The Tryptophan-Rich Region of HIV gp41 and the Promotion of Cholesterol-Rich Domains†

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ABSTRACT: The peptide *N*-acetyl-KWASLWNWFNITNWLWYIK-amide has a sequence that corresponds to the juxtamembrane region of the HIV-1 gp41 fusion protein. We have studied how cholesterol modulates the interaction of this peptide with membranes containing cholesterol using differential scanning calorimetry, circular dichroism, fluorescence spectroscopy, and nuclear magnetic resonance. We find that this peptide is less able to sequester cholesterol into domains than is *N*-acetyl-LWYIK-amide. On the other hand, the peptide *N*-acetyl-LASWIK-amide, which corresponds to a segment of HIV-2 and SIV gp41 fusion proteins, has intermediate potency between *N*-acetyl-KWASLWNWFNITNWLWYIK-amide and *N*-acetyl-LWYIK-amide in forming areas enriched in cholesterol, even though it does not have a cholesterol recognition/interaction amino acid consensus sequence (CRAC). We suggest that the difference between HIV-1 and HIV-2 in their requirements for glycosphingolipids in determining their tropism is related to their difference in partitioning to cholesterol-rich domains in biological membranes.

Domains in biological membranes that are enriched in cholesterol, play an important role in several steps of the reproductive cycle of HIV,[1] including the assembly and budding of the virus from infected cells (*1*, *2*). Evidence that HIV buds from raft domains includes the finding that the viral envelop is enriched in both lipids (*3*) and GPI-anchored proteins (*2*, *4*) found in "raft" domains of mammalian membranes. Viral entry into cells does not necessarily require the presence of cholesterol (*5*). However, the removal of cholesterol with methyl-β-cyclodextrin from cells with relatively low receptor densities reduces the capacity of HIV-1 to trigger fusion (*5*). Extraction of cholesterol from the envelop of HIV-1 or SIV also results in reduced infectivity (*6*). Several components that facilitate the entry of HIV into cells are suggested to be associated with raft domains in membranes, including cholesterol, CD4, the coreceptors CXCR4 and CCR5 as well as glycosphingolipids (*7*). In addition, actin is suggested to play an important role in the transport of HIV proteins. Actin polymerization is modulated, at least in part, by interaction with membrane rafts (*8*). There are thus several mechanisms by which raft domains could facilitate membrane interactions with HIV.

A consensus sequence, termed the CRAC motif, has been suggested to sequester proteins to cholesterol-rich domains of membranes. This motif has the pattern -L/V-(X)(1−5)-Y-(X)(1−5)-R/K-, in which (X)(1−5) represents between one to five residues of any amino acid (*9*). The HIV-1 fusion protein, gp41, has a segment, LWYIK, adjacent to the transmembrane helix that fulfills the requirements of a CRAC motif. We have shown that a synthetic peptide, *N*-acetyl-LWYIK-amide, induces the formation of cholesterol-rich domains in membranes comprised of cholesterol and phosphatidylcholine (*10*). The sequence LWYIK is located at the carboxyl-terminus of a longer membrane-proximal region that is rich in Trp. It has been shown by mutational studies that this region is important for membrane fusion and virus infectivity (*11*). This longer membrane-proximal sequence has also been studied as an isolated 20 amino acid, synthetic peptide and found to aggregate and promote fusion of liposomes containing sphingomyelin and cholesterol, the principal lipid components of membrane rafts (*12*, *13*). The amino-terminal region of this peptide is responsible for the formation of peptide oligomers (*14*).

In the present work we compare a peptide, *N*-acetyl-KWASLWNWFNITNWLWYIK-amide, corresponding to the full Trp-rich, membrane-proximal segment of HIV-1_{HXB2} with the carboxyl-terminal pentapeptide, *N*-acetyl-LWYIK-amide (*10*). The longer peptide corresponds to residues 665−683 of the gp41 protein. The presence of an aromatic domain adjacent to a transmembrane helix also occurs in the fusion protein of Coronavirus (*15*). Furthermore it was demonstrated that the membrane-permeabilizing activity per peptide bound for peptides corresponding to this aromatic domain in Coronavirus is very high in the presence of cholesterol. In the HIV-1 sequence database there are variants having a Ser at position at position 681 and that therefore do not formally

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[1] Abbreviations: HIV, human immunodeficiency virus; SIV, simian immunodeficiency virus; CRAC, cholesterol recognition/interaction amino acid consensus; PC, phosphatidylcholine; PO, 1-palmitoyl-2-oleoyl; SO, 1-stearoyl-2-oleoyl; MAS, magic angle spinning; DP, direct polarization; MLV, multilamellar vesicle; LUV, large unilamellar vesicle; SUV, small unilamellar vesicle; DSC, differential scanning calorimetry; T_m, transition temperature; ΔH, calorimetric enthalpy.

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have a CRAC motif. In addition, HIV-2 as well as several strains of SIV are also devoid of a CRAC motif. For example, for HIV-2CBL24, $SIV_{cpz(Q88004)}$, SIV_{mac251}, SIV_{agm} and SIVsm84, the segment 679−683, adjacent to the trasmembrane segment has the sequence LASWIK (*16*), similar to the sequence LWYIK of HIV-1, but not fulfilling the requirements of the CRAC motif because it has no Tyr (*9*). Consequently, the ability of the peptide *N*-acetyl-LASWIK-amide to induce the formation of cholesterol-rich domains in membranes, was also tested in this work.

EXPERIMENTAL PROCEDURES

Materials. The peptides, *N*-acetyl-LASWIK-amide and *N*-acetyl-KWASLWNWFNITNWLWYIK-amide were synthesized by SynPep Corporation (Dublin, CA) and purified by HPLC. Phospholipids and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL).

Preparation of Samples for DSC and NMR Experiments. Phospholipid and cholesterol were codissolved in chloroform/methanol (2/1, v/v). For samples containing peptide, an aliquot of a solution of the peptide in methanol was added to the lipid solution in chloroform/methanol. The solvent was then evaporated under a stream of nitrogen with constant rotation of a test tube so as to deposit a uniform film of lipid over the bottom third of the tube. Last traces of solvent were removed by placing the tube under high vacuum for at least 2 h. The lipid film was then hydrated with 20 mM PIPES, 1 mM EDTA, 150 mM NaCl with 0.002% NaN_3, pH 7.40 and suspended by intermittent vortexing and heating to 50 °C over a period of 2 min under argon. Samples used for NMR analysis were hydrated with the same buffer made in ^2H_2O and adjusted to a pH meter reading of 7.0 (pD = 7.4) and incubated at least 24 h at 4 °C to allow conversion of anhydrous cholesterol crystals to the monohydrate form. For the NMR measurements, a suspension containing about 10 mg of sample in 200 μL of buffer were spun in an Eppendorf centrifuge at room temperature. The resulting hydrated pellet was transferred to a 4 mm zirconia rotor with a 12 μL Kel-F insert, attempting to pack the maximal amount of lipid into the rotor while maintaining it wet.

Differential Scanning Calorimetry (DSC). Measurements were made using a Nano Differential Scanning Calorimeter (Calorimetry Sciences Corporation, American Fork, UT). The scan rate was 2 °C/min and there was a delay of 5 min between sequential scans in a series to allow for thermal equilibration. The features of the design of this instrument have been described (*17*). DSC curves were analyzed by using the fitting program, DA-2, provided by Microcal Inc. (Northampton, MA) and plotted with Origin, version 5.0.

Circular Dichroism (CD). The CD spectra were recorded using an AVIV model 61 DS CD instrument (AVIV Associates, Lakewood, NJ). The sample was contained in a 1 mm path-length quartz cell that was maintained at 25 °C in a thermostated cell holder. The CD data are expressed as the mean residue ellipticity. Lipid films to be used for CD were prepared as for DSC. The film was hydrated in 10 mM sodium phosphate buffer containing 0.14 M NaCl and 1mM EDTA at pH 7.4 and vortexed. The suspension was then sonicated to clarity to make SUVs.

Tryptophan Fluorescence Studies. The peptide was dissolved in 10 mM Hepes buffer, 0.14 M NaCl, 0.1 mM EDTA, pH 7.4. Dilutions were made from a 1 mg/mL stock solution. The concentration of peptide was determined by absorbance at 280 nm using an extinction coefficient calculated from the amino acid content (*18*). Excitation wavelengths of 280 and 295 nm were used, with 4 nm bandwidth in excitation and 8 nm in emission. Polarizers, set to 90° in excitation and 0° in emission, were used to reduce stray light. Fluorescence emission spectra were measured using ultrasensitive quartz mirror microcuvettes (N. L. Vekshin, Institute of Cell Biophysics, Pushchino, Russia). Spectra were determined at 25 °C using an SLM Aminco Bowman AB-2 spectrofluorimeter. The spectra were corrected for instrumental factors and a buffer blank was subtracted. Inner filter effect corrections were applied. When the fluorescence of the peptide was measured in the presence of lipid, the sample was prepared by hydrating the lipid film in Hepes buffer, vortexing and sonicating to make SUVs. The same concentration of SUVs of lipid without peptide was used as a blank. Concentration of the peptide in the cuvette for all samples was 150 μM.

^{1}H NOESY MAS NMR. High-resolution MAS spectra were acquired using a 4 mm zirconia rotor with a 12 μL Kel-F insert spinning at 5.5 kHz in a Bruker AV 500 NMR spectrometer. Probe temperature was 24 ± 1 °C. The 2D-NOESY spectra were obtained using mixing times of 50 and 300 ms. Resonances were assigned based on reports of phosphatidylcholine (*19*), cholesterol (*20*) and amino acid residues (*21*).

^{13}C DP/MAS NMR. A 4 mm zirconia rotor with the 12 μL Kel-F insert was placed in a Bruker Avance 300 spectrometer operating at 75.48 MHz for ^{13}C. The spectra were referenced to an external standard of glycine crystals, assigning a chemical shift of 176.14 ppm for the carbonyl carbon. Samples were spun at 5 kHz. The temperature inside the rotor was 25 ± 1 °C. Single pulse excitation with high power proton decoupling was used with a 4 μs pulse for ^{13}C and the proton frequency optimized for decoupling. A recycle time of 5 s was used.

RESULTS

DSC. DSC can be used to assess the extent to which a peptide causes segregation of cholesterol and phospholipid components (*22*). There are two criteria that indicate peptide-promoted phase separation of cholesterol-rich domains. One is that the formation of a cholesterol-rich domain has to be accompanied by the depletion of cholesterol from another region of the membrane. The cholesterol-depleted region would exhibit an increase in the enthalpy of the chain melting transition of SOPC in a mixture of SOPC and cholesterol. In this study we have used the low melting lipid SOPC since it is known that lipids with higher melting temperatures form domains with cholesterol even in the absence of peptide. This study is directed to show peptide-induced formation of cholesterol-rich domains. The other indication is that, when cholesterol passes its solubility limit in the cholesterol-rich domain, it will associate to form cholesterol crystallites. In the case of *N*-acetyl-LWYIK-amide there is a marked increase in the chain melting transition of SOPC upon addition of the peptide to mixtures of this lipid and cholesterol (*10*). In contrast, addition of *N*-acetyl-KWASL-WNWFNITNWLWYIK-amide to mixtures of SOPC with

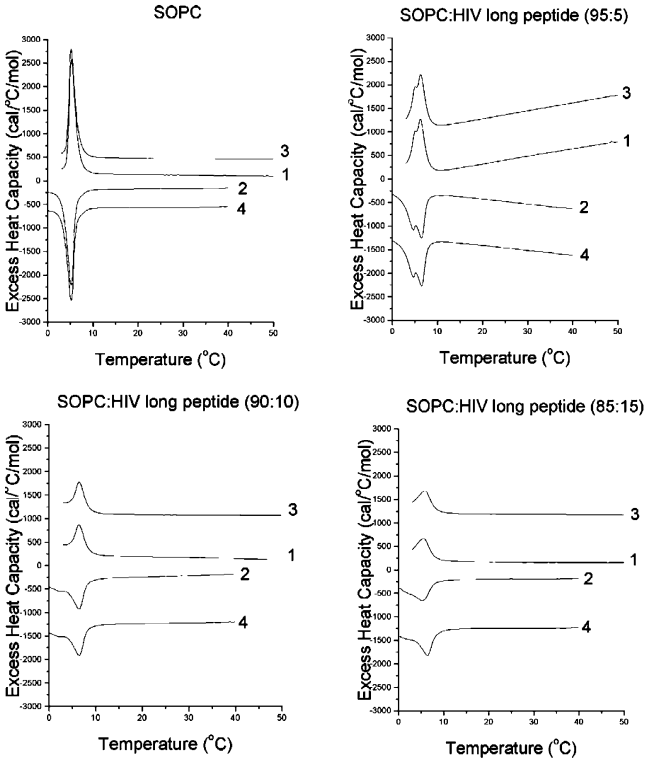

FIGURE 1: Differential scanning calorimetry of SOPC with 0, 5, 10 and 15 mol % *N*-acetyl-KWASLWNWFNITNWLWYIK-amide. Scan rate 2K/min. Lipid concentration 2.5 mg/mL in 20 mM PIPES, 1 mM EDTA, 150 mM NaCl with 0.002% NaN_3, pH 7.40. Sequential heating and cooling scans between 0 and 50 °C. Numbers are the order in which the scans were carried out, with scans 1 and 3 being heating scans, each of which was followed by one of the cooling scans 2 or 4. Scans were displaced along the *y*-axis for clarity of presentation.

Table 1: Effect of *N*-Acetyl-KWASLWNWFNITNWLWYIK-amide on the Gel to Liquid Crystalline Phase Transition of SOPC

% peptide	T_m (°C)	ΔH (kcal/mol of SOPC)
0	5.2	4.5
5	6.2[a]	3.1
10	6.4	2.2
15	5.8	1.9

[a] Temperature becomes progressively lower with each successive scan.

either 30, 40 or 50 mol % cholesterol results in a broadening and lowering of the enthalpy of the SOPC transition to an extent that it can no longer be accurately measured. This observation can be explained by the fact that the peptide also sequesters into cholesterol-depleted domains. This phenomenon is demonstrated directly by measuring the DSC of mixtures of *N*-acetyl-KWASLWNWFNITNWLWYIK-amide and SOPC without cholesterol (Figure 1). These scans show that the peptide causes a broadening of the phase transition and a lowering of the transition enthalpy (Table 1). The effect of the peptide is related to its mole fraction in the membrane. The finding that the SOPC phase transition is clearly split into two components in the presence of 5 mol % peptide suggests that the peptide is not uniformly distributed in the membrane. For the samples containing cholesterol, the polymorphic transition of anhydrous cholesterol crystals was observed only in the sample having the highest mol fraction of cholesterol (50 mol %) and the highest percent of peptide (15%) among the samples studied. This mixture gave a cholesterol crystallite transition at 35.6 °C on heating and 24.2 °C on cooling with an enthalpy of 70 cal/mol cholesterol. The formation of cholesterol crystallites at high concentrations of peptide and cholesterol demonstrates that *N*-acetyl-KWASLWNWFNITNWLWYIK-amide is not uniformly distributed between cholesterol-rich and cholesterol-depleted domains.

Table 2: Enthalpy of the Polymorphic Transition of Anhydrous Cholesterol in Mixtures of SOPC, Cholesterol, and *N*-Acetyl-LASWIK-amide[a]

mol % cholesterol[b]	mol % *N*-acetyl-LASWIK-amide	ΔH (cal/mol of cholesterol)
40	15	8
50	5	12
50	10	60
50	15	67

[a] Only samples showing cholesterol crystalline transitions with *N*-acetyl-LASWIK-amide are shown in this table. [b] Remaining lipid is SOPC.

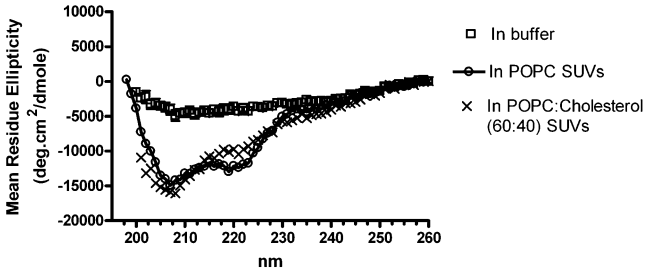

FIGURE 2: CD spectra of *N*-acetyl-KWASLWNWFNITNWLWYIK-amide. Peptide was in 10 mM sodium phosphate buffer containing 0.14 M NaCl and 1mM EDTA at pH 7.4. Lipid when present is in the form of SUVs at a concentration of 500 μM (100/1 lipid-to-peptide ratio). Measurements were made at 25 °C. Peptide in buffer without lipid (open square); Peptide in the presence of POPC (open circle); Peptide in the presence of POPC:cholesterol (6:4) (X).

The DSC of mixtures of 5, 10 or 15 mol % *N*-acetyl-LASWIK-amide with SOPC does not result in a change in the enthalpy of the phospholipid chain melting transition. This is similar to the behavior of *N*-acetyl-LWYIK-amide (*10*) but different than *N*-acetyl-KWASLWNWFNITNWLWYIK-amide (Figure 1). In mixtures of SOPC with 30 mol % cholesterol, *N*-acetyl-LASWIK-amide causes a small increase in the enthalpy of the chain melting transition of SOPC going from 500 to 600 cal/mole SOPC upon addition of 10 mol % *N*-acetyl-LASWIK-amide. However, at higher mol fractions of cholesterol the increase in the transition enthalpy of SOPC caused by *N*-acetyl-LASWIK-amide was not significant. There was, however, evidence for cholesterol crystallite formation at higher mol fractions of cholesterol and *N*-acetyl-LASWIK-amide (Table 2). Since this peptide does not interact with pure SOPC, it suggests that the formation of cholesterol crystallites results from the preferential interaction of the peptide with cholesterol-rich domains.

CD. The CD spectrum of *N*-acetyl-KWASLWNWFNITNWLWYIK-amide was measured in buffer and in the presence of SUVs of POPC and of POPC with 40 mol % cholesterol (Figure 2). There is a marked increase in helical structure in the presence of lipid. It is somewhat greater with

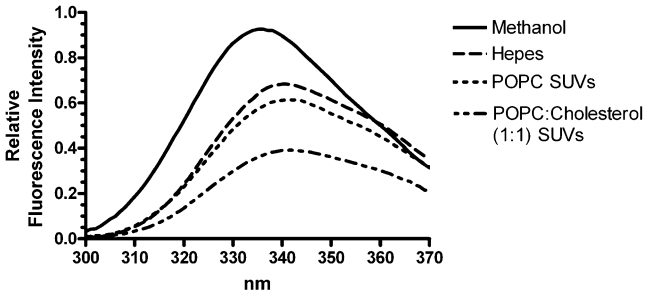

FIGURE 3: Fluorescence emission of *N*-acetyl-LASWIK-amide. Excitation wavelength 280 nm. Spectra are for a solution of the peptide in methanol (Solid line) or in 10 mM Hepes buffer, 0.14 M NaCl, 0.1 mM EDTA, pH 7.4 (Long dashes) or the peptide mixed with sonicated liposomes of POPC at a 1:1 peptide-to-lipid ratio (Short dashes) or the peptide mixed with sonicated liposomes of POPC:cholesterol (Long and short dashes) at a 1:1 peptide-to-lipid ratio. All samples contained 75 μM peptide. The spectra are plotted relative to the maximum emission intensity in methanol as 1.

POPC alone than with mixtures of POPC and cholesterol. The extent of secondary structure was not quantified due to possible spectral distortions caused by light scattering. The peptide has very low solubility in aqueous solutions where it aggregates.

Fluorescence Emission from Tryptophan. The peptide *N*-acetyl-LASWIK-amide has only one fluorescent group, the indole ring of Trp. The fluorescence properties of this peptide in various environments (Figure 3) is very similar to that previously reported for *N*-acetyl-LWYIK-amide (*10*).

^{1}H NOESY MAS NMR. The peptide *N*-acetyl-KWASLWNWFNITNWLWYIK-amide does not exhibit strong cross-peaks between the aromatic protons and those of the lipid in mixtures with POPC (Figure 4). In particular, the cross-peaks of the aromatic protons of the peptide with the methylene groups at 1.35 ppm or the terminal methyl groups of the acyl chains at 0.95 ppm are weaker than the cross-peak with the quaternary ammonium methyl group at 3.3 ppm. This suggests that this peptide does not insert deeply into POPC bilayers. Addition of equimolar cholesterol to the POPC results in more intense cross-peaks between the aromatic protons and protons from the acyl chains of the phospholipid. There is an inversion of sign of the cross-peaks using mixing times of 50 vs 300 ms (Figure 4). A negative sign of the NOESY cross-peak corresponds to rapid motion. The reversal of sign may be a result of slow exchange between two populations of the peptide. Another effect of cholesterol is to cause a splitting of the quaternary ammonium peak of the lipid headgroup in the presence of *N*-acetyl-KWASLWNWFNITNWLWYIK-amide.

The ^{1}H NOESY MAS NMR of *N*-acetyl-LASWIK-amide in POPC (Figure 5) shows a stronger cross-peak with the terminal methyl group of the phospholipid acyl chain, compared with *N*-acetyl-LWYIK-amide (*10*). However, even for *N*-acetyl-LASWIK-amide there appears to be greater insertion of the peptide into the membrane in the presence of cholesterol since in the slices from the NOESY with cholesterol, the 3.3 ppm peak is generally weaker and less well defined than that for the sample without cholesterol. In addition, using the 50 ms mixing time, the relative intensities of the cross-peaks to the methylene protons at 1.35 ppm compared with that of the terminal methyl groups at 0.95 ppm is lower in the sample with cholesterol, also indicating

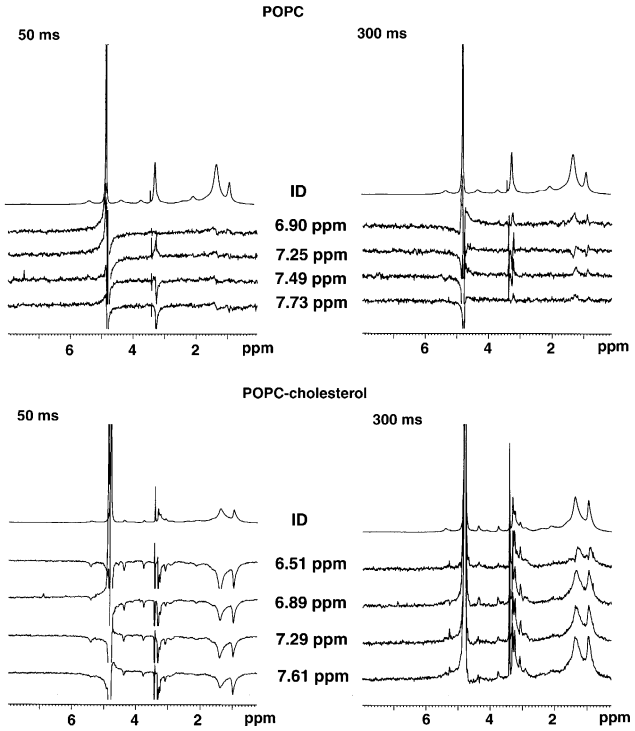

FIGURE 4: One-dimensional slices from the MAS ^{1}H NOESY spectrum at the chemical shifts of the aromatic protons using mixing times of 50 and 300 ms. Spectra are taken from samples of POPC or POPC:cholesterol (1:1), each containing 10 mol % *N*-acetyl-KWASLWNWFNITNWLWYIK-amide. Top spectra are conventional 1-D proton spectra of the samples.

that *N*-acetyl-LASWIK-amide inserts more deeply into membranes containing cholesterol. The motional properties of the peptide are also affected by cholesterol as indicated by reversal of the sign of most of the cross-peaks between aromatic and aliphatic protons using a 300 ms mixing time in the presence of cholesterol. Curiously this is opposite to the situation with *N*-acetyl-KWASLWNWFNITNWLWYIK-amide (Figure 4). The sign of the cross-peaks in both Figures are taken with respect to the peak for the aromatic proton being positive.

^{13}C DP/MAS NMR. Insertion of one of the peptides into a bilayer can result in a change of the chemical shift of certain resonances as a result of aromatic amino acid side chains of the peptide causing shielding of atoms above or below the ring as well as deshielding those at the edge of the aromatic ring. It should be kept in mind however, that other factors, such as changes in the polarity of the environment and/or H-bonding can also affect chemical shifts in bilayers containing cholesterol (*23*, *24*). When either of the two peptides used in this study are added at 10 mol % to POPC in the absence of cholesterol, in general the changes in chemical shifts of the ^{13}C of the lipid is not very large, although with *N*-acetyl-LASWIK-amide there are some changes for atoms near the center of the bilayer and for *N*-acetyl-KWASLWNWFNITNWLWYIK-amide there are two moderately large changes in the resonance position of one of the carbons of the C═C group as well as the C2 carbon of glycerol (Table 3). In the case of *N*-acetyl-LASWIK-amide, it is not likely that the peptide penetrates that far into the center of the bilayer, but rather there is a change in the packing of the lipid that affects the chemical shifts of atoms near the center of the bilayer. *N*-acetyl-KWASLWNWFNITNWLWYIK-

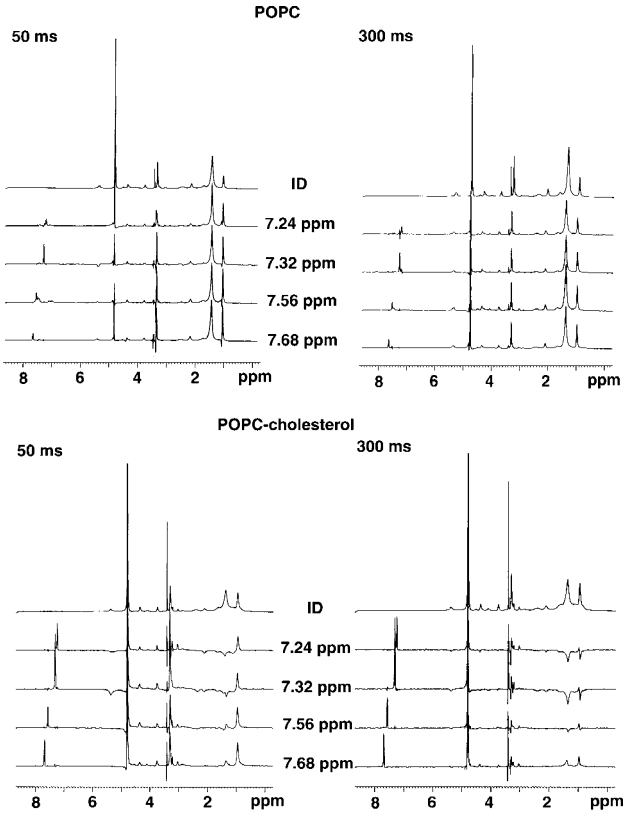

FIGURE 5: One-dimensional slices from the MAS ^{1}H NOESY spectrum at the chemical shifts of the aromatic protons using mixing times of 50 and 300 ms. Spectra are taken from samples of POPC or POPC:cholesterol (1:1), each containing 10 mol % *N*-acetyl-LASWIK-amide. Top spectra are conventional 1-D proton spectra of the samples.

Table 3: ^{13}C Chemical Shift Differences for POPC in the Presence and Absence of Peptide[a]

assignment	chemical shift (ppm)	± *N*-acetyl-LASWIK-amide	± *N*-acetyl-KWASLWNWFNITNWLWYIK-amide
acyl C═O	174	−0.06	0.05
acyl C═C	130.1	−0.05	*b*
acyl C═C	129.6	−0.06	0.14
glycerol C2	71	−0.02	−0.25
choline β	66	−0.04	−0.02
choline α	60	−0.05	−0.01
quaternary CH_3	54	−0.01	0.06
acyl C2	35	−0.06	0.01
acyl C15	23	−0.09	−0.03
acyl terminal methyl	14	−0.09	−0.03

[a] Differences in chemical shifts are given as resonance position of lipid without peptide minus resonance position with peptide. [b] Not determined because of poor resolution of the peak.

amide appears to have a stronger and more specific interaction with one of the carbons in the double bond of the oleoyl chain and with the C2 group of glycerol, but the direction of the shifts is opposite for these two resonance lines. If ring current effects caused the shifts, it would indicate that aromatic group lies over the C═C bond, possibly with π-electron interactions, and the edge of the aromatic ring would be near the glycerol C2. When cholesterol is present, there are significant changes in chemical shifts of several carbon atoms of the lipid (Table 4). In the case of *N*-acetyl-LASWIK-amide, these changes occur for carbon atoms in

Table 4: ^{13}C Chemical Shift Differences for POPC/Cholesterol (1:1) in the Presence and Absence of Peptide[a]

assignment	chemical shift (ppm)	± *N*-acetyl-LASWIK-amide	± *N*-acetyl-KWASLWNWFNITNWLWYIK-amide
acyl C═O	174	0.15	0.02
cholesterol C5	142	0.18	*b*
acyl C═C	130.1	0.10	*b*
acyl C═C	129.6	0.10	−0.05
glycerol C6	121	0.10	*b*
glycerol C2	71	0.23	0.19
choline β	66	0.13	0.11
choline α	60	0.13	0.12
cholesterol C14/17	57	0.11	0.06
quaternary CH_3	54	0.13	0.12
cholesterol C9	51	0.11	−0.01
cholesterol C13/4	43	0.09	0.07
cholesterol C10	37	0.08	0.06
acyl C2	35	*b*	0.06
cholesterol C19	20	0.07	0.03
cholesterol C21	19	0.07	0.03
acyl terminal methyl	14	0.06	0.02
cholesterol C18	13	0.09	0.10

[a] Differences in chemical shifts are given as resonance position of lipid without peptide minus resonance position with peptide. [b] Not determined because of poor resolution of the peak.

both cholesterol as well as POPC and at various depths within the bilayer. With *N*-acetyl-KWASLWNWFNITNWLWYIK-amide, the major effect is on the atoms in the headgroup region of the phospholipid.

DISCUSSION

We have previously shown that the small peptide, *N*-acetyl-LWYIK-amide, facilitates the formation of cholesterol-rich domains in membranes (*10*). In the HIV fusion protein, gp41, this amino acid segment is adjacent to the transmembrane helix of this protein. In the present work we have studied a longer segment of this juxtamembrane region in the form of the peptide *N*-acetyl-KWASLWNWFNITNWLWYIK-amide. This region of the protein is exceptionally rich in Trp residues and has been shown to be important for membrane fusion and virus infectivity (*11*). In addition, an isolated 20 amino acid, synthetic peptide was found to aggregate and promote fusion of liposomes containing sphingomyelin and cholesterol (*12*, *13*), confirming the importance of this region of the protein in promoting membrane fusion and suggesting that raft domains are the site of membrane interactions of this segment of gp41. Surprisingly, we find that the longer peptide, *N*-acetyl-KWASLWNWFNITNWLWYIK-amide, that contains LWYIK among other residues, such as other W that would promote membrane partitioning, is actually less effective than *N*-acetyl-LWYIK-amide in promoting the formation of cholesterol-rich domains. The segregation of cholesterol into a domain, induced by a peptide, will depend on the preferential partitioning of the peptide into a cholesterol-rich *or* a cholesterol-depleted domain (*22*). On the basis of DSC and of ^{1}H NOESY MAS NMR, we suggested that

N-acetyl-LWYIK-amide interacts almost exclusively with membranes containing cholesterol and not at all with pure phosphatidylcholine membranes (*10*). This is not the case for *N*-acetyl-KWASLWNWFNITNWLWYIK-amide that significantly reduces the enthalpy of pure SOPC (Figure 1, Table 1). This peptide interacts with, and appears to penetrate more deeply into, bilayers containing cholesterol as indicated by the stronger NOEs observed in the presence of cholesterol (Figure 4). In addition, at 15% peptide in a membrane that has an equimolar mixture of SOPC and cholesterol, there is formation of cholesterol crystallites, indicating that the peptide does not partition equally into cholesterol-rich and cholesterol-depleted domains. CD also shows that SOPC in the presence or absence of cholesterol induces similar amounts of secondary structure (Figure 2). Since *N*-acetyl-KWASLWNWFNITNWLWYIK-amide is more hydrophobic than *N*-acetyl-LWYIK-amide and has more W residues that strongly partition into membranes, it is less discriminating with regard to the lipid composition of the membrane into which it inserts. As a consequence it has less effect in causing rearrangement of the lateral distribution of lipids in the bilayer.

How do these findings relate to the properties of the intact gp41 protein, for which the full juxtamembrane region is thought to partition the protein into raft domains? There is evidence that the amino terminal portion of this segment is involved in the formation of oligomeric structures (*14*). It is likely that these oligomers are more stable and have a more specific structure in the intact gp41 protein where the C-terminal end is attached to the transmembrane segment that is also oligomeric. As a result, in the intact protein the only portion of KWASLWNWFNITNWLWYIK that is available to interact with the membrane is the LWYIK region that shows specificity for cholesterol-rich domains.

With the isolated long peptide, the oligomer is less stable and specific and, as a result, the amino terminal portion can also interact with membranes resulting in a loss of specificity for cholesterol-rich regions. Although viral infectivity is not synonymous with sequestering into raft domains there have been suggestions with regard to HIV that the two phenomena are associated. There are three mutants of HIV-1 that retain the LWYIK segment but have lost the inability to promote syncytia formation. These are the Δ671−677, the W(1−3)A and the +FLAG mutants (*11*). All of these mutants have modifications in the region on the N-terminal side of LWYIK and may affect the oligomeric state of gp41. In addition, the mutant +DAF is also inactive in syncytia formation (*11*) but it can still mediate dye transfer (*25*). In this mutant 9 amino acid residues are inserted between LWYIK and the transmembrane segment. These findings with the intact gp41 protein demonstrates that the presence of a segment with the sequence LWYIK is not the only requirement to produce fusogenic activity. One reason for this may be that this fusion protein also has some activity in membrane regions that are not enriched in cholesterol.

The segment LWYIK fulfills the requirements of a CRAC motif, i.e., -L/V-(X)(1−5)-Y-(X)(1−5)-R/K-, in which (X)-(1−5) represents between one to five residues of any amino acid (*9*). However, some strains of HIV-2 and of SIV do not contain a CRAC motif in the juxtamembrane region of gp41. In many strains of these viruses the sequence LASWIK replaces LWYIK. The similarity of these two sequences is obvious. Both fulfill the requirements of a CRAC sequence by having L and K residues in correct positions and LASWIK also has an aromatic amino acid, but it does not have a Y residue that is also a requirement for a CRAC motif. As an isolated peptide, we find that *N*-acetyl-LASWIK-amide has some of the same properties as *N*-acetyl-LWYIK-amide, but is somewhat less selective for partitioning into cholesterol-rich domains. Neither *N*-acetyl-LASWIK-amide nor *N*-acetyl-LWYIK-amide affect the phase transition of SOPC; however, *N*-acetyl-LASWIK-amide has more intense cross-peaks with aliphatic protons of the lipid (Figure 5) compared with *N*-acetyl-LWYIK-amide (*10*). Another indication that *N*-acetyl-LASWIK-amide induces some lateral phase separation of SOPC and cholesterol, but not as much as *N*-acetyl-LWYIK-amide does, is that addition of the former peptide to mixtures of SOPC and cholesterol causes only a small increase in the transition enthalpy of the phospholipid and only under certain conditions, while this change is much greater for *N*-acetyl-LWYIK-amide. *N*-acetyl-LASWIK-amide promotes the formation of cholesterol crystallites (Table 2) to a greater extent than *N*-acetyl-KWASLWNWFNITNWLWYIK-amide but less than *N*-acetyl-LWYIK-amide (*10*). Although the NOESY spectra suggest less dependence on the presence of cholesterol for the insertion of *N*-acetyl-LASWIK-amide into the membrane (Figure 5), both the fluorescence emission spectrum of Trp (Figure 3) and the peptide-induced changes in the ^{13}C chemical shifts of the lipid (Table 4) indicate an effect of cholesterol on membrane insertion. It is possible that *N*-acetyl-LASWIK-amide inserts into pure PC bilayers, as indicated by the ^{1}H NOESY, but because this peptide has only one aromatic group it is less perturbing to bilayer packing. As a consequence the peptide is not very effective in altering the phase transition properties of PC as measured by DSC. LWYIK has a greater contribution than LASWIK in sequestering the cholesterol to raft domains.

Interestingly, strains of HIV-1 that have LWYIK adjacent to the transmembrane domain require glycosphingolipids for viral entry (*7*, *26*, *27*), while HIV-2 that has other sequences in this region does not require the presence of glycosphingolipids in the target membrane (*26*). Furthermore, the site of binding of glycosphingolipids to the gp41 of HIV-1 includes the segment LWYIK (*28*). It thus may be less critical for the pathogenesis of HIV-2 that it be sequestered to raft domains, which would be consistent with our finding that LASWIK has less specificity for cholesterol-rich domains than does LWYIK.

Our studies show that a sequence comprising the entire Trp-rich domain of gp41, that contains LWYIK, is less effective than LWYIK alone in promoting the sequestering of cholesterol. The results with this peptide domain are related to those found with various length caveolin scaffolding domain peptides (*29*). In this case the longer peptide, with more aromatic groups present, is less capable of promoting the formation of cholesterol-rich domains. Perhaps being more hydrophobic it can no longer distinguish between specific lipid molecules in sequestering to membranes. Altering the sequence of LWYIK to LASWIK also reduced the lipid preference of the peptide, suggesting that segments that conform to the CRAC motif may have greater cholesterol-sequestering ability than similar peptides that do not conform to the requirements of this domain.

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