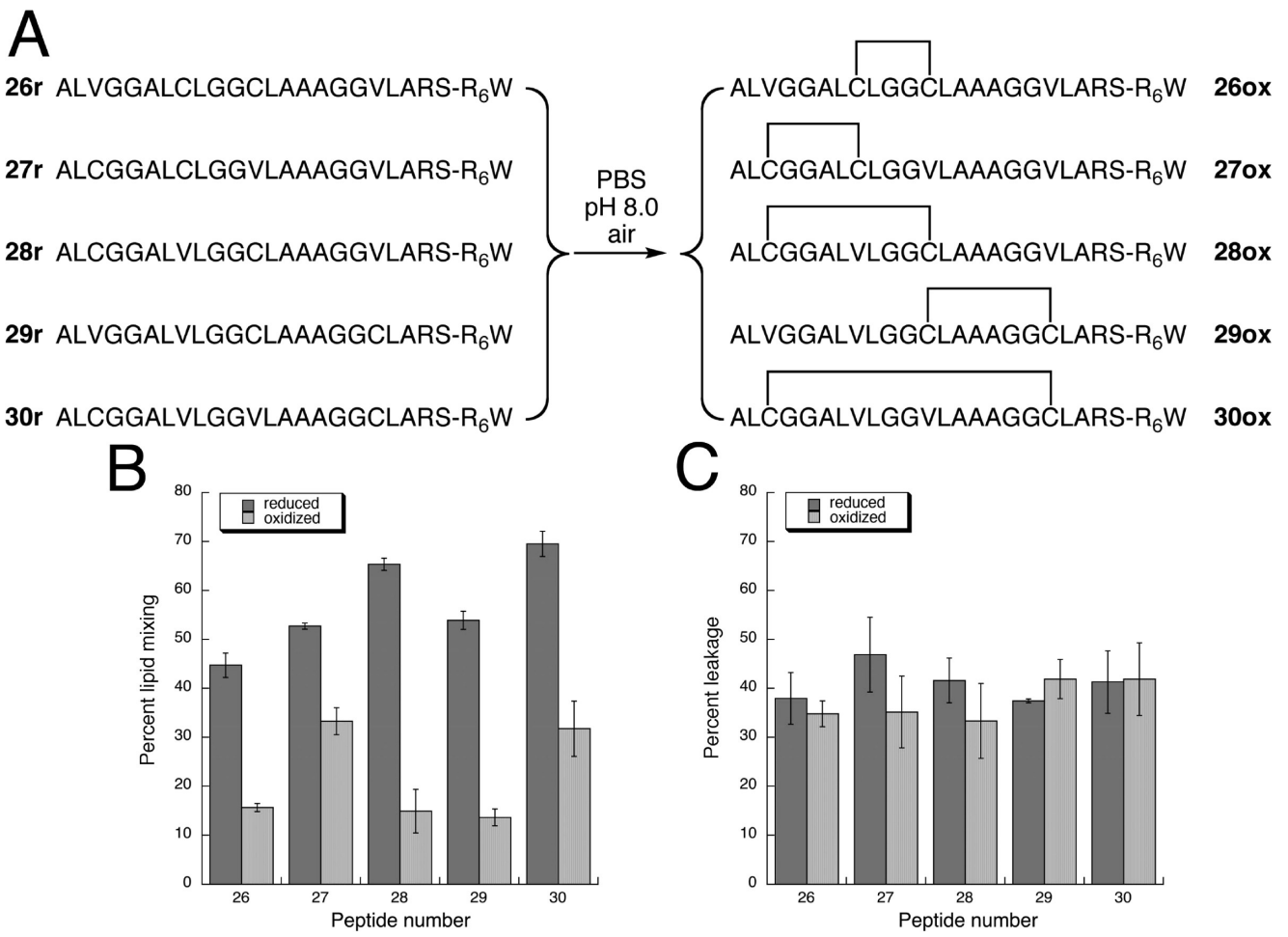

Figure 6. (A) Air oxidation of dicysteine peptides **26**−**30** yields the cystines shown. Percent lipid mixing (B) and leakage (C) induced by the reduced peptides (dicysteine, dark gray) and oxidized peptides (cystines, light gray). All differences between reduced and oxidized species are statistically significant (p value < 0.005).

membrane activity *in situ* but did not restore lipid mixing yield to that observed for the prereduced peptide, suggesting that the cystine is partially inaccessible due to membrane burial. Overall, these findings indicate that the linear peptide structure affords greater membrane activity than the cyclic peptide, possibly because the extended linear peptides can more efficiently facilitate vesicle−vesicle docking.

Importance of Glycine Content. Following the notion that overall hydrophobicity of the neutral peptide module is most critical feature, we questioned whether glycine residues were important for fusion at all. We prepared a peptide (**31**) with no glycine residues, only A and V. This sequence proved to be one of the most fusogenic (73%) and least lytic (2%) sequences we found, supporting the idea that fusion has no particular residue requirement. We also prepared a sequence with ten glycine residues, four more than found in the native sequence, and placed A, V, or L in the remaining (alternating) positions. The AG and VG peptides were outside and at the lower edge of the hydrophobicity window of activity, respectively; as expected, the AG peptide showed no activity, and the VG peptide was very weakly active. The LG peptide was right in the middle of the hydrophobicity window and showed strong fusogenic (61%) activity and weak lytic activity (7.1%) (Table 2). Thus, glycine content may be shuffled, increased or decreased to zero, while maintaining fusogenic function. It appears from our biophysical assays that a hydrophobic/cationic modular linear peptide with a favorable interfacial partitioning free energy (roughly $-1.6 \leq \Delta G \leq -7$ kcal/mol) possesses the minimal requirements to induce membrane fusion between anionic vesicles. Notably, as discussed below, the calculated interfacial partitioning free energy of peptide **31** is *unfavorable*, until a helical secondary structure is assumed.

Peptide Secondary Structure and Membrane Activity. Interestingly, the glycine-shuffled compositional isomers revealed significant fluctuations in membrane activity brought on by apparently minor sequence variations (Figure 2). We hypothesized that these sequence variations affected peptide folded state in the membrane, which affects function. We examined selected peptides by circular dichroism in native and SDS buffer, in search for a correlation between secondary structure and membrane activity. There has been considerable discussion on this topic with functional significance assigned to helical, sheet, and unstructured fusion peptides. It was not possible to study the peptides with lipid vesicles as peptide binding was accompanied by vesicle aggregation at peptide:lipid ratios greater than 1:200; thus, SDS was used to mimic the membrane environment. For all peptides, addition of SDS induced the folding of random coil structures; double minima

Table 2. Secondary Structure and Calculated Interfacial Partitioning Free Energy[a]

no.	fusion peptide variants	secondary structure	ΔG_{ip} (kcal/mol)	ΔG_{ip} (kcal/mol) helical
1	AVGIGALFLGFLGAAGSTMGARS	β sheet	−4.46	−13.66
2	GAVIGALGFLFGLAAGSTMGARS	helical	−4.46	−13.66
16	AAVGGVAAVGGVAAAVGGVAARS	poorly structured	+0.78	−8.42
17	AAVGGAAALGGLAAALGGLAARS	helical	−1.64	−10.84
21	ALLGGALLLGGLLAALGGLLARS	helical	−5.92	−15.12
29	ALVGGALVLGGCLAAAGGCLARS	ambiguous	−3.29	−12.49
30	ALCGGALVLGGVLAAAGGCLARS	ambiguous	−3.29	−12.49
31	AVAAAAVAVAAAVAAAAAVAARS	strongly helical	+1.84	−7.36
32	LGLGLGLGLGLGLGLGLGLRS	weakly helical	−7.29	−16.49

[a] Peptides and apparent secondary structure observed by CD (Figure 7), calculated interfacial partitioning free energy (ΔG_{ip}) for unstructured and assumed helical structure, hydrophobic module only. The C-terminal electrostatic anchor module (R_6W) is not shown for clarity.

(helix) and single minumum (sheet) signatures were observed, as well non-random coil CD signatures that were not clearly indicative of canonical secondary structures (Supporting Information). It was difficult to draw structure−activity relationships when modular and charge-distributed peptides were considered together. For instance, though both charge-distributed peptides **13** and **14** are primarily lytic (Table 1), they exhibit helical and sheet CD signatures, respectively. However, when the modular peptides are considered in isolation, it does appear that single minima CD spectra (sheet or mixed helix-sheet structures) are correlated with membrane permeablization while double minima CD spectra (helix) are correlated with fusion/lipid mixing. The native HIV fusion sequence **1** is both strongly fusogenic and lytic and exhibits a single minimum CD spectra suggestive of a β-sheet structure or a structure intermediate between helix and sheet. Notably, the glycine shuffled compositional isomers are all less lytic than **1** (with the exception of peptide **3**, which has a different charge distribution). Glycine-shuffled peptide **2** is markedly helical, more fusogenic than **1**, and considerably less lytic (Table 1, Figure 7); shuffled isomer **4** is also less lytic than **1**, and its CD spectrum has a stronger double minima than **1**. Furthermore, for reasons we cannot explain, the hexalysine anchored native sequence **1K** has comparable fusogenicity to **1** (hexaarginine anchored native sequence) but greatly diminished lytic activity (Table 1). The less lytic **1K** has a helical CD signature in SDS buffer while **1** has an intermediate secondary structure (Supporting Information). The observed helicity of **1K** is consistent with a structural study from Li and Tamm on a similar HIV fusion peptide derivative, though they did not study the lytic activity of this peptide.[33] This trend can be observed with the simplified hydrophobe variants as well: helicity and fusogenicity increase while decreasing in lytic activity (Figure 5). Certainly, glycine-free peptide **31** is by far the most helical peptide, as expected from removal of structure-breaking glycines, and also the most fusogenic and least lytic. Cystine-constrained peptide variants (**26**−**30**) did not exhibit increased fusogenicity with greater helicity, though it is possible that the constrained peptide backbone changes the relationship between structure and activity.

It also apparent that membrane inactive peptides do not structure significantly upon SDS treatment (**16**, Figure 7), suggesting weak binding. Peptide structuring upon membrane binding is highly favored, as this buries backbone amide functionality within a folded structure rather than exposing these polar groups to the lipid matrix.[64] Peptide folding upon membrane binding exacts an entropic penalty, which is exacerbated in glycine-rich sequences such as the fusion peptide variants. Possibly, the tighter interfacial anchoring that accompanies helical folding diminishes lytic function and favors fusion. This is most clearly shown in glycine-free variant **31**, which has an unfavorable calculated ΔG of partitioning to the bilayer−water interface of +1.84 kcal/mol when an unstructured peptide is assumed. However, the calculated interfacial partioning free energy becomes favorable (−7.36 kcal/mol), landing within our empirical "free energy partitioning window of activity" when a 100% helical structure is assumed; indeed, we observe a strong helical CD signature and fusogenic activity devoid of membrane permeablization.[64] It is tempting to conclude that helical structures are associated with fusion while mixed structures (with single minima CD spectra) are associated with lysis. Though many helical lytic peptides, including charge-distributed peptide **13**, have been reported,[44,47,79−81] within our library of peptides of *modular* structure, greater helicity does appear to be correlated with diminished lytic function (Figures 5 and 7). The connection between specific peptide structure and membrane function may derive simply from increased membrane partitioning of well-folded helical structures; increased surface binding should correlate with heightened membrane activity. This tighter peptide-membrane binding would then suppress lytic activity, though the mechanism for this suppression remains unclear.

N- and C-Terminal Electrostatically Anchored Peptides. Oriented or "oblique" peptide insertion of helical peptides, wherein one terminus is inserted more deeply into the membrane than the other, has been linked to the lytic membrane activity of helical antimicrobial peptides.[82,83] This mode of insertion has also been postulated to be of importance to the function of viral fusion peptides.[84−86] While not inconsistent with our hypothesized bridging model, oriented insertion did not appear to be obviously required by our model. We synthesized a fusion peptide variant flanked by two cationic membrane anchors (peptides **36**−**38**, Table 1) with the expectation that surface electrostatic anchoring at *both* termini would prevent deep insertion of one terminus of the fusion peptide domain due to the energetic cost of charge burial. Interestingly, the doubly anchored synthetic fusion peptides **36**−**38** clearly retained membrane activity with a significant but minor overall boost in both fusogenic and lytic function relative to their singly anchored analogues. Doubly anchored native HIV fusion peptide sequence (peptide **36**) was more active at lower concentration, achieving the same fusion and lysis yields at peptide−lipid ratios of 1:300 as the singly anchored peptides at ratios of 1:200. The increases in activity may simply be a result of improved electrostatic binding,

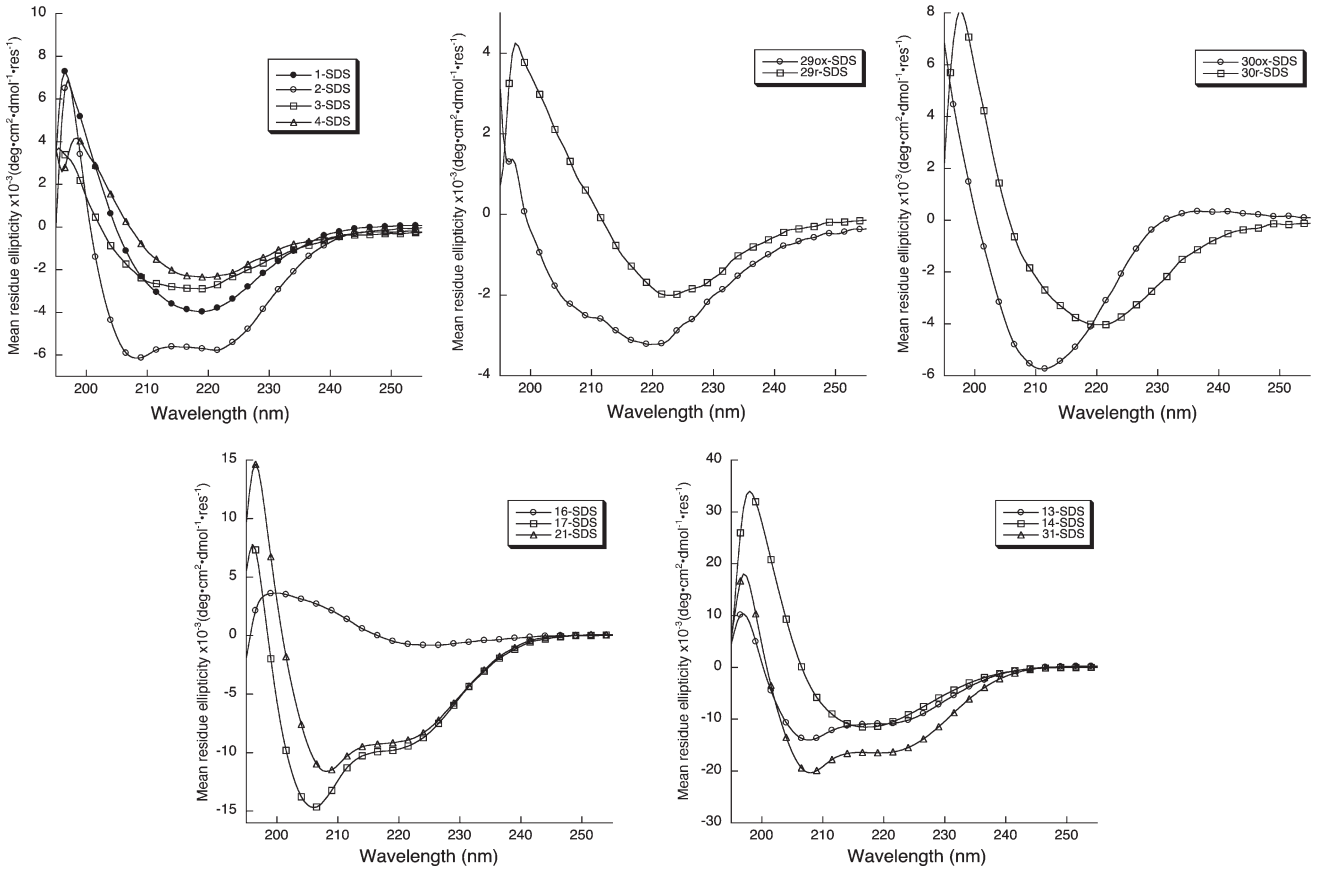

Figure 7. Circular dichroism spectra 1% SDS HBS solutions of (top, left) glycine-shuffled peptides **1**−**4**, (top, middle, right) dicysteine and cystine peptides **29r**, **29ox** and **30r**, **30ox**, (bottom, left) hydrophobic variants **16**, **17**, **21**, and (bottom, right) primarily lytic peptides **13**, **14** and exclusively fusogenic peptide **31**.

though vesicle precipitation at higher peptide concentration prevented quantitative comparison. Consistent with our hydrophobic−electrostatic bridging model, hydrophobic peptide modules with ΔG_{ip} outside the functional window remained inactive when flanked by two cationic modules, indicating the necessity of both hydrophobic and electrostatic binding. Doubly anchored fusion peptides may drive docking by the extrusion of the hydrophobic module into solvent as a loop domain that captures vesicles for reaction (Figure 8). Increased membrane activity of these peptides could arise from inhibited intramembrane loop burial due to unfavorable partial charge burial of the terminal cationic anchors or simply due to increased electrostatic binding of the peptide scaffolds, which has been previously observed. Interestingly, our double electrostatic anchor fusion peptides echo the structure and activity reported by Langosch and co-workers, and indeed, their findings are consistent with our interfacial bridging model.[40] They employed a system in which a hydrophobic 16-residue sequence was electrostatically anchored to an anionic membrane (∼20% phosphatidylserine, 20% phosphatidylethanolamine, 60% eggPC) by three cationic charges (lysine) on either terminus. Nonfusogenic peptides had partitioning free energies (−8.96 and +1.1 kcal/mol) outside our empirical window of function, while fusogenic peptides were within the window (−3.0 to −5.8 kcal/mol). Langosch and co-workers inserted a turn motif in the center of the hydrophobic peptide, anchored at either terminus, and observed increases in fusion rate and yield.

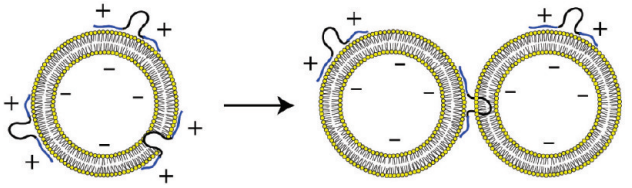

Figure 8. Possible mechanism for docking/bridging and fusion mediated by doubly anchored fusion peptides, represented as blue cationic modules flanking a black hydrophobic module.

Though this was correlated with the fusogenicity of sheet structures, their finding could also be viewed as an improvement in peptide surface presentation that facilitates docking, as a turn structure would favor loop presentation (Figure 8). Overall, the robust activity of doubly anchored peptides is fully consistent with the notion of interfacial bridging being key in peptide-induced fusion and suggests that oriented insertion may not be critical in determining biophysical function for fusogenic peptides.

Imaging Membrane Fusion in GUVs Induced by Selected Peptides. We found that inner membrane mixing[87] confirmed full fusion for peptides **1**, **1K**, **2**, and **31** (Supporting Information). It was our expectation that peptides that induced >50% fusion yields and low lysis should also exhibit vesicle contents mixing. However, use of known contents mixing assays did not indicate contents mixing in by any of our peptides.[88] Moreover, positive

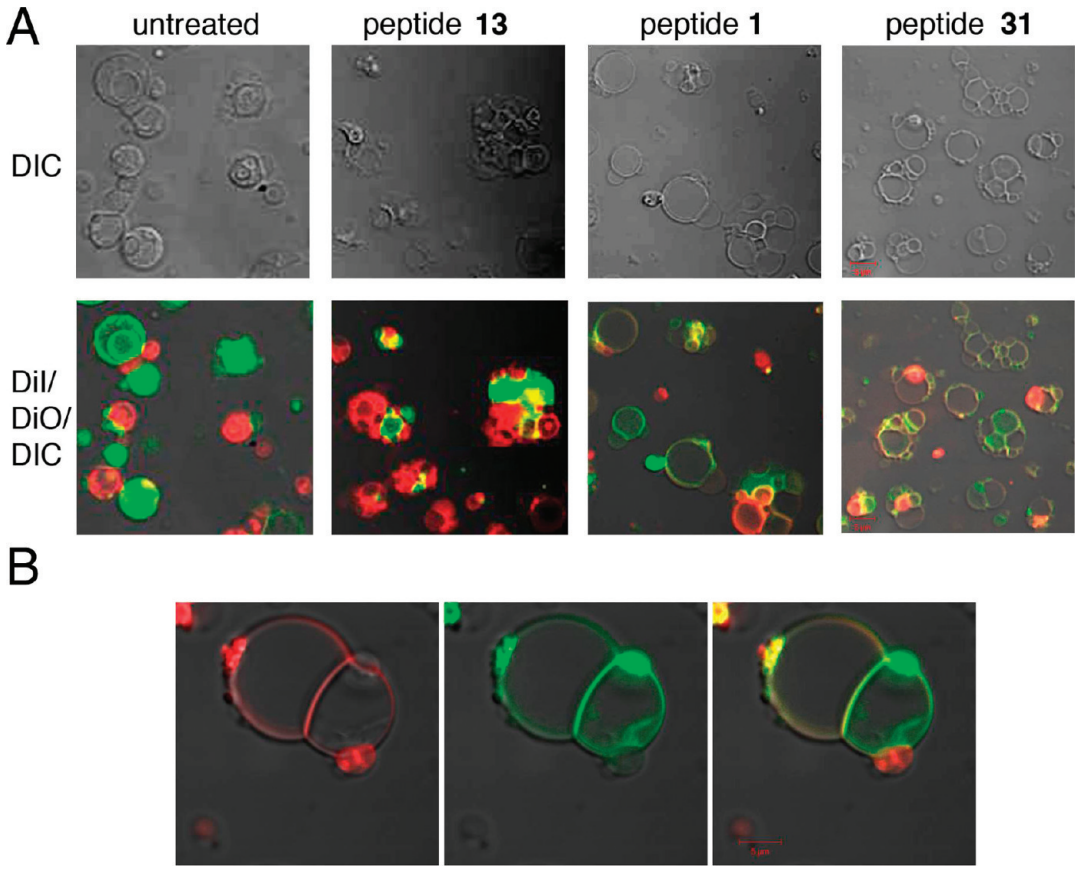

Figure 9. Confocal microscopy images of GUVS and treatment with the peptides indicated, imaged after 30 min incubation at 37 °C. (A) (top row) DIC images and (bottom row) an overlay of DIC, green and red fluorescence channels are shown. (B) Closer image of GUVs treated with peptide **31** viewed through the DiI (left) and DiO (middle) and overlay.

control experiments using high (fusogenic) concentrations of PEG also failed to produce a contents mixing signal;[89] even phosphatidylethanolamine-rich membranes were nonfusogenic by this assay in our hands, suggesting that the assay may be sensitive to conditions. We instead turned to fluorescence confocal microscopy to further evaluate the fusion process between two anionic giant unilamellar vesicle (GUV) populations, differing only in the dye used to label each population (red or green). Images were collected for GUVs treated with the native HIV-fp-R_6W peptide (**1**), peptide **31** (primarily fusogenic), peptide **13** (a compositional isomer of **1**, primarily lytic), and the electrostatic anchor, RSR_6W. Inspection of the peptide−GUV reactions over time using confocal microscopy revealed that the anchor did not appreciably cause vesicle aggregation or fusion, while the native HIV sequence **1** induced both aggregation and clear colocalization of red and green fluorescence in a single vesicle (Figure 9). Notably, the lytic peptide **13** did not induce lipid mixing of green and red GUVs but did cause some vesicle aggregation. The primarily fusogenic peptide **31** provided the highest apparent fusion yield, with many unilamellar vesicles containing both red and green fluorescence, as well as clusters of vesicles with lipid mixing at the interface and throughout the cluster. These data indicate that peptide-fusion catalysts necessarily induce vesicle aggregation (docking) which appears to resolve as full fusion, though the possibility remains that the majority of GUV lipid mixing occurs through hemifusion with fewer full fusion events. In contrast, lytic processes appear to mediate transient docking processes that result in contents leakage, though only nominal size changes are detectable by DLS (Supporting Information).

CONCLUSIONS

Beginning with the fusion peptide domain from HIV, we have extracted the essential features of a peptide membrane fusion catalyst with minimal lytic action. We find that peptide fusogenicity in a biophysical context can tolerate many modifications, provided that a modular peptide structure, wherein a hydrophobic sequence is coupled to a cationic sequence, is maintained. If the interfacial partitioning free energy of the hydrophobic portion is roughly between −1.6 and −7 kcal/mol, the peptide should catalyze lipid mixing between anionic vesicles. These observations fit well with a bridging model for peptide-induced fusion: hydrophobic and electrostatic anchoring tethers two anionic membranes together, facilitating both docking[18] and fusion. Within this model, peptide secondary structure may be generally tied to activity in that membrane-partitioning of a folded peptide is energetically favored over insertion of an unstructured peptide;[64] the most fusogenic compositional isomer of the HIV fusion peptide (**2**) was also found to be the most helical. Thus, any sequence with these general properties could serve as a fusogen against anionic membranes, and there appears to be no requirement for a glycine-rich sequence or specific amino acid composition. This broad determinant for fusogenicity

is consistent with prior reports of de novo peptide fusogens and unifies reports which identify both helix and sheet structures for viral fusion peptides.[33,37,53,90] Within this reported set of HIV fusion peptide variants, we have found that the mode of electrostatic anchoring may bifurcate function between highly potent lipid mixing and membrane permeablization, with distributed electrostatic interactions favoring permeation and block electrostatic anchoring favoring fusion (e.g., **13** and **31**). Overall, this study has provided a set of guidelines for design of membrane-fusion peptide catalysts; it is possible that these concepts also apply to non-peptide backbones.[45] Using these principles, we have found de novo fusogenic sequences that can mix membranes as efficiently as the native HIV fusion sequence, with suppressed lytic activity. These design principles may be used to generate synthetic fusogens useful as components of cellular delivery systems.

■ ASSOCIATED CONTENT

Supporting Information. Mass spectroscopic and HPLC characterization of peptides, cystine formation, additional CD, fluorescence, and microscopy data. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

HIV, human immunodeficiency virus; gp41, glycoprotein 41; LS resin, low substitution resin; Fmoc, fluorenylmethyloxycarbonyl; Rh-DHPE, rhodamine dihexadecanoylphosphatidylethanolamine; NBD-PE, *N*-4-nitrobenzo-2-oxa-1,3-diazole phosphatidylethanolamine; DiI, 1,1′-dioctadecyl-3,3,3′3′-tetramethylindocarbocyanine perchlorate; DiO, 3,3′-dioctadecyloxacarbocyanine perchlorate; TFA, trifluoroacetic acid; HPLC, high performance liquid chromatography; FRET, fluorescence resonance energy transfer; ePC, egg phosphatidylcholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycerophosphatidylglycerol; GUV, giant unilamellar vesicle; LUV, large unilamellar vesicle; HIVfp, HIV fusion peptide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HBS, HEPES buffered saline; TCEP, tris(carboxyethyl)phosphine; DLS, dynamic light scattering.

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