The C- and the N-terminal Regions of Glycoprotein 41
Ectodomain Fuse Membranes Enriched and Not
Enriched with Cholesterol, Respectively*

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To infect target cells, HIV-1 employs a virally encoded transmembrane protein (gp41) to fuse its viral envelope with the target cell plasma membrane. We describe the gp41 ectodomain as comprised of N- and C-terminal subdomains, each containing a heptad repeat as well as a fusogenic region, whose organization is mirrored by the intervening loop region. Recent evidence indicates that the gp41 directed fusion reaction proceeds to initial pore formation prior to gp41 folding into its low energy hairpin conformation. This implies that exposed regions of the gp41 ectodomain are responsible for the bulk of the fusion work, probably through direct protein-membrane interactions. Prevalent fusion models contend that the gp41 ectodomain initially interacts with the target cell surface through its highly hydrophobic N terminus, which is believed to insert into the target membrane, thereby linking the virus to the target cell. This arrangement allows the N-terminal subdomain to interact with the target cell surface, whereas the C-terminal subdomain remains proximal to the virion, allowing interaction with the viral envelope. The composition of the viral envelope and the target cell surface differ due to the virus budding from raft microdomains. We show here that constructs corresponding to the C-terminal subdomain specifically destabilize ordered and cholesterol rich membranes (33 molar %), whereas the N-terminal subdomain is more effective in fusing both unordered cholesterol-free membranes and those containing lower amounts of cholesterol (10 molar %). Moreover we show that, in the context of the C-terminal subdomain, the heptad repeat contributes helical structure, which may describe the enhanced inhibitory effect of the C-terminal subdomain relative to the C-terminal heptad repeat (C34) alone. Our results are discussed in light of recent findings that showcase the role of exposed gp41 regions in effecting membrane fusion.

Infection of human cells by HIV-1† requires fusion between the viral envelope and plasma membrane whereby the virion transfers its nucleocapsid into the cytoplasm of the host cell. Virally encoded envelope glycoproteins play a major role in driving the membrane fusion reaction by helping to overcome the free energy barriers associated with this process (1). The envelope glycoprotein complex of HIV-1 is initially synthesized as a gp160 precursor molecule that is subsequently processed by a cellular protease into a mature surface, gp120 subunit noncovalently attached to a transmembrane gp41 subunit (2–4). The subunits are assembled as trimers (5), which are directed to the plasma membrane and then incorporated into budding virions. Recent studies (6, 7) show preferential budding of the virions through the so-called raft microdomains of the plasma membrane, which provides an explanation for the unusually high content of cholesterol and sphingomyelin in the viral envelope (8). Binding of gp120 with CD4 and a co-receptor (reviewed in Ref. 9) cooperatively initiates a series of conformational changes in the gp120/gp41 complex (10), which eventually promotes the actual merging of the viral and target cell membranes via structural rearrangements of the gp41 subunits.

Two main functional regions separated by a loop are identified within the extracellular 175-residue ectodomain of the gp41 subunit: N-terminal and C-terminal subdomains (see Fig. 1). The N-terminal subdomain is made of the N-terminal hydrophobic fusion peptide (FP) region and an adjacent α-helical leucine/isoleucine zipper termed the N-terminal heptad repeat (NHR). The FP is believed to play a pivotal role in the fusion event by inserting into the target membrane and directly effecting the fusion of apposing bilayers (reviewed in Refs. 11 and 12), although the mode of action remains illusive. The adjacent NHR is believed to be involved in structural re-arrangements that draw together opposing viral and target cell membranes (13). Recent studies show that the NHR acts synergistically with the FP to fuse vesicles, implying that these regions interact directly with membranes (14). We describe the C-terminal subdomain as reflecting its N-terminal counterpart. Specifically, it contains a heptad repeat termed the C-terminal heptad repeat (CHR) followed by a hydrophobic region called the tryptophan rich pre-transmembrane region, which was proposed to serve as an internal fusion peptide (IFP) (15). Peptides corre-
Membrane Targets of gp41 Fusion Peptides

réponding to the IFP are effective in inducing lipid mixing of model vesicles (15–17). The CHR, similar to the NHR, is believed to be involved in structural re-arrangements of gp41 that results in virus-target membrane apposition (13).

Current models describe the fusion process through a progression of conformational changes in the gp41 ectodomain. In the native state, gp41 is held in a metastable state by interaction with gp120 (13, 18). Binding of gp120 to receptors releases gp41 from metastable constraints, allowing it to transform (19) to an extended conformation termed the pre-hairpin intermediate (PHI). Although not observed, the PHI is characterized by exposed NHR and CHR regions (20–22), and exists transiently (20, 22), and the N-terminal fusion peptide region is believed to be inserted into the target membrane (11) thereby bridging the gap between the two membranes. Furthermore, the PHI is believed to be the target of peptide inhibitors derived from the gp41 ectodomain that presumably act by binding to regions in the PHI and competitively inhibit further conformational changes (21, 23–26). One example is C84, which is derived from the CHR of gp41 and inhibits cell-cell fusion at nanomolar concentrations by binding the NHR coiled-coil (25). Folding of the PHI into a stable hairpin conformation is believed to coincide with fusion. Contrary to the widely accepted belief that hairpin folding drives the fusion reaction by initiating pore formation, it was recently proposed that fusion pore formation occurs prior to hairpin formation and that hairpins are required for pore stabilization (27). The hairpin conformation was contrived based on studies of soluble fragments of the ectodomain, which revealed a thermostable core made of a parallel trimeric coiled-coil of NHR peptides with $\alpha$-helical structure, which may explain both the enhancement in lipid mixing of C56 relative to C20 (IFP) as well as the enhanced inhibitory effect of C56 compared with C34.

EXPERIMENTAL PROCEDURES

Materials—Rink-amide methylbenzyldihydramine resin and 9-Fmoc amino acids were purchased from Calbiochem-Nova-Biochem AG (Laufelfingen, Switzerland). Other reagents used for peptide synthesis included trifluoroacetic acid (Sigma), piperidine (Merck), N,N-diisopropylhydrazine (Sigma), benzyl alcohol (Acros), 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, and dimethylformamide (peptide synthesis grade, BioLab) were purchased from Sigma. Egg phosphatidylcholine (PC), phosphatidylserine, egg sphingomyelin (Spm), cholesterol (extra pure), and lysophosphatidylcholine (LPC) were purchased from Sigma as well. 5- and 6-[(4-chloromethyl)benzoyl]-aminotetramethylrhodamine (CMTMR) and calcine-AM were purchased from Molecular Probes (Eugene, OR). All of the additional reagents were of analytical grade. Buffers were prepared using double-distilled water.

Peptide Synthesis—The peptides were synthesized by the Fmoc solid phase method on Rink-amide methylbenzyldihydramine resin using an ABI 433A automatic peptide synthesizer and the resin was resuspended using trifluoroacetic acid:double-distilled water:TES at 18.5:1:0.5% (v/v). N70 was prepared as described previously (14). The peptides were purified by reverse-phase high pressure liquid chromatography on C4 reverse-phase Vydac semi-preparative and analytical columns to >98% homogeneity. The purified peptides were homogenous (>98%) by analytical high pressure liquid chromatography. The peptides were further subjected to amino acid analysis and to Platform LCZ electrospay mass spectroscopy to confirm their composition and molecular weight.

Preparation of Large Unilamellar Vesicles (LUV)—The lipids were dissolved in a 2:1 (v/v) mixture of chloroform:methanol and then dried under a stream of nitrogen while rotating, thereby deposing a thin film of lipids on the wall of a glass vial. Excess solvent was removed by overnight lyophilization, and the lipid films were stored under argon to prevent oxidation. Dry lipid films were suspended in PBS buffer by vortexing to produce large multilamellar vesicles. The lipid suspension was further processed with five cycles of freezing and thawing followed by extrusion through polycarbonate membranes initially with 1-µm diameter pores and then with 0.2-µm diameter pores to generate LUV. LUV were used within 24 h following preparation.

Peptide-induced Lipid Mixing—Lipid mixing of LUV was measured using a fluorescence-probe dilution assay (34). LUV containing 0.6 molar % each of NBD-PE as the energy donor and Rho-PE as the energy acceptor were prepared in PBS as described above. A 1:4 mixture of labeled and unlabeled phosphatidylcholine (10 µl total phospholipid concentration) was suspended in 400 µl of PBS, and a small volume of peptide from a stock solution was added. The increase in NBD fluorescence at 530 nm (8-nm slit) was monitored with the excitation set at 467 nm (8-nm slit). The fluorescence intensity before the addition of the peptide was referred to as 0% lipid mixing, and the fluorescence intensity upon addition of reduced Trion X-100 (0.05% (v/v)) was referred to as 100% lipid mixing. All of the fluorescence measurements were done on an SLM-AMINCO Bowman series 2 luminescence spectrometer.

ATR-FTIR Measurements—Spectra were obtained with a Bruker Equinox 55 FTIR spectrometer equipped with a deuterated triglyceride sulfate detector that was coupled to an ATR device. For each spectrum, 150 scans were collected with a resolution of 4 cm$^{-1}$. Samples were prepared as described previously (35). A mixture of lipids (0.5 mg) alone or with peptide was deposited on a ZnSe horizontal ATR prism (80 × 7 mm). Prior to preparing the samples, the trifluoroacetate (CF$_2$COO$^-$) counterions, which strongly associate with the peptide, were replaced with chloride ions by washing in 0.1 M HCl and lyophilization. This eliminated the strong C = O stretching absorption band near 1763 cm$^{-1}$ (36). Peptides were dissolved in MeOH, and lipids were dissolved in 1.2 MeOH/CH$_3$Cl mixture. Lipid-peptide mixtures or lipids alone were spread with a Teflon bar on the ZnSe prism and then dried under vacuum for 15 min. Polarized and non-polarized spectra were recorded. For analysis of the peptide amide I contribution alone, the non-polarized spectra were low-pass filtered at 4.5 cm$^{-1}$. For analysis of the phospholipid acyl chain order, symmetric (1820 cm$^{-1}$) and antisymmetric (1854 cm$^{-1}$) stretching vibrations were analyzed of spectra following polarization (both parallel and perpendicular). The ratio between parallel and perpendicular
pendicular polarized absorbance maxima for these two stretching vibrations was compared for samples of phospholipid alone and phospholipid + peptide. The background for each spectrum was a clean ZnSe prism. The multilayers formed, composed of PC:Spm:chol at 1:1:1 molar ratio, produce significant background signal in the amide I region due to absorption from the amide bond in sphingomyelin. Procuring an accurate peptide amide I signal necessitated the use of relatively high peptide:lipid molar ratios (1:50). For measurements conducted of peptides in PC multilayers, a peptide:lipid molar ratio of 1:100 was used. Deuteration of the sample was achieved by introducing an excess of deuterium oxide ($^{2}$H$_{2}$O) into a chamber placed on top the ZnSe prism in the ATR casting and incubating for 5 min before acquisition of spectra. The H/D exchange was considered complete due to a complete shift of the amide II band. Any contribution of CO vapor to the absorbance spectra near the amide I peak region was eliminated by subtraction of the spectrum of pure lipids equilibrated with $^{2}$H$_{2}$O under the same conditions.

**ATR-FTIR Data Analysis**—Prior to curve fitting, a straight base line passing through the ordinates at 1700 and 1600 cm$^{-1}$ was subtracted. To resolve overlapping bands, the spectra were processed using PEAKFIT$^{TM}$ (Jandel Scientific, San Rafael, CA) software. Second- and fourth-derivative spectra were calculated to identify the positions of the component bands in the spectra. These wave numbers were used as initial parameters for curve fitting with Gaussian component peaks. Positions, bandwidths, and amplitudes of the peaks were varied until: (i) the resulting bands shifted by no more than 2 cm$^{-1}$ from the initial parameters; (ii) all of the peaks had reasonable half-widths ($\sim$20–25 cm$^{-1}$); and (iii) good agreement between the calculated sum of all of the components and the experimental spectra was achieved ($r^{2} > 0.999$).

The relative contents of different secondary structure elements were estimated by dividing the areas of individual peaks, which were assigned to a particular secondary structure, by the whole area of the resulting amide I band. The results of two independent experiments were averaged.

**Analysis of the Polarized ATR-FTIR Spectra**—The ATR electric fields of incident light were calculated as shown in Equation 1 (37),

$$E_{\text{x}} = \frac{2\cos\theta \sqrt{\sin\theta - n_{21}^2}}{\sqrt{1 - n_{21}^2/[1 + n_{21}^2] \sin^2\theta - n_{21}^2}}$$

$$E_{\text{y}} = \frac{2\sin\theta \cos\theta}{\sqrt{1 - n_{21}^2/[1 + n_{21}^2] \sin^2\theta - n_{21}^2}}$$

(Eq. 1)

where $\theta$ is the angle of a light beam to the prism normal at the point of reflection (45°) and $n_{21} = n_{21}\text{N}\text{m}$ ($n_{1}$ and $n_{2}$ are the refractive indices of ZnSe taken as 2.4 and of the membrane sample taken as 1.5, respectively). Under these conditions, $E_{\text{x}}$, $E_{\text{y}}$, and $E_{\text{z}}$ are 1.09, 1.81, and 2.32, respectively. The electric field components together with the dichroic ratio ($R_{\text{VTR}}$), defined as the ratio between absorption of parallel to a perpendicular to the electric field, are used to calculate the orientation order parameter, $f$, by the following formula shown in Equation 2 (38),

$$f = \frac{2(E_{\text{x}}^2 - R_{\text{VTR}}E_{\text{y}}^2 + E_{\text{z}}^2)}{h(3\cos^2\alpha - 1)E_{\text{x}}^2 - R_{\text{VTR}}E_{\text{y}}^2 - 2E_{\text{z}}^2}$$

(Eq. 2)

where $h$ is the fraction of transition dipoles in the molecule that belong to the ordered structure of the absorption band whose order parameter has been calculated. Lipid order parameters were obtained from the symmetric ($\sim$2833 cm$^{-1}$) and antisymmetric ($\sim$2922 cm$^{-1}$) lipid-stretching modes using the same equations, setting $\alpha = 90^\circ$ (37). The average angle of orientation of the helical axis with respect to the bilayer normal, $\gamma$, is calculated based on the order parameter with the formula shown in Equation 3 (38),

$$f = 1/2(3\cos^2\gamma - 1)$$

(Eq. 3)

**Dye Transfer Fusion Assay**—Peptide inhibition of cell-cell fusion was assayed by monitoring the redistribution of a water-soluble fluorescent probe from the effector cell to the target cell upon their co-incubation with each other. SupT target cells were labeled with calcien-AM at 10 $\mu$M for 60 min at 37°C and then washed and resuspended in RPMI 1640 medium. The plated HeLa cells infected with the recombinant vaccinia virus construct VPE16, which expresses HIV-1 IIB gp120–41 (4), were labeled with CMTMR at 20 $\mu$M for 1 h at 37°C, washed several times, and combined with the target cells (1:3 effector-target cell ratio). Different concentrations of peptides, dissolved in PBS buffer, were then added. The cells were co-cultured for 2 h at 37°C in 24-well plates (Costar, Cambridge, MA). C34 and C56 inhibition were tested in parallel on the same day. These experiments were repeated several times, and the general trend was found to be the same. Both phase and fluorescent images were collected using an Olympus IX70 with a ×20 objective lens and a CCD camera (Princeton Instruments, Trenton, NJ). A 82,000 optical filter cube (Chroma Technology Corporation., Brattleboro, VT) was used for the excitation of calcine (494/517) and CMTMR (541/565). Three images per well were collected, and dye transfer from the donor to the acceptor cell was counted via Metamorph software (Universal Imaging, West Chester, PA). The scoring of fusion events was conducted as described previously (22).

**CD Spectroscopy**—The CD spectra of the different peptides (10 $\mu$M) in 1% LPC were determined in an Aviv 202 spectropolarimeter in a quartz optical cell with a 1-mm path length at 20°C in the range of 193–260 nm (1-nm steps, 10-s averaging). The spectrum of 1% LPC was subtracted from the spectra of the peptides in LPC.

**RESULTS AND DISCUSSION**

The C- and N-terminal Subdomains Potently Induce Lipid Mixing of LUV either Enriched in or Devoid of Cholesterol, Respectively—To compare the specificity with which peptides from the C- and N-terminal subdomains perturb membranes, we tested the ability of C20, C56, FP33, and N70 (refer to Fig. 1) to induce lipid mixing of LUV designed to reproduce the disparate membrane order conditions on the virion and target cell surface (following cholesterol depletion) (Figs. 2 and 3). PC LUV were chosen to mimic the cholesterol-depleted target cell surface because of their wettability and the fact that the outer leaflet of blood cell membranes is enriched in PC and sphingomyelin (reviewed in Ref. 39), both of which share the same head group. PC alone does not self-organize into ordered versus unordered regions and therefore represents a homogenous membrane surface (40). To reproduce the well ordered viral membrane surface, we chose a lipid composition based on the work of Aloia et al. (8) who quantified the lipid composition of virions and target cells. For the viral envelope, they identify the dominant outer leaflet choline lipids PC and Spm to be in approximate equal abundance and show an approximate 1:1 ratio of cholesterol to phospholipid. Accordingly, we model the virion surface with a PC:Spm:chol (1:1:1) lipid composition. It has been shown that cholesterol promotes lateral organization in membranes at physiologic temperatures when combined with Spm (40). Organization of raft structures follows the association of Spm and cholesterol. In in vitro vesicle preparations of Spm and cholesterol, optimal inhomogeneity in lipid dispersions occurs with 25–40 molar % cholesterol (40). Therefore, in addition to modeling the virion lipid surface, PC:Spm:chol (1:1:1) vesicles have a strong propensity to spontaneously form ordered regions.

Comparing Fig. 2 with Fig. 3, we show a clear correlation of the ability of C- and N-terminal constructs to perturb cholesterol-rich and cholesterol-free vesicles, respectively. Fig. 2 confirms our previous finding that N70 is a potent fusogen toward PC LUV, reiterating the important role that the NHR plays in membrane destabilization (14). In addition, we checked the ability of the N-terminal subdomain to disrupt vesicles whose lipid composition models that found in the outer leaflet of blood cells (Spm:PC:PE:chol:phosphatidylserine at a 11:10:4:3:1 ratio (reviewed in Refs. 39 and 41). This mixture of lipids includes the microdomain inducing sphingomyelin and cholesterol, although the cholesterol content at ~10% is much less than that in Spm:PC:chol (1:1:1). Evidently, the absence or presence of ~10% cholesterol does not affect the fusogenic function of the N-terminal subdomain. Comparable to FP33, C20 has relatively low activity toward PC LUV, whereas inclusion of the CHR (C56) enhances lipid-mixing activity (Fig. 2). In addition, C34 is ineffective at inducing lipid mixing of PC LUV (data not

18528
shown). Our results here confirm the lipid-mixing ability of C20 (15–17) alone. More importantly we show that, in the context of the C-terminal subdomain, the function of C20 is preserved with the continuous CHR now contributing to membrane destabilization.

Interestingly, we find that C-subdomain peptides are superior to N-subdomain peptides in perturbing cholesterol-rich vesicles (Fig. 3). C56 and C20 vigorously perturb PC:Spm:chol LUV with C56 showing enhanced activity compared with C20 (as in PC LUV). It has been previously reported that C20 effectively induces lipid mixing of PC:Spm:chol (1:1:1) LUV (17). We confirm this finding and show that, in identical vesicles, the CHR contributes to the lipid-mixing function of the C-terminal subdomain. On the other hand, FP33 is ineffective at inducing lipid mixing of PC:Spm:chol LUV over the concentration range tested, whereas the activity of N70 is significantly reduced compared with PC LUV. Collectively, these results delineate the membrane-destabilizing potency of gp41 ectodomain fragments. C-terminal subdomain fragments (C20 and C56) destabilize cholesterol-rich LUV preferentially, whereas N-terminal subdomain fragments (FP33 and N70) preferentially fuse vesicles that are either cholesterol-free or contain ~10% cholesterol. The finding that FP33 is ineffective in perturbing PC:Spm:chol LUV may represent a safety mechanism that prevents the FP from destabilizing the viral envelope shortly after metastable release of gp41 from gp120 caused by receptors binding or highly labile gp120-gp41 contacts due to the hypermutability of HIV in the absence of receptor binding.

For the two subdomains, the heptad repeats differ in the relative degree to which they contribute to lipid mixing of both cholesterol-rich and cholesterol-free vesicles. The NHR contrib-
The non-covalent N70-C56 parallels the additive effect of the peptides alone can be finding that the lipid-mixing function of the pre-mixed N70 and decreased ability of this complex to induce lipid mixing relative peptides within the context of the assay. Formation of the that there is neither synergism nor interference between free N70 act additively to induce lipid mixing of PC LUV, meaning peptides, we compared the lipid-mixing ability of N70 and in the Presence of N70 —Acting alone, both C56 and N70 perturb PC LUV. To understand the interactive effect of these peptides, we compared the lipid-mixing ability of N70 and C56 alone and pre-mixed (Fig. 4). The data indicate that C56 and N70 act additively to induce lipid mixing of PC LUV, meaning that there is neither synergism nor interference between free peptides within the context of the assay. Formation of the N70-C56 core would sequester the membrane-interacting NHR and possibly also the IFP regions, probably resulting in a decreased ability of this complex to induce lipid mixing relative to the combined effects of the free N70 and C56 peptides. Our finding that the lipid-mixing function of the pre-mixed N70 and C56 parallels the additive effect of the peptides alone can be interpreted in different ways. (i) The non-covalent N70-C56 core is formed when the peptides are pre-mixed in Me$_2$SO and then disassociates upon interaction with membranes as shown for the analogous N36-C34 core (44). (ii) The N70-C56 core is unable to form in Me$_2$SO, with the kinetics of lipid mixing of free peptides, much faster than core formation in LUV. (iii) The N70-C56 core is not formed in either Me$_2$SO or upon addition to membranes. It should be noted that we know of no reports showing HIV-1 non-covalent core formation using constructs that include the FP. It could be that for the N70 construct in solution, the hydrophobic FP folds back to interact with the hydrophobic groove of the NHR, thereby inhibiting CHR binding.

The ability of free peptides to induce lipid mixing and fusion of model vesicles follows the ability of the peptides to bind to and induce aggregation and fusion of vesicles. In the case of N70, we have shown that the peptides bind to vesicles and induce aggregation and subsequent fusion of the vesicles (14). Elucidation of the details by which membrane-associated peptides conduct the final fusion step promises to resolve central questions in protein-induced membrane fusion.

Circular Dichroism Spectroscopy Indicates That C56 Has Higher $\alpha$-Helical Content Than C20 in a Membrane-mimetic Environment—The structure of fusogenic peptides has been shown to be important for their ability to promote fusion (45). Because C56 is more effective than C20 at inducing lipid mixing of both PC and PC:Spm:chol (1:1:1) LUV, we compared their secondary structure in membrane-mimetic LPC micelles using CD. Light scattering caused by the liposomes in suspension in the range of wavelengths essential for secondary structure determination prevented the usage of any type of vesicles in the CD measurements. Therefore, LPC at micellar concentrations (1% w/v) was chosen as an applicable membrane mimetic environment with low light scattering effect. Both peptides displayed spectra with minima at 208 and 222 nm (Fig. 5), typical of $\alpha$-helix structure. Under these conditions, it has been reported that $\alpha$-helical contents are underestimated (46). The fractional helicity of a peptide is proportional to its mean residue ellipticity at 222 nm. Given the 1.5-fold increase in absorbance of C56 over C20 at 222 nm along with the fact that C56 is far longer than C20, the CD data clearly demonstrate that the $\alpha$-helical content of C56 is significantly higher than that of C20.

FTIR Spectroscopy Indicates That C56 Has Predominantly $\alpha$-Helical Content in Cholesterol-enriched Membranes—The enhanced fusogenic function of C56 compared with C20 may be related to differences in structure and/or peptide-membrane interactions. We used FTIR spectroscopy to study peptide structure in association with PC:Spm:chol (1:1:1) membranes and the ability of the peptides to disrupt acyl chain order of the membranes. The experimental conditions of peptides interacting with deuterated phospholipids multilayers closely approximate biological conditions. For structural characterization, deconvolution of the amide bond absorbance in the amide I region (1600–1700 cm$^{-1}$) allows identification of the main secondary structural components, which are characterized by regions of resolvable absorbance as described under the “Experimental Procedures.” The spectra corresponding to C20, C56, and C34 are shown in Fig. 6. The assignments, wave numbers, and
spectra superimpose on the experimental FTIR spectra. The fitted
together with the experimental FTIR spectra. The fitted
spectra superimpose on the experimental FTIR spectra with good agreement ($r^2 > 0.999$) and
are therefore not shown. The individual
fitted components are represented below by dashed lines. The fourth derivatives, calculated to identify the positions of the components bands in the spectra, are shown in panels A2, B2, and C2 for
C20, C56, and C34, respectively.

![Graph](image-url)

**TABLE I**

Secondary structure content of the peptides in deuterated PC:Spm:chol (1:1:1) multilayers determined by ATR-FTIR spectroscopy

<table>
<thead>
<tr>
<th>Peptide</th>
<th>$\beta$-Aggregate, $1620-1625$ cm$^{-1}$</th>
<th>$\beta$-Sheet, $1625-1640$ cm$^{-1}$</th>
<th>Random coil, $1640-1664$ cm$^{-1}$</th>
<th>$\alpha$-Helix, $1650-1655$ cm$^{-1}$</th>
<th>$\beta$ Turn, $1655-1670$ cm$^{-1}$</th>
<th>$\beta$ Turn, $1670-1685$ cm$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>C20</td>
<td>1621 (11%)</td>
<td>1637 (19%)</td>
<td>1656 (58%)</td>
<td>1670 (11%)</td>
<td>1678 (12%)</td>
<td></td>
</tr>
<tr>
<td>C56</td>
<td>1622 (12%)</td>
<td>1636 (14%)</td>
<td>1654 (57%)</td>
<td>1664 (16%)</td>
<td>1681 (6%)</td>
<td></td>
</tr>
<tr>
<td>C34</td>
<td>1622 (2%)</td>
<td>1643 (60%)</td>
<td></td>
<td>1664 (16%)</td>
<td>1677 (&lt;5%)</td>
<td></td>
</tr>
</tbody>
</table>

* The wave number of each component peak in the spectral deconvolution is indicated with the percent area relative to the sum of contributing component peaks in parentheses.

relative areas of the component peaks are listed in Table I. The different secondary structural elements were assigned according to Jackson and Mantch (47). The spectrum between 1700 cm$^{-1}$ and 1730 cm$^{-1}$ in which proteins do not absorb was practically a straight line, confirming no contribution of residual water to the spectrum (data not shown).

Comparing C20 with C56 in Fig. 6, we see that their absorbance spectra in the amide I region are very similar. Deconvolution reveals the major component peak for C20 and C56 as being centered at 1656 and 1654, respectively, indicating predominant $\alpha$-helical structure for both membrane-associated constructs. In this initial structural analysis of C20 and C56, we see only small differences. Specifically, a component peak at 1676 corresponding to $3_{10}$-helical contribution is revealed in the fourth derivative of C56 and not C20 with a relative contribution of 11%. Otherwise, the two peptides share similar and dominant $\alpha$-helical contributions (>55%) with lesser $\beta$-sheet and $\beta$-aggregate contributions. Under similar conditions, Nieva and co-workers (15) report that C20 adopts mainly $\alpha$-helical structure. Because C56 is 36 residues longer than C20, it has more residues contributing to $\alpha$-helical structure. Examining the structure of the CHR (C34) alone in membranes, we see that its amide I absorbance spectra differs from that of C20 and C56 by being more broad with the maximal absorbance shifted to the left, away from the $\alpha$-helical band. Deconvolution reveals that the major component peak is centered at 1643 in the random coil region, indicating that C34 is largely unstructured in deuterated membranes.

The findings that the C-terminal subdomain is more effective than the IFP at inducing lipid mixing of both cholesterol-rich and cholesterol-free LUV may be explained by cooperative stabilization of the CHR and IFP regions in the context of the C-terminal subdomain. As suggested by FTIR experiments in deuterated PC:Spm:chol multilayers and supported by CD measurements in membrane-mimetic LPC micelles, the C-terminal subdomain has an $\alpha$-helical fragment that is longer than that of the IFP. Because C56 is predominantly $\alpha$-helical with no random coil contributions, helical structure is induced in the CHR when conjugated to the largely helical IFP since the free CHR peptide (C34) is largely unstructured in deuterated membranes as in solution (48), which may explain its lack of lipid-mixing function. Therefore, interaction with the deuterated membranes does not induce or stabilize $\alpha$-helical structure in the CHR as does interaction with other regions in the gp41 ectodomain (IFP and NHR). It seems likely that the primary and/or secondary structural arrangement of the IFP as an adjacent region is required to stabilize the CHR secondary structure toward a predominantly helical state, similar to the one it assumes in the hairpin. As the CHR structure is stabilized by the IFP, alternatively, the CHR probably stabilizes the IFP to a degree, specifically at the N terminus of the IFP. The complementary ability of the two regions to stabilize one another will affect their functional roles as discrete regions in the context of the protein. Stabilization of the IFP by the CHR will enhance the membrane-perturbing ability of the IFP. Stabilization of the CHR by the IFP will enhance the ability of the CHR to interact with the coiled-coil NHR regions, facilitating hairpin formation.

Both C20 and C56 Disrupt phospholipid Acyl Chain Order in Cholesterol-enriched Membranes—Polarized ATR-FTIR spectroscopy was used to analyze the effect of the peptides on membrane order of both PC and PC:Spm:chol (1:1:1) membranes. The orientation of each multilayer with and without peptide was determined. The symmetric ($v_{\text{sym}} = 2853$ cm$^{-1}$) and antisymmetric ($v_{\text{antisym}} = 2923$ cm$^{-1}$) vibrations of lipid methylene C–H bonds are perpendicular to the molecular axis of fully extended hydrocarbon chains in membranes. Measuring the dichroism of infrared light absorbance can reveal the order and orientation of the membrane sample relative to the prism surface. The $R$ value corresponding to both the symmetric and anti-symmetric vibrations for the lipids in the absence of peptides were determined, and the order parameters $f$ were
calculated. The order parameters based on the symmetric and anti-symmetric vibrations are both similar in magnitude and follow an identical trend in variation between pure multilayers and peptides in multilayers. Therefore, we show only the order parameter derived from the symmetric vibrations. The effect of the peptides on the acyl chain order was estimated by comparing the CH2-stretching dichroic ratio of pure multilayers alone with that obtained for peptide bound to identical multilayers with membrane-bound peptide. The calculated ratios given as \( R \) values with corresponding order parameters \( f \) are shown in Table II. The data reveal that both C20 and C56 disrupt the acyl chain order of PC:Spm:chol membranes to a similar degree with C34 slightly more disruptive. These results suggest that the differential lipid-mixing ability of C56 and C20 is not a function of their ability to disrupt the order of the membranes with which they interact. Our findings that the CHR is largely unstructured in deuterated membranes but adopts helical structure when conjugated to C20 suggest that free CHR peptides are artifactual in structure. Therefore, the effect of C34 alone on membrane order should be considered with caution. In correlation to their ability to induce lipid mixing of PC:Spm: chol vesicles, N70 disrupts the same multilayers to a lesser degree relative to C20 and C56, whereas FP33 is ineffective in perturbing the membrane order.

The ability of the peptides to affect membrane organization of PC multilayers runs parallel to their ability to induce lipid mixing of the same vesicles. FP33 and C20 are marginal in inducing lipid mixing of PC LUV, and each peptide in PC multilayers shows no measurable change in lipid order relative to pure PC multilayers. The same is true for C34, which displays no lipid mixing of PC LUV. For the constructs corresponding to the two subdomains, we see that both N70 and C56 significantly perturb PC multilayers.

When exposed and interacting with membranes during fusion \( in vivo \), it is probable that the CHR region preferably occupies an \( \alpha \)-helical conformation similar to its hairpin helix. This amphipathic \( \alpha \)-helical arrangement provides a hydrophobic interface for associating with the membrane, with the potential to affect membrane destabilization. However, our results comparing peptide-membrane interactions show that both the IFP and C-terminal subdomain disrupt the lipid acyl chain order to the same degree for ordered multilayers. Although not conclusive, these results suggest that \( \alpha \)-helical regions of the CHR are not contributing to membrane destabilization directly and that their primary role may be interdigitation with the coiled-coil to form the hairpin. Collectively, our findings point to the IFP, stabilized in context of the C-terminal subdomain, as being primarily responsible for the membrane fusion function of the C-terminal subdomain.

C56 Is an Inhibitor of HIV-1-induced Cell-Cell Fusion—Free peptides derived from the C-terminal subdomain, such as C34 and T20, have been shown to inhibit cell-cell fusion at nanomolar concentrations (23, 25). The peptides are believed to act through a dominant-negative mechanism to interfere with formation of the "hairpin"-like conformation of the ectodomain by specific binding to the hydrophobic groove on the surface of the N-helical coiled-coil (13, 18, 23, 44, 49). Peptide binding to the exposed coiled-coil presumably prevents gp41 folding into the hairpin, which is believed to correspond with fusion. Modifications to C34 and T20 that stabilize the \( \alpha \)-helical structure improve their inhibitory activity (50, 51), presumably by enhancing their interaction with the exposed viral coiled-coil in the PHI conformation. Because of the strong \( \alpha \)-helical signal of C56 in a membrane-mimetic environment, we postulated that C56 will be an effective inhibitor of cell-cell fusion. We performed separate experiments with labeled cells testing the inhibition activity of C56 with C34 used as a control. The plotted data were fit to a hyperbolic decay function (Fig. 7). The concentration at which 50% inhibition is reached (IC\(_{50}\)) can be extracted from each curve. The IC\(_{50}\) for C34 and C56 were estimated at 125 ± 34 nm \( (R^2 = 0.98) \) and at 66 ± 38 nm \( (R^2 = 0.97) \), respectively. The difference in IC\(_{50}\) between the two peptides is ~2-fold. Student’s \( t \) tests showed that the difference in the patterns of inhibition shown in Fig. 7 is statistically significant \( (p = 4 \times 10^{-7}) \). It should be noted that the previously reported value of IC\(_{50}\) for C34 in a similar assay was lower than the one determined here (42). The main reason for the difference is that the vaccinia system utilized in our experiment expresses a higher amount of gp120/gp41 on the cell surface. Accordingly, more C34 is required to inhibit fusion. Our results show that the C-terminal subdomain preserves the highly potent inhibitory activity of C34. Moreover, the data suggest that the C-terminal subdomain is even a slightly better inhibitor than C34. This leads us to postulate that the C-terminal subdomain maintains the required structure to carry out its function throughout the fusion event.

Concluding Remarks—Membrane fusion can be described through a succession of stages: (i) apposition of membranes; (ii) hemifusion of outer leaflets to form a stalk; (iii) fusion pore formation through fusion of both bilayers at the locus of the stalk; and (iv) fusion pore enlargement with full content mixing. Recently, Markosyan et al. (27) showed that fusion pore

### Table II

<table>
<thead>
<tr>
<th>Peptide</th>
<th>( R^\circ )</th>
<th>( f^\circ )</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC</td>
<td>1.41 ± 0.02</td>
<td>0.28 ± 0.01</td>
</tr>
<tr>
<td>PC + C20</td>
<td>1.42 ± 0.01</td>
<td>0.27 ± 0.01</td>
</tr>
<tr>
<td>PC + C56</td>
<td>1.58 ± 0.02</td>
<td>0.19 ± 0.01</td>
</tr>
<tr>
<td>PC + C34</td>
<td>1.37 ± 0.02</td>
<td>0.29 ± 0.01</td>
</tr>
<tr>
<td>PC + FP33</td>
<td>1.42 ± 0.01</td>
<td>0.26 ± 0.01</td>
</tr>
<tr>
<td>PC + N70</td>
<td>1.55 ± 0.06</td>
<td>0.20 ± 0.02</td>
</tr>
<tr>
<td>PC:Spm:chol (1:1:1)</td>
<td>1.15 ± 0.02</td>
<td>0.42 ± 0.01</td>
</tr>
<tr>
<td>PC:Spm:chol + C20</td>
<td>1.52 ± 0.02</td>
<td>0.21 ± 0.01</td>
</tr>
<tr>
<td>PC:Spm:chol + C56</td>
<td>1.49 ± 0.02</td>
<td>0.23 ± 0.01</td>
</tr>
<tr>
<td>PC:Spm:chol + C34</td>
<td>1.52 ± 0.01</td>
<td>0.21 ± 0.01</td>
</tr>
<tr>
<td>PC:Spm:chol + FP33</td>
<td>1.15 ± 0.01</td>
<td>0.42 ± 0.01</td>
</tr>
<tr>
<td>PC:Spm:chol + N70</td>
<td>1.28 ± 0.02</td>
<td>0.34 ± 0.01</td>
</tr>
</tbody>
</table>

- \( R \) is the ratio of parallel to perpendicularly polarized light at the indicated wave number.
- \( f \) is the order parameter based on \( R \).
formation occurs prior to hairpin formation. These results suggest that exposed regions of the PHI are responsible for the bulk of the fusion work (up until fusion pore initiation) through protein-membrane interactions because incubation with free NHR and CHR peptides freezes fusion presumably by sequestering exposed viral NHR and CHR regions through peptide-
protein interactions (27). Prior to hairpin folding, the two subdomains have the opportunity to interact with their adjacent membranes. Specifically, the C-terminal subdomain can interact with the adjacent viral envelope, whereas the N-terminal subdomain can interact with the target cell membrane. Membrane fusion requires membrane destabilization, and it has been shown that regions from both C- and N-terminal subdomains perturb membranes (14, 16, 17, 45, 52–54). Based on our results, we propose that the C- and N-terminal subdomains exert their effects following PHI formation and up until the hairpin formation through direct protein-membrane interactions (refer to Fig. 8). We believe that the role of the C-terminal subdomain, similar to the N-terminal subdomain, has the dual function of binding to and destabilizing membranes and folding into the hairpin conformation. These actions are necessarily sequential. For the C-terminal subdomain, the IFP binds to and destabilizes the viral membrane as an α-helix, which is stabilized by the CHR. The CHR is also helical, stabilized by the IFP, but seems to be less involved in membrane interaction and more reserved for interdigitation with the NHR coiled-coil to form the hairpin at later stages when these regions become juxtaposed through membrane apposition or changes in membrane architecture. For the N-terminal subdomain, the NHR binds to membranes, localizing inserted FPs, and both regions combine to destabilize the target cell membrane. The N-terminal subdomain is as effective in destabilizing both unordered cholesterol-free membranes and cholesterol-containing membranes whose lipid composition mimics that of the blood cell surface but has diminished effect on inhomogenous cholesterol-rich membranes. Our observations imply that in addition to the known function of the CHR to interact with the NHR to form the hairpin, in the context of the C-terminal subdomain, the CHR can contribute to the HIV-1 fusion process by coordinating with the IFP to affect the organization of the highly ordered cholesterol-rich viral membrane.

REFERENCES

The C- and the N-terminal Regions of Glycoprotein 41 Ectodomain Fuse Membranes Enriched and Not Enriched with Cholesterol, Respectively
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